Review of the Bovine TB Research Programme

3rd-6th July 2006

Veterinary Exotic Diseases, Research and Official Controls Division

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Review of the bovine tuberculosis (bTB) research programme– July 2006

1 Review of the bovine tuberculosis (bTB) research programme – July 2006

1.1 Introduction

Defra funds a substantial programme of research to contribute to Defra's objective to protect the public's interest in relation to environmental impacts and health and ensure high standards of animal health and welfare. The results generated by these research programmes are used to underpin existing policy, to identify the need to develop new policy, and to support Defra's regulatory roles.

1.2 Defra's aim and strategic priorities

Defra's overarching aim is sustainable development. The goal of sustainable development is to enable all people throughout the world to satisfy their basic needs and enjoy a better quality of life, without compromising the quality of life of future generations.

Defra have identified 5 strategic priorities to assist the Department in the achievement its overarching aim:

1. Sustainable consumption and production: Breaking the link between economic growth and environmental degradation and resource use through promoting and enabling more sustainable patterns of consumption and production;

2. Protecting the countryside and natural resource protection: Creating a robust policy framework and evidence base in order to promote the sustainable use and enhancement of the country's natural heritage and ecosystems;

3. Sustainable rural communities: Encouraging sustainable regeneration in disadvantaged rural areas, promoting social inclusion and reducing deprivation. Ensuring higher quality, more accessible public services to rural communities;

4. Sustainable farming and food, including animal health and welfare: Helping to create a sustainable food and farming supply chain serving the market and the environment; putting in place systems to reduce risks of animal diseases, and being ready to control them when they occur; and

5. In addition to this, Defra has on-going responsibility for emergency preparedness, including planning for emergencies in animal and plant diseases, flooding, food supply, water supply, dealing with the consequences of a Chemical, Biological, Radiological or Nuclear incident.

The research commissioned by Defra is intended to assist the animal health and welfare directorate to meet the overarching Departmental aim through its Strategic Priorities, and to implement policy effectively through statutory and non-statutory means and finally, to ensure that policy is based on a sound and current scientific footing. The directorate serves as a customer for the research programme and acts on the basis of advice from veterinary, scientist and policy teams.

1.3 Strategic objectives of the bTB research programme

The overall objective of the bTB research programme is to provide a scientific evidence base on which policies; strategy and negotiations relating to bTB are based. This is achieved by:

- Defining research requirements; and
- Applying scientific knowledge to the study of uncertainties.

Research is commissioned by Defra to achieve policy and scientific objectives as set out in the ROAME A (***R**ationale, **O**bjective, **A**ppraisal, **M**onitoring, **E**valuation*) (see page 434). The ROAME A provides a formal description of the rationale behind the programme. The statement defines the customer's objectives firstly in policy terms, and secondly in terms of scientific objectives to support the policy need. The ROAME A for this programme will be re-appraised following this review.

The policy issues and drivers are:

1. To gain a better understanding of the epidemiology of *M. bovis* infection in cattle and wildlife;
2. To evaluate the benefits, costs and effectiveness of the present strategies that attempt to reduce occurrences of bTB in cattle; and
3. To develop better strategies to reduce occurrences of bTB in cattle.

Policy issues and drivers are addressed via R&D research, research from other countries and public opinion.

Specific objectives and priorities of the bTB research programme are to:

- Improve diagnosis and detection of *M. bovis;*
- Develop additional control strategies to reduce the incidence of TB in cattle;
- Develop effective vaccines for cattle and badgers;
- Assess wildlife populations with respect to the prevalence of *M. bovis*; and
- Understand the pathogenesis, transmission and modelling of TB in cattle and wildlife species.

1.4 TB review terms of reference

Defra research programme reviews are held every three to five years as part of Defra's on-going research management and evaluation process. The purpose of these reviews is to assess Defra funded R&D in relation to its:

- Scientific quality;
- Delivery of benefits to policy;
- Delivery towards the overall objectives of the research programme;
- Additionally, research programme reviews examine the scope and direction of the programme, and aim to identify future research priorities and needs. This will consider the programme in the context of research programmes in the field that are financed by other UK sponsors, and considering the implications for redirecting Defra research.

1.5 Reviewers terms of reference

- To consider the appropriateness of the research programme to the ROAME A.
- Consider whether the scientific approaches are appropriate for the objectives of the programme and if they are being taken forward competently.
- Assess the conduct of the work, with particular reference to value for money and best laboratory/field practice.
- Assess the effectiveness with which appropriate opportunities for technology transfer are being addressed and in particular whether reports and publications are being delivered to an appropriate standard.
- Examine the proposals for future scientific direction of the work.
- Consider the collaboration with other institutes and universities both nationally and internationally.
- Complete the project appraisal forms, comment verbally at the review meeting and make written recommendations accordingly.

The bTB research programme

2 The bTB research programme

2.1 Introduction

A wide-ranging programme of research has been put in place following recommendations from the Krebs Report (1997[1]). A major component of this was a randomised badger culling trial (RBCT) to test the effectiveness of badger culling as a means of controlling bTB. This trial was managed and funded directly through the TB Division of the Animal Health and Welfare Directorate rather than via the R&D programme. Consequently, the RBCT followed a different management and reporting process compared to other R&D projects on the R&D programme. The culling trial was overseen by the Independent Scientific Group on cattle TB (ISG), chaired by Professor John Bourne. The RBCT is not covered by this review. More information about the RBCT can be found at http://www.defra.gov.uk/animalh/tb/index.htm.

The remainder of the bTB research programme, which this review will cover, is also advised on by the ISG as part of a holistic approach to achieve a sustainable control policy for bTB.

Extensive peer review processes ensure the scientific quality of the research; using expert independent referees at all stages of research procurement and evaluation. Independent advisors, steering groups and/or project management groups oversee most areas of the programme. The majority of research is also commissioned through open competition to ensure fairness and as wide a contractor base as possible. Contractors bidding for Defra research funding are expected to establish international and national collaborative links so bringing in additional expertise.

The major components of the bTB research programme are:

- Further developments of a vaccine for either cattle and/or badgers;
- Studies to improve diagnostic techniques;
- Studies to gain a greater understanding of the natural pathogenesis of the disease in cattle and badgers; including post genomic research to determine which parts of *Mycobacterium bovis* (*M. bovis*) are responsible for its pathogenesis and those that could be useful for either vaccine or diagnostics development;
- Epidemiological factors influencing the prevalence and persistence of the disease in cattle; including the identification of risk factors contributing to the development of the disease in cattle and deer, transmission routes between and within species; and
- Studies to investigate badger/cattle interactions and find effective ways to minimise these in the farmyard and buildings. There is also work to gain more information on the ecology of wildlife hosts, and the ecological effects of culling badgers.

All the projects covered by this review are listed on page 438-441.

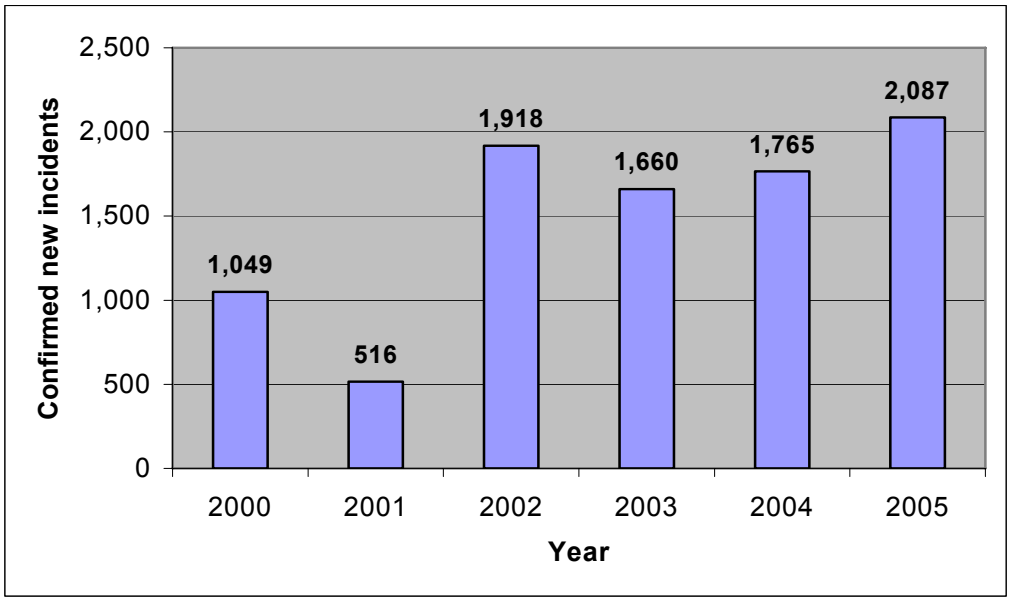
[1] Krebs JR, Anderson RM, Clutton-Brock T, Morrison WI, Young D, Donnelly CA: Bovine tuberculosis in cattle and badgers. In London: MAFF Publications, PB3423; 1997.

2.2 Bovine TB in GB

The incidence of bTB in cattle has been rising since the late 1980's. The increase in confirmed new incidents of disease from 2000-2005 is shown in Figure 1.

Fig 1 Confirmed new incidents of Bovine TB in cattle between 2000-2005.

The figure for 2001 is artificially lower than expected due to the cessation of routine TB testing due to FMD. In 2002 the confirmed incidents were correspondingly higher than expected as the backlog of delayed tests was cleared. The numbers above each bar indicate the number of new incidents each year.

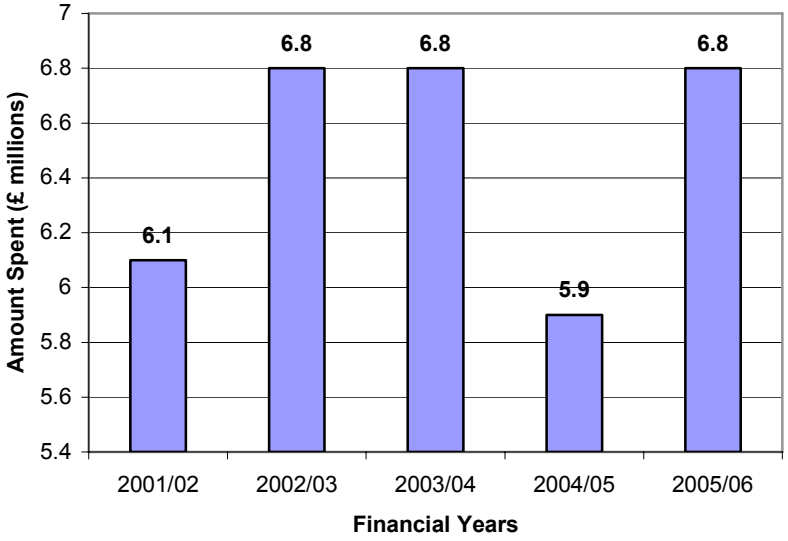


The cost of control of bTB, which includes both the annual tuberculin testing (when testing is anywhere from one to four yearly) and compensation for the compulsory slaughter of reactors and dangerous contacts, was £56.6M in the 2002/03 financial year. In 2004/05 these costs had risen to £71.4M. In 2005, the total numbers of reactors that were slaughtered was 19,972 in GB with this figure rising to 22,566 when the number of dangerous contact animals is included.

2.3 Costs of research

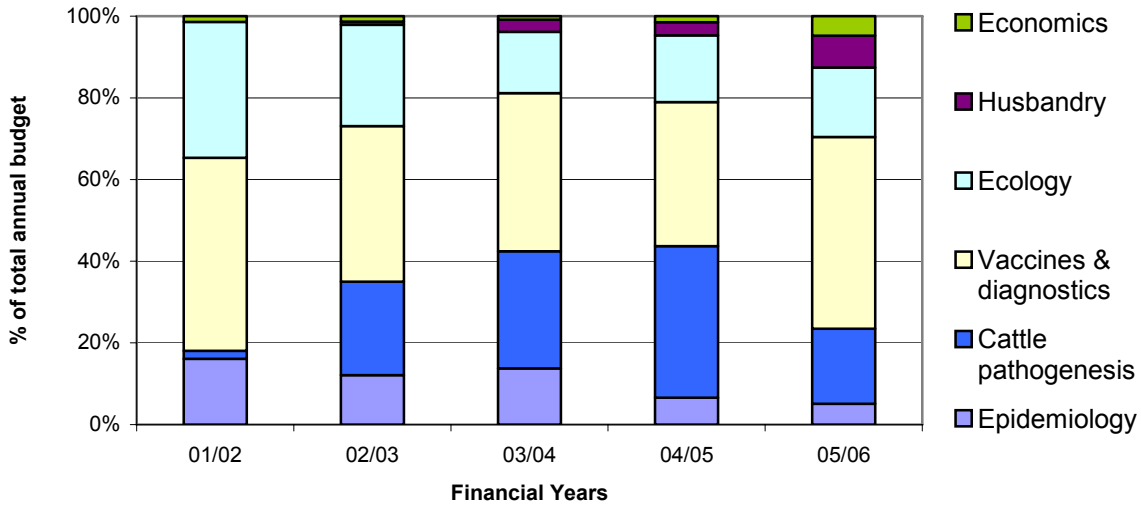
Since the Krebs report in 1997 there was a large increase in funding for bTB research, and this has remained fairly consistent since 2001 (Figure 2). In 2006/07 the predicted spend on R&D contracts is £6M. However, there is a large body of vaccine research that is covered by TB division directly (TB vaccine development budget), and this amounts to a further estimated £2M in 2006/07.

Fig 2 Research funding into bTB since 2000 (excluding the RBCT and its associated work and the work funded via the TB vaccine development budget). Numbers above the bars indicate the total R&D spend in millions in each financial year.



A historical view of the distribution of the bTB R&D spend over the last 5 financial years is shown in Figure 3.

Fig 3 Distribution of bTB R&D spend between different areas (excluding the RBCT and its associated work and the TB vaccine development budget).



The bTB review document

3 The bTB review document

The objectives of the review were achieved with the participation of research contractors, independent external referees, TB division, Veterinary Exotic Disease, Research and Official Controls Division (VEROD) and the Independent Scientific Group on cattle TB (ISG).

This document includes summary reports that were prepared by research contractors, for on-going and completed projects under review on 3rd-6th July 2006. The only exception is SE3109, which was prepared by VEROD and this project was not presented at the bTB review. The summary reports are designed to describe the aims, methods, results and conclusions in a clear and readable format.

The project proposal, available annual/final report and the summary reports were provided to referees prior to the bTB review. These documents were used by referees to evaluate the projects. The project appraisal form detailed in Annex A was subsequently completed by referees. Scores are attributed to various sections of the appraisal form. The range of scores varies from 0 to 4 with 0 = poor, 1 = partial, 2 = satisfactory, 3 = good and 4 = extremely good. Mean scores, derived from the average of the external and internal referees score, give a final score that is weighted equally for internal and external reviewers' comments.

All comments from referees have been taken into consideration by Defra and have been relayed to contractors to help direct on-going problem projects.

Finally, a list of members of the appraisal panel (Annex B) and details of bTB research supported by other funders (Annex E) are included in this document.

Summary of general observations

4 Summary of general observations

- Overall, 86% (43/50) of the research projects reviewed received scores of satisfactory or greater (≥2). Of the 7 projects that received less than satisfactory scores (<2), three did not have final reports available in time for review and one was in a very early stage and was difficult to properly evaluate.

- Overall, projects reviewed in the 'Vaccines/Diagnostics/Immunology' and 'Pathogenesis/Genomics' sections of the programme received higher overall scores than those in the other sections. Overall, the projects reviewed in the 'Ecology/Husbandry' section of the programme received lower scores. This may, in part, be explained by the greater practical difficulties in conducting field based research and by the fact that different groups of referees were used to evaluate these groups of projects. Some areas of weakness were identified which will be addressed.

- The trend towards multidisciplinary and collaborative research should be encouraged in the future as it is beneficial both in terms of outputs and scientific investment.

- Good progress is being made interrogating the large, complex, national datasets available (e.g. CTS and VetNet) to understand better the epidemiology of bTB in cattle. A general recommendation is to continue with this work as the epidemiological information generated is of direct relevance to the disease control programme.

- The establishment of an overarching economic framework is desirable to determine where current resources are being utilised, where changes would have the most impact on disease control, and to assess the economic implications where changes have occurred. In line with this, the research programme would benefit from more integrated economic input.

- Although it has not been established whether BCG will give adequate protection to act as an effective vaccine for cattle, the current research emphasis is on attempting to improve the protection given by BCG by using a prime boost approach. Vaccine development is an iterative process and past candidates identified and found effective in small animal models may need to be revisited if the current candidates under consideration fail. Several vaccine candidates developed in the human medical field are being evaluated in cattle for protective efficacy.

- The evaluation of protective efficacy using a natural transmission model in cattle is underway. A decision is needed on what level of protection is deemed sufficient in order to progress a vaccine through to market authorisation.

- BCG vaccine in wild badgers is not expected to induce sterile immunity, therefore the longer term issue of shedding of *M. bovis* following vaccination and challenge should be addressed.

- The programme of research on diagnostics has progressed from utilising genome sequence information to the experimental testing of selected reagents. Identification of further reagents through antigen mining continues. These reagents will need to be evaluated in the field once decisions are taken on how they are to be integrated into the current bTB control programme.

- No immune correlate of protection has yet been found for *M. bovis*. However, results presented at this review have highlighted that further work in this area is required and could have important implications for diagnostics.

- Further work to standardise and validate PCR to detect *M. bovis* in animal tissues and the environment, and assessment of potential applications of this technique would be desirable.

- Research has been undertaken in the area of strain typing and the distribution of molecular types across GB. Further efforts should be made to translate these results into routine use by the SVS for disease control. It does not appear necessary yet to evaluate further techniques for greater discrimination of molecular types as the policy need has been addressed.

- Significant and robust research has been conducted on the infection kinetics of *M. bovis* in cattle using experimental models. These results have generally complemented those of parallel studies using naturally infected field reactors. Both have raised evidence that undetected infected cattle exist and further work in this area is warranted.

- Greater detail is required to understand how perturbation in badger populations affects bTB transmission. This will require greater collaboration between ecologists and epidemiologists.

- In general more research effort could have been placed on quantification of these risks so that a) strategies can be devised to abrogate them, and b) these factors could have usefully been incorporated into disease epidemiology models to help refine the outputs when considering species interactions.

- Specific recommendations for research management:

    - Research requirement specifications should be very clearly defined so that prospective contractors can understand and address policy issues more easily.
    - Research proposals should continue to be independently peer reviewed to ensure that their aims and objectives are met and that work carried out is based on sound and robust science. Research should not be funded unless issues identified by peer review are adequately met.
    - A small minority of projects were identified as needing more direct management and steering by Defra to prevent contractors straying from their original aims and objectives.
    - Further research may not be needed in some of the research areas reviewed e.g further develop tools for molecular typing of strain. Research outputs to date may be sufficient for Defra policy needs and resources could be diverted to other areas of the programme.

Monday 3rd July 2006

Epidemiology/Economics/Modelling/Risk Assessment

Monday 3rd July 2006

Venue: The Diskus, Transport House, 128 Theobald's Road, London WC1X 8TN

09:00 – 09:30	Registration and Coffee		
09:30 – 09:40	Introduction		Chairman- *Gabrielle Edwards*
	Code	**Title**	**Speaker(s)**
09:40 – 09:55	SE3001	A spatial analysis using GIS of risk factors associated with TB Incidents in cattle herds in England and Wales	ERGO
09:55 – 10:25	SE3020	An integrated approach to the application of *M. bovis* genotyping for the control of bovine tuberculosis in GB	VLA
10:25 – 10:45	SE3034	Exploratory investigation of cattle movement records in Britain to enhance animal disease surveillance and control strategies	VLA/ERGO
10:45 – 11:00	SE3229	Enhanced modelling and prediction of the spread of bovine tuberculosis in mainland Britain: impacts of cattle movements, climate and spoligotype	VLA/ERGO
11:00 – 11:20	Coffee		
11:20 – 11:35	SE3006	Quantification of the risk of transmission of bovine TB from badgers to cattle within localised areas	VLA
11:35 – 11:55	SE3007	Integrated modelling of *M. bovis* transmission in badgers and cattle	CSL
11:55 – 12:25	SE3117	Cost-Benefit analysis of badger control	CSL
12:25 – 12:55	SE3026	Bovine TB transmission in restocked herds: risk factors and dynamics	Warwick
12:55 –13:40	Lunch		
13:40 – 14:05	SE3112	Assessment of the economic impacts of TB and alternative control policies	Reading
14:05 – 14:30	SE3116	The economic value of changes in badger populations	Reading
14:30 – 14:50	SE3036	A quantitative risk assessment on the role of wild deer in the perpetuation of TB in cattle	CSL
14:50 – 15:10	SE3037	A quantitative risk assessment of the role of wild deer in the perpetuation of TB in cattle	Risk Solutions
15:10 – 16:30	Coffee		
15:30 – 18:30	Closed session – confidential project specific issues. Proposed way forward of the research area		

SE3001 A spatial analysis using GIS of risk factors associated with TB incidents in cattle herds in England and Wales

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/01/99
End date 31/03/03

Total cost £188,373

Abstract
To describe the spatial distribution of bTB in England and Wales, and explain why it might persist in certain "hot spot" areas, a GIS-based spatial analysis was attempted. The basic methodology was to assemble three spatially referenced databases: of the disease, the population at risk and the environment, and to integrate these within a geographical information system (GIS). The collation of the environmental data – undertaken mostly through a subcontract with Imperial College – was successfully achieved within the project's first 12 months, but significant problems were encountered with the bTB mapping, particularly with geo-referencing errors. This required considerable effort to be expended on developing methods to successfully identify and resolve this critical problem. In addition, a key spatial set required for the mapping – that of the agricultural parishes was lacking, and to reconstruct it necessitated digitizing from paper maps. Because of these delays, the project was not able to fully realize a true spatial analysis, and its major output was confined to a detailed mapping of the course of bTB in England and Wales over the period. However, the project did lay the foundations for a number of follow-on projects, which might not have been possible without it.

Aims
01. Collate bTB data and the cattle herd population at risk, and use these data to calculate a robust spatial index of disease incidence.
02. Produce a bTB disease atlas summarising the data produced in aim 01.
03. Collate spatially explicit datasets on purported environmental risk factors, including climate, landuse, soil type, and badger density.
04. Conditional upon the success of aim 03, undertake a spatial correlation analysis to determine if associations exist between purported risk factors and the incidence of bTB within hot-spot areas.

Relevance to Defra
As stated in the original project proposal:
- The detailed description of the situation as regards bTB at a parish level for England and Wales will facilitate the planning of control measures and resource allocation at both a national and divisional level;
- The existence of a readily updateable mapping database will facilitate the communication of the current TB situation to decision makers, journalists and the public;

- The setting up of a detailed bovine population database will similarly enable Defra to facilitate resource planning and implementation of policy concerned with livestock; and
- Scientific data (eg population at risk at a parish level) will be generated which should improve the rigour of the other Defra commissioned work on the aetiology and control of bTB incidents, particularly that concerned with risk assessment.

Methods

For the collation of the agro-ecological datasets a systematic search was made of the literature to discover pre-existing sources. Those selected as being both available and relevant to the bTB "hot-spot" hypothesis were soil and stream sediment geo-chemistry, topsoil properties physical and chemical properties, long-term climatic averages (rainfall, temperature, wind etc) and land cover (the ITE 1-km^2 modal vegetation). Each dataset was provided at a different spatial scale, and to achieve consistency, these were all harmonised at a standard 10-km^2.

A key dataset, which was not possible to obtain, was that of badger density, as although data was found, none was of sufficient quality, or extent, or accessibility to be used by the project. To overcome this, a specific follow-on project was undertaken, using surveyed areas from the Kreb's trial to provide quality sett location data, and a range of habitat variables, many of them derived from the above work. The analysis from this project indicated that although significant associations occurred sett density and many variables, the resulting model had poor predictability (Durr *et al* 2004). Badger sett density was therefore not modelled or mapped, though an indirect measure of density obtained from a road-kill survey of bTB in badgers was used for the regression modelling stage.

The source data for constructing a herd level spatially referenced denominator dataset was the SVS's VetNet" animal health information system, which provided an exact Ordnance Survey map reference, and an approximate one by the country-parish-holding (CPH) identifier. After conversion of the OS map ref to eastings and northings, these were plotted within the GIS, *ArcView 3.2*. This detected a large number of errors, with farms being in the sea or not within their referenced county. Similar difficulties were countered with the parish level geo-referencing, as the majority of CPH identifiers were assigned at the 1974 county reorganisation, for which a digitised map was not available. To overcome this, paper maps of the parishes for each county within England and Wales for 1974-76 were systematically compared to the 2000 OS digital map, and where changes were found, the original border was recreated through GIS-based digitisation.

To overcome the problem of erroneous OS map references in VetNet, the use of postal codes was explored as a more precise geo-referencing tool. A specific sub-study was undertaken using actual boundaries for a subset of farms in Cornwall, which had been originally digitised to facilitate Badger Removal Operations (BROs) by the then Wildlife Unit of MAFF. Eastings and northings were assigned to each CPHH using the *MatchCode* package and these were compared to those of the main farm building and the farm "centroid". This study established methods to detect erroneous map references by a three-way comparison between them, postal codes and parish centroids.

As for the work constructing the population at risk dataset, the main data source for herd-level disease incidents ("breakdowns") was VetNet. The data was generally found to be reliable when cross-checked against the summary statistics found in Defra's annual reports of the *Chief Veterinary Officer*. Defining a confirmed bTB incident as occurring when movement restrictions were imposed, a parish-level annual Incidence Rate of the number of new breakdowns per herd years at risk was calculated and mapped.

The original intention was to produce a paper "atlas" of bTB for the period. However, advances in internet GIS technologies meant that an on-line digital atlas became feasible, using a then novel XML-based technology, Scalable Vector Graphics (SVG). The development of this atlas (*Spida*: "SVG Presented Interactive Disease Atlas") was undertaken though a satellite Defra-funded project, and the resulting successful deployment described in Adcock and Durr (2004).

Upon completion of the bTB mapping, a spatial analysis was undertaken, focusing initially on exploration of the spatial dataset, using a number of established techniques such as dot plots, kernel density ratio maps, cartograms and choropleth quantile maps (Pfeiffer, 2004). These were then supplemented with more advanced methods including scanning statistics (SatScan), difference K-functions and LISA cluster maps to describe the spatial structure of the disease. Finally, standard non-spatial regression modelling was undertaken to relate the environmental variables with the incidence of the disease, via a forward stepwise Poisson regression for each 10-km^2 of England and Wales.

Results

- The complexity of geo-referencing farms was confirmed, and the inherent problems with precision and accuracy of using single point identifiers were quantified (Durr and Froggatt, 2002). However, pending the collation of national farm boundary datasets, single point identifiers remain the only practical method of farm geo-referencing, and methods to ensure their accuracy were developed, based upon a comparison of all available data (parish centroid, OS reference and postal code).
- The practicability of collating a harmonised environmental covariate dataset for animal health studies was confirmed, and the key issues relating to data gaps and deficiencies described (Durr *et al* 2000). A national map of badger density remains a critical gap, with subsequent work showing that this cannot be resolved by prediction mapping using pre-existing environmental datasets (Durr *et al* 2004).
- A digital dataset of 1974 agricultural parish boundaries was successfully constructed, and has since been used extensively to map parish testing intervals, which are a useful surrogate of the disease (eg Anon 2005).
- The marked spatial patterning of bTB was confirmed by both the disease mapping visualisation and the exploratory spatial statistics. However, the exact nature of this spatial structure and the underlying processes causing it were not fully explored, and remains work outstanding.
- The regression modelling showed the incidence of bTB to significantly related to a number of environmental variables, particularly climate and soil, and was in broad agreement with the findings of Wint *et al* (2002). However, these explanatory variables were shown to be strongly correlated (ie show multicollinearity), and

result in unstable regression co-efficients. When compounded by a lack of adjustment for spatial autocorrelation, it was concluded that the regression modelling was inherently flawed, and the results needed to be viewed as provisional.

Conclusions

- Precise and accurate farm geo-referencing is essential to any spatial analyses, and as was shown in FMD epidemic, may be critical to successful animal disease control, especially if predictive models based upon single-point identifiers are developed and applied (eg Ferguson *et al* 2001).
- The strong spatial patterning of bTB makes GIS-based visualisation an ideal tool for both exploration and management of the disease. Although this has yet to be fully realised by Defra, the potential of real time bTB data integration via Internet GIS and digital disease atlases has been demonstrated (Adcock and Durr, 2004).
- Although it is possible to show significant relationships between environmental variables and the incidence of bTB by regression techniques, the problem of multi-collinearity, means that such results should be treated as provisional pending more rigorous analyses.
- The limits of regression approaches are compounded by the lack of accurate spatial data on badger density, and resolving this is essential to further research attempting to define the role of the environment in contributing to the current bTB epidemic.

Future research

Two priority areas for further research were highlighted by this project:

- Development and implementation of robust methods of spatial analyses of large and complex national datasets, such as that of bTB in GB; and
- Development of novel methods to estimate and map badger density not dependent upon pre-existing environmental datasets.

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Review comment

This project successfully collated, displayed and analysed several spatially referenced datasets on the cattle population at risk, TB incidents and covariate information. The information provided was essentially descriptive in nature but the aim was clearly worthwhile and important. The project began to explore spatial analytic techniques for the epidemiology of the bTB epidemic. However, the project was compromised by lack of good data, particularly on population at risk and on a key covariate, badger density. In addition, while herd-level data on bTB incidents was good, data on individual animals was rather less good and had to be reconstructed in various ways. The researchers showed ingenuity to resolve problems and this project has laid the foundation for others although it is not clear how to interpret all of the findings from the project. However, the evaluation and subsequent validation, correction and updating of various large databases is an extremely useful output. In terms of future work new and accurate methods to estimate and map badger densities in GB are required, and there is a major need for the development and implementation of robust statistical methods for spatial analysis of large and complex national datasets, such as that of bTB in GB. If the bTB datasets, VetNet data, and data on molecular typing of *M. bovis* isolates, can be integrated with the CTS database; an interactive internet-based GIS-mapping tool could be produced. This could inform policy-making significantly by producing a tool

with which to rationalise and standardise the use of molecular typing data by the SVS.

Scores
Conclusions based on sound evidence: 3.0
Quality of science: 2.6
Overall rating: 2.6

SE3020 An integrated approach to the application of *Mycobacterium bovis* genotyping for the control of bovine tuberculosis in GB

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/2001
End date 30/09/2004

Total cost £869,960

Abstract
The aim of this project was to answer the question posed by in the MAFF research requirement R4 – 'How can new molecular typing tests and reagents be used to address questions on the epidemiology of TB in cattle and wildlife?' In order to do this we exploited the VLA database comprising spoligotyping information on ~32,000 *Mycobacterium bovis* strains isolated from 1975-2005 and VNTR (ETR) profiles from ~8000 typed *M. bovis* isolates from 1997 to date.

Analysis of the population structure of *M. bovis* in GB revealed spoligotype 9 is the progenitor strain of approximately 94% of all strains of *M. bovis* in GB and that different daughter strains have emerged in different geographical areas as a result of 'clonal expansion'. Thus the epidemic of bTB in GB may be seen as a series of local epidemics caused by different strains emerging in different areas of the country. Moreover, the spoligotype and VNTR profile of *M. bovis* AN5, a strain that was originally isolated in England circa 1948 and is used worldwide for bovine PPD production is not shared by any extant strains in the database. This raises the possibility that the AN5 strain may not be optimal for the detection infection by *M. bovis* strains currently prevalent in GB and highlights that further work should be done to resolve this matter.

The geographical localization of different strains could be exploited to determine the source of *M. bovis* as a result of farmers purchasing infected animals. For example, new breakdowns occurring in geographically separate and previously TB-free regions of the country could be linked by combining genotyping and cattle movement data to the movement of cattle from TB hotspot areas as a result of restocking herds following the FMD outbreak in 2002.

Typing data was also used to support the ISG in interpreting findings from the Randomised Badger Culling Trial (RBCT). In one such study, *M. bovis* infections in badgers and cattle were shown to be spatially associated on a scale of 1-2km. Badgers and cattle infected with the same strain type of *M. bovis* were particularly closely correlated.

Finally, using FT-IR, we showed that clonal groupings of *M. bovis* share distinct phenotypic characteristics, possibly cell wall differences that may result in differences in virulence, transmission or ability to evade the tuberculin skin test.

Aims

To maximise the usefulness of molecular typing techniques, it is necessary to combine them with traditional epidemiology, population genetics, molecular biological and phenotypic studies, and advanced biostatistics. Although separate studies may be undertaken with each of these disciplines, most progress will be made by a holistic approach. Through such a study, we aimed to address fundamental questions about how *M. bovis* in the cattle - and by extension wildlife - populations varies in space and time and understand the nature of the environmental forces driving this variability. Specific objectives were to:

- Identify *M. bovis* types of varying genotype-phenotype characteristics based on their epidemiological behaviour within the GB cattle population;
- Undertake molecular biology and whole cell metabolomic studies to characterize the genotype-phenotype variation associated with these types;
- Carry out population genetic studies to understand the mutation dynamics of *M. bovis* in space and time;
- Develop spatial statistical procedures appropriate for the typing data set; and
- Combine all the information into an integral whole to provide advice and decision support for the use of molecular epidemiology at the national and local level.

Relevance to Defra

Genome variation studied by molecular epidemiology has made new insights into disease transmission and evolution possible. The information gathered from studying strain variation can be used for modelling disease dynamics, predicting epidemics, policy planning, monitoring interventions, gaining insight into the life processes of the organism and the nature of the selective pressures driving strain variation. However, to maximise the usefulness of current molecular typing techniques, it is necessary to combine them with traditional epidemiology, population genetics, whole genome mutations, phenotypic studies and advanced biostatistics. Moreover, there is a need for techniques that give greater discrimination of *M. bovis* isolates at the local epidemiological level. The objective of this project was to develop and utilise these additional approaches and combine the data generated in a holistic manner.

Four immediate uses of the results for practical TB control were envisaged: 1. Decision support for the SVS to permit an assessment of whether infection in purchased animals was more likely to be pre or post introduction to the farm; 2. Identification of genetic and phenotypic differences associated with field disease parameters such as lesion localization and spread; 3. Models of disease diffusion in the cattle population and by extension, if data is available, badger and other wildlife populations. 4. Improved molecular epidemiological support for the Independent Scientific Group (ISG).

Methods

1. *M. bovis* strains were selected based on the population structure defined as part of this project. These strains were characterized on the basis of the nature of their genetic and phenotypic variation. Genetic methods included spoligotyping, VNTR analysis, SNP detection through sequence analysis and whole genome microarray analysis. Phenotypic typing were also employed to analyse the same *M. bovis* isolates. Specifically Fourier Transform-Infra Red was used as a whole-organism fingerprinting tool.

2. The variability of *M. bovis* types in space and time was collated, and this information was used to undertake a detailed population genetics study.
3. The analytical problems presented by intrinsically multivariate and spatial nature of the *M. bovis* typing dataset were be resolved by developing appropriate statistical procedures using multivariate kernel regression methods and latent Gaussian process models.
4. R code was written to implement the multivariant kernel smoothing methodology which also allowed integration into a GIS environment to facilitate its routine use by veterinary epidemiologists.

Results

Genome variation studied by molecular epidemiology has made new insights into disease transmission and evolution possible. The information gathered from studying strain variation can be used for modelling disease dynamics, predicting epidemics, policy planning, monitoring interventions, gaining insight into the life processes of the organism and the nature of the selective pressures driving strain variation.

At VLA the current methods of choice for the molecular typing of *M. bovis* isolates are spacer-oligonucleotide typing (spoligotyping) and variable number of tandem repeat (VNTR) typing. The VLA spoligotype database that currently holds typing information on ~32,000 *M. bovis* strains isolated from 1975-2005 (with ~95% of data for strains isolated since 1997). The VNTR method uses 7 ETR targets (A-F) originally described by Frothingham and Meeker-O'Connell (1998) and the VNTR database currently holds data for ~8,000 isolates.

The aim of this project was to answer the question posed in the MAFF research requirement R4 – 'How can new molecular typing tests and reagents be used to address questions on the epidemiology of TB in cattle and wildlife?' The *M. bovis* genome sequence was used to develop complementary strain typing tests to VNTR and spoligotyping ie SNP (SNM) typing and whole genome deletion typing which helped to clarify the genetic relationships between strains found in GB. The tests were targeted to strains of epidemiological importance including archival material collected over the past 20-30 years. The data generated was used to analyse transmission of TB types among farm and wildlife species using a combination of population genetic analysis, spatial statistical analysis, molecular sequencing, phenotypic and epidemiological analyses.

Analysis of the population structure of *M. bovis* in GB revealed Spoligotype 9 is the progenitor strain of approximately 94% of all strains of *M. bovis* in GB and that different daughter strains have emerged in different geographical areas. Thus the epidemic of bTB in GB may be seen as a series of local epidemics caused by different strains emerging in different areas of the country.

The most plausible explanation for the population structure of *M. bovis* in GB is that *M. bovis* has undergone a series of 'clonal expansions' either caused by the spread of a favourable mutation, or as a result of a 'founder effect' as a clone invades a previously uninfected host or geographical area (Smith *et al* 2003; Hewinson *et al* 2005). In reality both selection and population sampling effects are probably involved in generating clonal expansion and geographical localization of *M. bovis* in GB. It is both possible and worthwhile to tease apart the relative contributions of these

population level forces to the geographical localization of *M. bovis* in GB in order to target disease control strategies more effectively. This remains an important challenge for future research so that control strategies can be targeted most effectively.

As part of this project, we demonstrated that the geographical localization of different strains could be exploited to determine the source of *M. bovis* as a result of farmers purchasing infected animals. For example, new breakdowns occurring in geographically separate and previously TB-free regions of the country could be linked by combining genotyping and cattle movement data to the movement of cattle from TB hotspot areas as a result of restocking herds following the FMD outbreak in 2002. This approach highlighted that the restocking of herds had resulted in the introduction of *M. bovis* isolates with spoligotypes that had not been seen in these areas of the country before. These data were used to underpin a policy of pre-movement testing of cattle to prevent movement of infected animals, especially those that are to be moved from areas of the country that are at high risk of *M. bovis* infection to areas of low risk for the disease.

These studies also demonstrated that the combined use of the State Veterinary Service's TB99 epidemiological questionnaire and Vet Net (disease management database) TB recording data, the British Cattle Movement Service's Cattle Tracing System (CTS) data, and the VLA's molecular typing database provides a powerful method for the investigating the source of confirmed bTB breakdowns. However, detailed discussions with SVS on uses of molecular epidemiology highlighted that the development of a "point and click" Intranet application that could display all these data along with probability estimates of this spoligotype being detected – on the basis of past data – on this farm, would have a significant impact on implementing this approach. The development of statistical analysis for the complex spoligotyping dataset as part of this project along with other VLA initiatives including introduction of a web-based database system for project decision support and the use of XML-based technology to successfully display maps over the Defra intranet will facilitate the development of such a decision support system and following the development of a prototype system, a concept note for the development of this system was submitted to Defra in 2004.

In general, the Spoligotype and VNTR patterns obtained from badger isolates between 1972-1976 were the same as those observed in badgers and cattle in the same geographical areas today. This suggests that the geographical clustering of strains has not changed since the first isolation of *M. bovis* from badgers over thirty years ago. This data is in sharp contrast with the rapid movement of strains to geographical areas outside their normal range that was observed as a consequence of the restocking of cattle herds after the FMD epidemic of 2002.

Typing data was also used to support the ISG in interpreting findings from the Randomised Badger Culling Trial (RBCT). In one such study, *M. bovis* infections in badgers and cattle were shown to be spatially associated on a scale of 1-2km. Badgers and cattle infected with the same strain type of *M. bovis* were particularly closely correlated. These observational data support the hypothesis that transmission occurs between the two host species; however they could not be used to evaluate

the relative importance of badger-cattle and cattle-badger transmission (Woodroffe *et al* 2005).

Epidemiological analysis designed to look at associations between spoligotypes and the epidemiological features of an outbreak was performed. Preliminary analysis revealed some intriguing differences across different molecular types of *M. bovis*. This analysis showed differences in numbers of inconclusive reactors across spoligotypes, and revealed that particular spoligotypes are more frequently detected on repeat testing. This suggests that clonal groups of *M. bovis* have distinct phenotypes that may be relevant to the control strategy (Goodchild *et al* 2003).

Therefore the phenotypic differences between spoligotypes was investigated further. Fourier transform infrared (FT-IR) spectroscopy was used to examine 100 blinded strains of *M. bovis* of diverse spoligotype. FT-IR is a rapid whole-organism fingerprinting method that generates a biochemical signature of the bacteria. Cluster analyses of the resulting spectra generated strain-groupings that closely mirrored the phylogeny generated from a combination of spoligotype and single nucleotide mutations. Hence, fingerprinting methods based on phenotype or genotype grouped the strains into similar clusters. These results indicate that clonal groupings of *M. bovis* share distinct phenotypic characteristics, possibly cell wall differences that may result in differences in virulence, transmission or ability to evade the tuberculin skin test.

M. bovis AN5, a strain that was originally isolated in England circa 1948 and is used worldwide for bovine PPD production. However, the spoligotype and VNTR profile of this strain is not shared by any extant strains in the database (Inwald *et al* 2003). This raises the possibility that the AN5 strain may not be optimal for the detection infection by *M. bovis* strains currently prevalent in GB and highlights that further work should be done to resolve this matter.

A test panel of *M. bovis* isolates was made up of 10 isolates of each of the 10 most frequently encountered spoligotypes of *M. bovis*. Using sequencing we analysed 91 potentially polymorphic sites in 89 isolates representing the diversity of spoligotypes most frequently recovered in GB. Forty-four the potentially polymorphic sites were found to be phylogenetically informative.

From this analysis we have identified a series of polymorphisms that are consistent with the previously suggested phylogenetic history of the *M. tuberculosis* complex including *M. bovis* (Brosch *et al* 2002). Furthermore, the single nucleotide mutations clearly show that, within strains of bovine adapted *M. bovis* recovered in GB, strains of spoligotype 20, 25 and 35 are distinct from all others analysed (strains of spoligotype 9 and its children). These two groups of strains differ in at least 9 SNMs. This results is consistent with the phenotypic analysis that also clustered strains of these spoligotypes away from strains of spoligotype 9 and its children. It also suggests that spoligotype 9 may have emerged in GB as a result of an evolutionary bottleneck. There was no evidence for recombination in this data set.

Conclusions

- The genotyping of *M. bovis* in GB has revealed that the population consists of a small number of readily identifiable clones and that these clones are geographically localised.
- Within geographical sub-divisions a single clone is dominant or has reached fixation (endemic clones).
- An analysis of VNTR polymorphism within the two most common spoligotypes concluded that the population structure was incompatible with a process of random mutation and drift and could best be described as a series of clonal expansions in which different genotypes rose to high frequency either as a result of sampling (founder effect) or by selection.
- The geographical localization of different strains can be exploited to determine the source of *M. bovis* as a result of farmers purchasing infected animals.
- Badgers and cattle in the same area share identical genotypes.
- The geographical clustering of strains has not changed since the first isolation of *M. bovis* from badgers over thirty years ago.
- Fingerprinting methods based on phenotype or genotype grouped the strains into similar clusters indicating that clonal groupings of *M. bovis* share distinct phenotypic characteristics.
- AN5 strain may not be optimal for the detection infection by *M. bovis* strains currently prevalent in GB.

Future research

1. Produce decision support for the SVS to permit an assessment of whether infection in purchased animals was pre or post introduction to the farm. (Spida 2).
2. Identification of genetic and phenotypic differences associated with field disease parameters that may assist vaccine candidate selection and validation.
3. Now that data from the cattle tracing system (CTS) has become available, the combination of CTS data with the molecular typing data set has the potential to throw greater light on the routes and causes of *M. bovis* transmission. This approach should be used to address the following questions:

a. To define the contribution of cattle-to-cattle transmission in the spread of *M. bovis* within GB;

b. To clarify the importance of 'amplification' of *M. bovis* within restocked herds and provide clear evidence of the long distance transfer of *M. bovis* by cattle;

c. To determine whether differences in cattle movements can explain differential rates of clonal expansion;

d. To identify epidemiologically important phenotypes that may be associated with different strains (eg the ability to evade detection by skin test before successful transmission, the ability of the strains to persist, disseminate etc); and

e. As highlighted by the ISG, typing of isolates obtained from badger RTA surveys around areas of re-stoked herds would help elucidate the direction of transmission of *M. bovis* and the time it takes to get any infection established in wildlife.

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In addition a Review article has been requested by Nature Reviews in Microbiology. This manuscript is in preparation.

Review comment

A high quality and well organised but mostly academic research project which has produced some interesting findings. The project was ambitious and combined innovative plans in several areas including molecular biology, statistical methods, epidemiology and population genetics. The project's seven main aims were well focused on the Defra research requirement and all seven objectives appear to have been substantially met. The key recommendations of the project are that these typing methods should be used routinely on new isolates as a striking geographical focusing of particular genotypes, evident in both cattle and badgers occurs. These methods should become an established part of the epidemiological toolkit. If implemented, the recommendations are likely to have a major impact on our understanding of the spread of bTB. The cost-effectiveness of the routine use of both spoligotyping and VNTR typing should be assessed. Further work also needs to take place on the technology transfer aspect. There is still much to do to make full use of the data accumulated, and the technology developed, in the course of this project before it can be used to solve practical problems. The lack of tools for the systematic and standardised analysis of molecular epidemiological data by the SVS is an obvious example.

Scores

Conclusions based on sound evidence: 3.5
Quality of science: 3.7
Overall rating: 3.5

SE3034 Exploration of cattle movement records in Britain

Organisation Environmental Research Group Oxford Limited (ERGO) Oxford

Start date 01/06/03
End date 31/03/04

Total cost £84,780

Abstract
A streamlined set of protocols has been developed to extract and analyse cattle movement records from the British Cattle Movement Service's Cattle Tracing System database, so that movements can be characterised, mapped and modelled. Cattle movements, particularly from established disease foci, play a dominant role in determining the presence of the disease, most especially in areas away from long-term disease centres. The analyses also confirm that a combination of movement, climatic and agro-environmental indicators can be used to project bTB distribution in the short term, and demonstrate "proof of concept" techniques to extend these into the medium term.

Aims
The primary objective of this study was to examine the records of cattle movement in Britain and assess their relevance as predictors, or risk factors, in the spread of bTB and the wider utility of such information.

Relevance to Defra
Monitoring and modelling the spread of bTB in Britain and assessing the impact of movement controls are priority concerns of Defra.

Methods
Desk and computer based exploration of various large, independent databases relating to: cattle movements (CTS data); cattle distribution (agricultural census data); bTB (VetNet surveillance data); and a range of environmental variables, derived from satellite imagery and other sources.

Data extraction, analysis and modelling involved the following sequential steps:
- Database scrutiny, cleaning and validation;
- Data transformation, geo-referencing and derivation of movement variables;
- Statistical analyses and characterisation of cattle movement patterns;
- Development and testing of mathematical models of cattle movement;
- Statistical comparison of cattle movement and bTB distribution; and
- Development and testing of mathematical models of the spread of bTB distribution.

Results
The main findings of this study have been published in two papers:

Mitchell, A., D. Bourn, J. Mawdsley, W. Wint, R. Clifton-Hadley and M. Gilbert (2005). Characteristics of cattle movements in Britain – an analysis of records from the Cattle Tracing System. *Animal Science*. **80**, 265-273.

Abstract
This paper reviews the main temporal and spatial characteristics of cattle movements in Britain, based on an analysis of records in the British Cattle Movement Service's Cattle Tracing System (CTS) database, focusing on the period 2001 to 2003, during which notification of cattle movements was mandatory. Movements vary weekly and seasonally according to the production cycle, with peaks in late spring (April) and early autumn (October), and an average 1·63 million farm-to-farm movements per month, equivalent to 19·6 million per annum. The geographical distribution of these movements appears to be relatively stable from year to year, with the great majority of animals moving less than 100 km per journey, although many tens of thousands move over far greater distances of up to 1000 km. The procedures developed to extract, match, geo-reference, analyse and display movement records have greatly enhanced the utility of the CTS database, in that it is now feasible to assess, monitor and map the spatial dynamics and geographical distribution of cattle movements, and provide this information in standardized format on a regular basis.

Gilbert, M., A. Mitchell, D. Bourn, J. Mawdsley, R. Clifton-Hadley and W. Wint (2005). Cattle movements and bovine tuberculosis in Great Britain. *Nature.* **435**, 491-496.

Abstract
For 20 years, bTB has been spreading in Great Britain (England, Wales and Scotland) and is now endemic in the southwest and parts of central England and in southwest Wales, and occurs sporadically elsewhere. Although its transmission pathways remain poorly understood, the disease's distribution was previously modelled statistically by using environmental variables and measures of their seasonality. Movements of infected animals have long been considered a critical factor in the spread of livestock diseases, as reflected in strict import/export regulations, the extensive movement restrictions imposed during the 2001 foot-and-mouth disease outbreak, the tracing procedures after a new case of bTB has been confirmed and the Government's recently published strategic framework for the sustainable control on bTB. Since January 2001 it has been mandatory for stock-keepers in Great Britain to notify the British Cattle Movement Service of all cattle births, movements and deaths5. Here we show that movements as recorded in the Cattle Tracing System data archive, and particularly those from areas where bTB is reported, consistently outperform environmental, topographic and other anthropogenic variables as the main predictor of disease occurrence. Simulation distribution models for 2002 and 2003, incorporating all predictor categories, are presented and used to project distributions for 2004 and 2005.

Conclusions

- It has been demonstrated unequivocally that the movement of animals, especially those from cells where bTB is present, and particularly for locations outside the core disease areas, is a critical factor in the current exponential increase in bTB. The more potentially infective movements there are into a cell, the higher the chance of bTB occurring there.
- However, there are a number of regions into which substantial numbers of animals are imported, where the disease appears regularly, yet does not appear to become established. Reasons for this, eg the imported animals may only remain in the areas for a short time before being slaughtered or are moved elsewhere, could easily be investigated.
- Patterns of cattle movement are essentially consistent from year to year, which means that they can be used together with other variables to project the likely distribution of bTB in the short-to-medium term future.
- Distribution modelling has demonstrated the utility of incorporating movement indicators into short-term projection procedures, and the "proof-of-concept" simulation analyses have constructed and validated the procedures needed for medium-term projections. The reliability of these medium term projections could be improved, however, by incorporating an estimate of the proportion of inward movements from infected areas at the time of each simulation run.
- Similar modelling and mapping techniques could be employed to investigate the distribution and spread of bTB spoligotypes.
- Provided that some additional field data could be acquired to update existing training distributions, it would be a comparatively simple process to use multivariate regression methods, akin to the modelling techniques used in the current analyses, to model and map potential badger distribution, as demonstrated for many other species and disease vectors (Hay *et al* 2000).
- The protocols developed for CTS data extraction, mapping and modelling cattle movement and disease spread are considered to be of direct relevance to veterinary surveillance and Defra's Rapid Analysis and Detection of Animal-related Risks (RADAR) programme.

Future research

A follow up study with Veterinary Laboratories Agency (Weybridge) on: "enhanced modelling and prediction of the spread of bTB in mainland Britain: impacts of cattle movements, climate and spoligotype" has been commissioned by Defra to:

- Investigate and predict the potential impacts of various cattle movement control scenarios on the spread of bTB in Britain;
- Model the spread of individual bTB spoligotypes using cattle movement records from known focal points;
- Upgrade bTB distribution and spread predictor archive and incorporate cattle movement control impacts and spoligotype modelling into improved short and medium term disease prediction models; and
- Conduct an in-depth study of the TB history in an area where TB has developed as a new problem mainly during the period when cattle movement data is available.

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Publications generated from the project

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Mitchell, A., D. Bourn, J. Mawdsley, W. Wint, R. Clifton-Hadley and M. Gilbert (2005). Characteristics of cattle movements in Britain – an analysis of records from the Cattle Tracing System. *Animal Science*. **80**, 265-273.

Review comment

This was a sound and well conducted project where the results were unsurprising. The modelling approach was well structured, elegant and well prosecuted. The movement from statistical analyses to simulation (albeit the latter was proof of concept only) was innovative. The demonstration of the link between cattle movements and geographic spread is important, and provides a useful basis to begin thinking about movement controls as part of bTB policy. The project appears to have been extremely cost effective; producing some useful results for a modest overall cost. It is also one of the first to make use of large scale national cattle movement and environmental databases, demonstrating the value of these databases for epidemiological studies. It has established the value of distribution modelling as a useful tool for investigating risk factors for diseases. The evaluation and subsequent validation of the CTS database is an extremely useful output of this project. Further work should investigate links between patterns of TB strain types and movement parameters and also calculate social network type parameters to be included in regression models. Further work is funded in SE3229 to assess (using models and economic analysis tools) the potential impact of different movement controls on the incidence and geographic spread of bTB. The findings have considerable implications for policy and support recent Defra initiatives on pre-movement testing.

Scores

Conclusions based on sound evidence: 3.4
Quality of science: 3.3
Overall rating: 3.3

SE3229 Enhanced modelling and prediction of the spread of bovine tuberculosis in mainland Britain: impacts of cattle movements, climate and spoligotype

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/11/2005
End date 31/10/07

Total cost £588,361

Abstract
This project capitalises on the exploratory studies (SE3023: Exploratory study to model the distribution and spread of bovine tuberculosis using multi-temporal satellite imagery, 2002) and (SE3034: Exploratory investigation of cattle movement in Britain, June 2004) and will supply practical applications to underpin policy decisions on bTB control options. The study is specifically intended to provide a series of policy driven outputs, including:

1. Short and medium term predictions of bTB spread for the next 2-8 years;
2. Assessments of the potential impacts of different levels and types of cattle movement control and results for chosen scenarios;
3. Evaluations of the utility of the models for assessing the actual impact of cattle movement controls after they are introduced; and
4. Identification of linkages between these outputs and Defra economic models of movement control.

Aims
A key element for success will be close liaison with Defra policy staff, veterinary advisors and economists. Specific attention will be given to:

- The contribution of cattle movement to new foci of disease;
- The role of cattle movement in the local spread of bTB;
- Ideas on how to assess the impact of pre-movement testing; and
- The modelling of different pre-movement scenarios.

Relevance to Defra
The project will provide Defra with short and long term predictions of bTB spread, and the identification of the key cattle movements that contribute to bTB.

Methods
Statistical inputs will include further development and refinement of previous analysis of cattle distribution, cattle movements and bTB incidence data, using a range of multivariate, spatial and temporal analyses, including: cluster analysis, time series analysis, stepwise multiple logistic regression and stochastic simulation modelling, as described in Gilbert *et al* (2005).

Results
To date – none

Conclusions
To date – none

References cited in text
M. Gilbert, A. Mitchell, D. Bourn, J. Mawdsley, R. Clifton-Hadley & W. Wint (2005). Cattle movements and bovine tuberculosis in Great Britain. Nature **43**, 491-426.

Review comment
This project is a logical continuation of the two earlier projects SE3023 and SE3034 and will provide the necessary linkages with strain typing data and cattle movements, as well as evaluate different movement policy scenarios. The two teams involved in this study have the capability to take this work forward and introduce other elements, including environmental and climatic information, and changed bTB testing strategies. This should provide very valuable information on factors affecting spread of bTB which will influence policy decisions and support the development of models to test control options and strategies. The project priority now, in policy terms, is to monitor and assess how effective the new pre-movement testing policy is in preventing the spread of TB, and how it could be modified to make it more effective in the light of those findings.

Scores
Conclusions based on sound evidence: 2.5
Quality of science: 3.2
Overall rating: 3.2

SE3006 Quantification of the risk of transmission of bovine tuberculosis from badgers to cattle within localised areas

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/99
End date 30/09/02

Total cost £167,000

Abstract
A quantitative model was developed to assess the risk of transmission of *Mycobacterium bovis* from badgers to cattle in parishes within the proactive triplets of the Randomised Badger Culling Trial. It first estimates the probability that a randomly selected badger in a given parish is infected, and subsequently excretes *M. bovis* into the environment. The procedure used data from the Badger Removal Operation, supplemented where not available by RTA survey data. It then estimates exposure of cattle to *M. bovis* of badger origin and any subsequent infection. The former uses a model describing a simplified random farm by rectangular grids. The grid cells represent pasture, badger marking areas, cattle sheds or milk parlours. Badger excretion onto these cells is simulated daily for one year. Badger preference for excretion at latrine sites and linear boundary features gives an inhomogeneous behaviour. Differences in excretion of faeces, urine, respiratory excretion and wound exudates are also modelled. Natural decay of *M. bovis* is also modelled. This calculates a spatial and temporal distribution of contamination. When this distribution is calculated, the grid cells are then populated with cattle according to seasonal variation and husbandry practices, and a dose response model is used to estimate the numbers infected. An expert opinion workshop was held to estimate badgers' preferential marking behaviour, quantity of excreta and levels of bacteria present. The resulting model can then be used to study the effects of possible control policies, such as culling, vaccination, and changes in husbandry practices.

Aims

- Develop a model framework to describe transmission of bTB from badgers to cattle within defined locations in GB.
- Develop individual quantitative risk assessment models and identify crucial areas of data deficiency, which require further investigation to reduce uncertainty.
- Present a series of practical control strategies for each of the defined locations.
- Submit risk assessment models for publication in peer-reviewed journals.

Relevance to Defra
The continued increase of bTB in areas of GB is a major animal health concern. To understand the results of the Randomized Badger Culling Trial and related work, a framework is required by which such results can be understood. The model framework developed in this project allows the effects of the basic parameters of bTB epidemiology – badger and cattle behaviour, numbers and locations of bacteria excreted, cattle does response – to be integrated to predict the expected observations. The level of agreement between the model and observations points out the sufficiency of the elements of the model in explaining the observations, and offers suggestions of what other phenomena might need to be considered in studying the infection of cattle with bTB, and well as the effects of possible control strategies.

Methods
Inputs to model.
- Likelihood of badgers traversing pasture boundaries, pasture, cattle sheds and milk parlours, with any seasonal variation.

- Degree of excretion according to type of excretion – urine, faeces, wound exudates, respiratory excretion – according to location and time.

- Degree of bacterial load of the excreta.

- Likelihood of cattle traversing the same locations and coming into contact with excreta, with seasonal variation and variation in husbandry practices.

- Dose response of cattle to infected badger excreta.

Model.
- Simplified representation of farm – areas represented by rectangular grids.

- Badger model.
  - *Nb* badgers present in neighbourhood, move across grid according to input behaviour pattern, and excrete infected excreta according to their input behaviour pattern, and prevalence of infection.
  - Bacteria on grid decay with time and are replenished from new excreta.
  - Output – bacterial distribution on grid with time.

- Cattle model.
  - *Nc* cattle roam across the grid according to variation with season and husbandry practice, according to input cattle behaviour.
  - Cattle encounter infected excreta.
  - Dose response determines whether cattle become infected.

Assessment of risk.
- Baseline model run with best estimate input parameters – determine predicted amount of infection in cattle.

- Vary inputs to model to represent husbandry practices, culling, vaccination, to determine the effect of control strategies.

Results

Results from the model.

1. The risk of badger to cattle transmission is highest from bite wound exudate followed by urine, bronchial secretions and finally faeces.
2. When transmission occurs, the number of cattle infected within a herd is generally small and is very likely to be less than 10% of the herd.
3. The time until first infection within a herd is very variable. This means that the potential for cattle to cattle transmission will also be very variable.
4. The effect of reducing badger prevalence is very dependent on the quantity of *M. bovis* in excreta. If large amounts are excreted, a reduction in prevalence will have a negligible effect on risk whereas if the levels are intermediate or low, a marked reduction in risk will be observed.
5. Similarly, reducing cattle access to latrines will be dependent on the level of *M. bovis* excreted; in reality there is likely to be mixed levels of excretion, depending on the stage of disease.

Identification of data gaps and suggestions for future work.

1. Studies to provide the following types of data are particularly important to facilitate reliable quantification of risk:
    a. Measurements of badger prevalence in different areas of GB;
    b. Sensitivity of *M. bovis* detection methods in badgers;
    c. Quantities of *M. bovis* excreted via various routes at different stages of disease;
    d. Dose–response for different routes of infection; and
    e. Likelihoods of excretion via different routes in isolation and combination at different stages of disease.
2. The quantities of *M. bovis* and the dose-response parameter are of particular importance because they determine the effects of disease control.
3. Analysis of past Woodchester Park data and the implementation of future studies relating to infection in both cattle and badgers which have known locations in the Park may be a useful both in terms of model validation and in general terms of understanding badger to cattle transmission. In addition quantification of bacterial load in consecutively collected clinical samples from badgers in the Park by methods which have known sensitivities may enable intermittent excretion to be defined and allow the quantification of bacterial load at various stages of disease in naturally infected badgers.
4. If and when more data becomes available, the model could be expanded and rerun to:
    f. Provide local estimates of risk corresponding to specific levels of infection; and
    g. More realistic scenarios relating to the combination of different excretion routes.

Conclusions

A QRA model for transmission of *M. bovis* from badgers to cattle by exposure to infected badger excreta was developed, which simulates *M. bovis* excretion by badgers, its survival in the environment, cattle exposure to the organism, and the potential for cattle infection following exposure. Results for four exposure routes (urine, faeces, bronchial secretions, bite wound exudates) were obtained individually, as data to combine them was not available, although the model can combine them. Bite wound exudate gave the highest transmission risk, followed by urine, bronchial secretions and faeces. Infection time will be extremely variable, as will the potential for cattle to cattle spread.

The model predicts that low numbers of cattle will be infected, in line with Cheeseman (1981), who indicates that infection in cattle herds exposed to excreting badgers is low, and other data indicating mean cattle numbers reacting in a breakdown herd to be 3.7, and mean cattle numbers positive per reactor herd as 1.7.

The control measures investigated (cattle density reduction, restricting cattle access to latrines, reducing badger prevalence) have negligible effect when *M. bovis* levels in excreta are high. If levels are intermediate, restricting access to latrines and reducing badger prevalence have a noticeable effect, and there is a reduction in cattle numbers infected and time until first infection. Reducing cattle density appears to have a very limited effect.

Future research

The following issues on data availability and consequent validation of the model were noted at the time of the project:

Development of individual models for specific areas of GB to derive absolute estimates of risk was a goal of this project. However, due to very limited reliable badger prevalence data, it was decided that the model should be run for hypothetical levels of *M. bovis* in badgers and thus that the primary objectives would be the identification of key data gaps and the investigation of control measures. When prevalence data from the RBCT becomes available, they should be used within the model to derive local estimates of risk. The use of proactively culled triplets of the RBCT as a spatial unit in order to estimate the proportion of badgers infected and excreting *M. bovis* in the first part of the model was intended to allow validation using the data obtained from the RBCT itself. However this data was not made available at the time of the project, and it is not clear if they will include information relating to the excretion status or just the disease status of the badgers trapped. Several of the parameters of this model had very limited data available for quantification. The quantity of *M. bovis* excreted and the dose-response parameter had a substantial effect on model predictions. Given that the effects of control measures were dependent on the levels of *M. bovis*, reliable data to quantify the significant parameters is essential. The model includes the concept of intermittent excretion of *M. bovis* by infected badgers, with a probability of 0.5 each day. This was considered to be a worst case scenario, although levels and frequency of intermittent excretion are not currently known, and should be investigated further.

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Cheeseman CL, and Mallinson PJ (1981). Behaviour of badgers (*Meles meles*) infected with bovine tuberculosis. *J.Zoology*, **194**, 284-289.

Publications generated from the project

Gallagher, E., Kelly, A., Pfeiffer, D.U. and Wooldridge, M. (2003). A quantitative risk assessment for badger to cattle transmissions of *Mycobacterium bovis*. Proceedings of the Society for Veterinary Epidemiology and Preventive Medicine, 31 March 2003, Warwick.

Review comment

The ambitious aims of this project were to quantify the risk of transmission of bTB from badgers to cattle in a variety of settings, identify data deficiencies, and devise practical control policies. Unfortunately, the only objective substantially achieved has been to identify the considerable data deficiencies relating to several key parameters, particularly those describing aspects of the badger epidemic, such as prevalence and shedding rates. Many parameters had to be guessed, many others were elicited using expert opinion which might not be wholly reliable. Under these difficult conditions the experimenters did the best they could. Ultimately, what is required is an estimate of a single parameter, the conditional probability of transmission to a cattle herd, given exposure to an infected badger population. Perhaps greater effort should be devoted to direct estimation of this number, by replicating the data obtained from Woodchester park or the RBCT which is now complete, rather than the elaborate reconstruction of the transmission process attempted here. The project has delivered little in the way of insight or derivation of practical TB control policies. However, later projects are addressing these areas and are less theoretical in nature.

Scores

Conclusions based on sound evidence: 1.3
Quality of science: 1.6
Overall rating: 1.4

SE3007 Integrated modelling of *M. bovis* transmission between badgers and between badgers and cattle

Organisation Central Science Laboratory
York

Start date 01/04/99
End date 31/03/03

Total cost £902,757

Abstract
Previous models were developed to permit heterogeneity of social group area and size, thus permitting them to become linked to a GIS, and simulate real landscapes. Model predictions show that proactive control strategies are much better than reactive strategies, although for vaccination combined policies can be very effective.

Model sensitivity analysis indicated that a limited number of badger population parameters were responsible for the maintenance of TB within the badger population. These parameters are mostly related to female recruitment (eg cub mortality). Understanding and measuring the temporal variation in these parameters would be very beneficial for further modelling. Habitat and climate data are important drivers of badger density and for disease persistence and an improved understanding of these relationships are required.

Simulation models produced by CSL, and York and Newcastle Universities can be linked to a GIS structure to simulate population growth and disease spread in real landscapes. These can now be used to simulate larger scale scenarios, subject to the limitations of population parameter and density estimates.

Benefit: cost analysis of different control policies agrees with predictions of cattle herd breakdown rates in that proactive control is most beneficial for endemic disease scenarios, but not new focal outbreaks.

The work has already led to eight published papers and a further six have been submitted. These not only cover the analyses above, but also present new methodologies for sensitivity analysis and advances in the application of GIS interrogation for simulation modelling.

Aims
- Further improve and develop the CSL/VLA badger/TB model to predict the effect of badger management strategies on cattle herd breakdown rates.
- Analyse badger, cattle, habitat and climate data to extract relevant data in order to produce GIS-based versions of the badger/TB models.
- Further develop spatially explicit GIS-based stochastic simulation badger/TB models to determine factors important to the spread of *M. bovis* in the badger, and extrapolate such models to a larger scale.

- Calculate the cost-effectiveness, cost-benefit ratios and broader social welfare effects of previous and potential future badger management strategies.

Relevance to Defra
This proposal addressed the research requirement understanding the causes of herd breakdown, part a. Since the early 1990s, the number of cattle herds succumbing to TB infection has dramatically increased. The majority of these breakdowns are in the south-west, and the European badger is believed to be responsible for many of these infections. This project brought together experts in fields of computer modelling, GIS and bio-economics with the aim of producing integrated predictive computer models which could be used to examine the costs and benefits of any proposed badger management strategy, and to advise Defra on identified management options.

Methods
The work was performed independently at three centres: CSL, York University and Newcastle University.

CSL: A review of TB modelling was performed, and an estimate of the basic reproductive number calculated by using standard population level approaches (Anderson & May, 1991). A mathematical model based on an earlier model (Anderson & Trewhella, 1985) was constructed and used to investigate lethal control, vaccination and fertility control. The Individual-Based (IB) model developed as CSL (Smith, Cheeseman & Clifton-Hadley, 1997; Smith *et al* 1995) was further developed to investigate badger control strategies, including vaccination, and this model has been used to investigate social perturbation and simulate the outcome of the RBCT. Heterogeneity was introduced to the simulation model by the use of a carrying capacity, set for each social group individually, which defined the maximum number of breeding females per social group. Finally, CSL used results from all three centres to perform a model cross-validation study to investigate whether the results form the three independent models were in agreement.

York: The work at York University focussed on further developing an earlier IB model (White & Harris, 1995a, 1995b) and updating it, data analysis and economic analysis. York University developed a procedure for incorporating realistic environmental heterogeneity into a spatial modelling framework. Field survey data of badger distribution and habitat from two 10km x 10km areas in Gloucestershire and Wiltshire in south-west Britain were used to parameterise the landscapes within a spatial stochastic simulation model. Discriminant function analysis (DFA) was developed using ground-surveyed data from Wiltshire, to predict badger group sizes. A Geographic Information System (GIS) was trained to recognise reflectance patterns of different habitat types from remotely sensed data using a multi-linear regression model. The spatial stochastic model was used to investigate the theoretical relative efficacies of proactive and reactive control of TB in badger populations.

Newcastle: The work at Newcastle University focused on the development of a GIS version of an individual-based spatially-explicit model to investigate the dynamics of badger population dynamics and TB epidemiology in a real landscape. The sensitivity of the model to input parameters was tested using a Latin Hypercube Sampling (LHS), and analysed using partial correlation and binary logistic regression. An LHS strategy was used to select input parameters for the model from the known or

estimated ranges of the different variables in the model. The model was run a sufficient number of times to encompass the potential range of conditions that occur naturally, rather than simply worst and best case scenarios. A uniform distribution was assumed for each variable with upper and lower limits derived from the literature. Variables were also assumed to be independent of each other. This approach will lead to an overestimate of the size of the likely universe of possible values that each life history parameter could take. Partial correlation coefficients between each model parameter and the badger population size after 16 years were calculated after 1000 simulations. The power of the partial correlation coefficients was calculated exactly. The data resulting from the sensitivity analysis were transformed into presence/absence of TB in a modelled badger population after 20 years. Binary logistic regressions were performed on the Latin Hypercube variable set to identify variables contributing significantly to the presence of infected badgers after 20 years.

Results

CSL:

The review of TB models in badger, possums and cattle was published (Smith, 2001), as was a more recent updated version (Smith, 2005). This demonstrated that the badger/TB situation in the UK is the best-understood wildlife TB situation, and has the longest dataset (Woodchester Park). This data was used to estimate the basic reproductive number (R0) using both population prevalence and age at infection to give a value of between 1.1 and 1.2. This value has been recently supported with an independent model from the ISG (Cox *et al* 2005), although these methods all assumed homogeneity and increasing heterogeneity lead these methods into underestimating R0.

A simple mathematical model investigated different control strategies, including temporary and permanent sterilisation. The model showed that the theoretical advantages of lethal control is due to the birth of susceptibles during a vaccination campaign, which makes it harder to keep the population below the critical threshold density. This difference in efficacy of lethal control to vaccination was very marked for bTB, although no account was taken of social perturbation. The inclusion of fertility control within a vaccine meant that the chance of disease eradication was very similar to that of lethal control, since the birth of susceptibles is now much reduced.

The IB model has been adjusted to include cattle herds, investigate the use of a live test, and different control strategies (Smith *et al* 2001a; Smith *et al* 2001b), including vaccination (Wilkinson *et al* 2004). This was the first badger-TB model to examine sex differences in disease epidemiology and the transmission of TB from badgers to cattle. Analysis of herd breakdowns rates and badger population size in the mid 1980s and mid 1990s suggested an annual finite transmission rate of between 3.4 and 6.0% per super-excreting badger. The prevalence of TB, and the number of simulated cattle herd breakdowns was reduced for all control strategies using a live-test, namely localised culling, ring culling, and proactive culling. However, only proactive culling resulted in a marked reduction in these values within a few years. If trapping efficacy is increased above its current value (80%), this does not improve the effectiveness of these culling strategies. If the number of individual badgers caught and tested per social group is doubled from two to four animals per group then the overall level of effectiveness of these strategies could be doubled. The

effectiveness can be improved if the sensitivity of the live-test is increased, but does not continue to show an improvement above a sensitivity of about 70% for this control method.

The model appeared to underestimate the rate of population recovery following widespread culling, but this has since been addressed in project SE3117.

Of the historical methods of badger control, gassing and the 'clean ring' strategies were the most effective at reducing disease prevalence in the badger and cattle herd breakdown rates. These results agree with those of earlier models. The proactive badger removal operation as part of the current RBCT should cause a decrease in the number of cattle herd breakdowns within the culled area (by about 20-50% of all herd breakdowns caused by badgers) but it also has the greatest effect on the badger population size.

The model predicted that vaccinating 80% of groups at a low vaccine rate (10% successfully immunised) was more effective at reducing prevalence of TB in badgers, than vaccinating 10% of the groups at a higher rate (80% successfully immunised). Although a lower cost option, vaccinating badgers purely in reaction to cattle herd breakdowns took longer to reduce badger-TB prevalence to 50%, than proactive strategies (7 to 20 years compared with 3 to 5 years). The model indicates that vaccination of badgers is a viable alternative to badger culling for the control of TB in cattle, but combined control policies were not investigated.

York:
Discriminant function analysis (DFA), predicted badger group sizes as low, medium or high based on habitat data from the Gloucestershire area with an overall accuracy of 88%, although it was less good at predicting high and medium than low group sizes. There are several likely reasons for the lack of accuracy in predicting locations with higher badger densities. Firstly, the two study areas used had few high-density areas within them, which therefore weakened the power of the DFA for these densities. Habitat composition is also unlikely to be the only determinant of badger density. Main sett locations are often associated with linear features such as hedgerows and tree-lines. Such linear features cannot be identified using LANDSAT imagery and so represent other parameters that may strongly influence badger density distribution but which cannot be assessed using this platform alone.

The GIS correctly predicted the major habitat types from reflectance data with 92% accuracy. However, predictions of badger density based on remotely sensed habitat data were less reliable, especially for the medium and high group size categories. Despite the fact that some data were not included, the overall prediction accuracies were high. The individual predictions for the different areas could be greatly improved with additional training from other geographical areas (both habitat and badger density).

It has been argued that attempts at eliminating TB in cattle have not been effective because environmental and human factors that may be conducive to the persistence of TB have been ignored. Quantifying the link between TB incidents in cattle (herd breakdowns) and these other factors could be of considerable value in developing reactive, and especially, proactive measures to curtail or minimise the risk of herd

breakdowns. We used data from 211 parish groups in six counties over the period 1988-1996 and employed a logistic regression, descriptive, and multiway frequency analysis to investigate the extent to which various agriculture-related variables could be used to predict the probability of herd breakdowns in different parts of south-west Britain. The results showed that the size of the farm labour force (number of full time farmers, partners and directors), the number of cattle per unit of labour and the number of cattle per hectare of grassland within a parish group were significantly associated with the frequency of herd breakdowns. The results show that these variables are significant influences on herd breakdowns in southwest England. We demonstrated how the model could be used to predict the probability of a specific parish group, experiencing zero, one, two or three or more herd breakdowns based on these agricultural and farm management characteristics.

The spatial stochastic model investigated control in badger populations occupying two specific landscapes in southwest England. Different control strategies were compared in terms of benefits, measured as the reduction of numbers of infectious badgers and costs, measured as the numbers of badgers killed. With a background of endemic disease, proactive control produced greater benefits than reactive control. With no endemic disease background, the highest benefits were obtained from those controls (proactive and reactive) closest to the time of the local infection. Greater culling efficiency resulted in greater benefits. With endemic background disease, the more proactive the control, the stronger was this effect in terms of absolute benefits. With endemic background disease, proactive control tended to result in better benefit:cost ratios than reactive control. However, without endemic background disease, reactive control tended to produce better benefit: cost ratios.

The above model was run with medium overall badger density. This work was then extended by running the model at three overall badger densities (low, medium and high), and introducing a small local area control option. The results support the findings at medium overall density and suggest that the levels of benefits and benefit: cost ratios are highly dependent on the overall badger density of the extended area and the level of background TB in an area.

Newcastle:
The GIS model was an individual-based model with the age, TB status and sex of each badger in each social group as the state variables. The model interrogated each badger at six-month time steps to determine stochastically the life history of the individual. The LHS strategy indicated which parameters were significant in the model (Table 1).

From the sensitivity analysis, there was a 93% concordance between persistence of TB in the simulation model and the regression line fitted to it based on the 22 significant predictors.

The sensitivity analysis revealed that the simulated badger population size after 20 years was most dependent on five parameters affecting female recruitment (probability of breeding, mortality of adult females in the first half of the year, mortality of juvenile females in the second half of the year and mortality of female cubs in both halves of the year). The simulated prevalence of TB was most affected by the population size, the rate at which infectious badgers transmit the disease to other

members of their social group, and the rate at which the disease is spread outside of the social group.

The spatial and temporal predictions of the model were tested with field data of badger demography and TB prevalence. The TB parameters best fitted the demographics of badgers and the epidemiology of TB observed at Woodchester were used to produce spatial output of TB distribution. One hundred simulations of the model were performed, using the badger population and disease status of badgers in Woodchester Park in 1981 to initialise the model. Values for the number of years that an infected badger was present (termed TB prevalence), the number of times that a group becomes infected (termed TB incidence) and the average duration of infection (prevalence divided by incidence) were calculated for each social group in the 100 simulations. The means of all the simulations were then compared to values of prevalence, incidence and average duration derived from the field data. There was a good match between the simulation model and the field data for both population dynamics and TB prevalence under a number of parameter values for within- and between-social group transmission. However, there was little overlap between the areas where the model closely predicts both badger population size and TB prevalence. Validation in time allowed the prediction of values for transmission parameters for which we have no information. When validated in space, the model generated population sizes and disease incidence that were consistent with the observed field population.

We conclude that modelling TB dynamics must include spatial *and temporal* heterogeneity in life history parameters, social behaviour and landscape. It is clear from the results that the model was not capable of generating all of the observed spatial variation in badger and TB demographics. Key features that were missing were environmental heterogeneity arising from spatial variations in habitat composition and temporal variations in weather. Since badgers rely on a food resource, whose availability is strongly dependent on weather and TB persistence in the environment has a strong environmental correlate, these need to be included in the next phase of modelling. Very little is known about disease transmission parameters within badger populations, and this approach will further the understanding of TB epidemiology. More explicit modelling of social interactions between individuals and their impact on TB transmission rates within and between social groups is likely to enhance the accuracy of predictions and the overall utility of the model.

Conclusions

The original CSL badger/TB model has been adapted both by CSL and by Newcastle University and now permits heterogeneity in social group area and size, permitting simulations to be performed for real landscapes. By using data from 1981 from Woodchester Park, the revised models have been validated by predicting the population growth over the following 20 years. Novel sensitivity analysis has identified the most important parameters in the maintenance of TB in the badger population: those mostly related to female recruitment. In addition, this work helped demonstrate support for the existence of social-perturbation, which may undermine the effects of culling.

The models have examined historical and potential badger control strategies. The current model of cattle herds is simplistic and does not include cattle infection, movement, and differential stocking rates or management. The results demonstrate that reactive control is less effective than proactive control. This is also supported by the benefit: cost analysis from York. York University have produced models to predict badger density, based on ground survey data, and are working on methods to predict badger density based purely on remote-sensed data. Predictions of areas of high density could also be used to adjust bait density for vaccination campaigns.

Future research

The results of this project strongly suggest that further work is required in the following areas.

1. Refined field measurement of female recruitment population parameter values, and their temporal variation. This can be partly achieved by analysis of trial data.
2. Further analysis of the relationship between environmental factors and prevalence of TB.
3. Further development of the cattle model to produce a combined badger/cattle/TB model (see SE3117).
4. Analysis of trial data to give further estimates of badger and social group density.
5. Analysis of trial data to give estimates of the level of social perturbation and badger movement following culling (see SE3117).
6. Development of the modelling in tandem with the vaccine research.
7. A full cost-benefit analysis (see SE3117).

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Review comment

This project brought together a group with very strong modelling skills and GIS expertise. The group had access to the best set of data available on a disease in a wildlife population which is transmissible to domestic livestock and was a good application and evolution of existing models. The project produced some well validated models and fully met its objectives. Some novel results were produced, for example, discovery of the importance of female recruitment with regard to maintenance of bTB in the badger population, and one of the first descriptions of the affect of social perturbation on level of disease in badgers and consequences with regard to herd breakdowns. The project has generated a large number of scientific papers for peer reviewed journals and produced models which produced valuable information for policy formulation and are available for further study of bTB in badgers and cattle in a variety of scenarios with spatial, temporal and economic parameters. The difficulty is ensuring that the outputs are not overly relied on (without due recognition of the associated prediction uncertainty). Further refinement and development of work on this area is taken forward in SE3117.

Scores

Conclusions based on sound evidence: 2.8
Quality of science: 2.8
Overall rating: 2.8

Table 1 Partial correlation coefficients and associated F-values after 1000 simulations, relating the predicted total number of badgers to the different life-history parameters used in each model run. The F-values have 1 and 972 degrees of freedom. Power is calculated assuming a significance level for the F-distribution at 0.01, and predicted number of simulations to achieve a power of 0.8 is given for the significant life history parameters. +++ indicates p<0.001; ++ indicates p<0.01; + indicates p<0.05.

Variable	Partial correlation coefficient	F-value	P-value		Power at α= 0.01	Number of simulations to achieve power = 0.8 at α = 0.01
Probability of producing 1st litter	0.690	881.671	0.000	+++	1.000	291
Probability of producing 2nd litter	-0.006	0.031	0.860		0.008	
Probability of producing 3rd litter	-0.004	0.019	0.889		0.008	
Probability of producing 4th litter	0.022	0.456	0.500		0.008	
Female cub mortality season 1	-0.678	824.895	0.000	+++	1.000	323
Female juvenile mortality season 1	-0.507	335.974	0.000	+++	0.373	1797
Female adult mortality season 1	-0.528	375.063	0.000	+++	0.519	1442
Female cub mortality season 2	-0.816	1942.241	0.000	+++	1.000	83
Female juvenile mortality season 2	-0.612	582.876	0.000	+++	0.984	614
Female adult mortality season 2	-0.600	548.151	0.000	+++	0.963	707
Male cub mortality season 1	-0.229	53.872	0.000	+++	0.010	68597
Male juvenile mortality season 1	-0.125	15.437	0.000	+++	0.008	854078
Male adult mortality season 1	-0.088	7.572	0.006	+++	0.008	3534422
Male cub mortality season 2	-0.252	66.012	0.000	+++	0.011	46211
Male juvenile mortality season 2	-0.079	6.062	0.014	+	0.008	5441545
Male adult mortality season 2	-0.060	3.553	0.060		0.008	
Female movement probability	-0.030	0.865	0.353		0.008	
Male movement probability	-0.250	64.752	0.000	+++	0.011	47770
Super-excretor infection rate	-0.275	79.718	0.000	+++	0.012	31850
Excretor infection rate	-0.067	4.349	0.037	+	0.008	10572572
Between-social group transmission, females	-0.079	6.058	0.014	+	0.008	5451653
Between-social group transmission, males	0.001	0.001	0.973		0.008	
Excretor females to latent	0.046	2.104	0.147		0.008	
Excretor males to latent	-0.031	0.933	0.334		0.008	
Latent to super-excretor	-0.046	2.106	0.147		0.008	
Latent to excretor	-0.048	2.268	0.132		0.008	
Additional super-excretor mortality, females	-0.031	0.937	0.333		0.008	
Additional super-excretor mortality, males	0.001	0.001	0.970		0.008	

SE3117 Cost-Benefit analysis of badger control

Organisation Central Science Laboratory
York

Start date 16/08/2004
End date Phase I 31/10/2004 Phase II 31/03/2007

Total cost Phase I £41,738 Phase II £401,976

Abstract
In Phase I, the Badger/TB simulation model devised by the Central Science Laboratory (SE3007) and the Cost Benefit model devised by Reading University (SE3112) were amended to enable output from the former to be used directly in the latter.

Information on the costs of badger culling were estimated from data supplied primarily by the Defra Wildlife Unit and the cost of a cattle herd breakdown from data supplied by the VLA and the Reading University study. Preliminary assessment of cattle movement data suggest that between 26% and 85% of cattle herd breakdowns were not caused by cattle moving into the index herd, and thus, this percentage could have been caused by wildlife.

Badger trapping, and gassing, was simulated at different scales. The results indicated that some badger culling strategies might result in an economic benefit in an ideal world (ie no social perturbation, full compliance, efficient control, cause of breakdown correctly attributed). The results of this project are not yet sufficiently robust to inform a national policy decision on badger culling. Limited simulations that included 'social perturbation' of badgers were performed, and these agree with results from the RBCT.

Aims
Phase I
- Integrate the CSL and Reading models.
- Simulate some badger control strategies and produce a Cost-Benefit Analysis.
- Produce a scientific and lay report on the outcomes and potential future steps.
- Produce an independent ongoing review of the process and results.

Phase II
- Integrate the Reading CBA within the CSL model.
- Analyse available badger data, update the CSL model and validate the results.
- Evaluate the approach for, and program, the additional cattle simulation within the CSL model.
- Produce a GIS version of the badger/cattle model.
- Evaluate badger and cattle management strategies as identified during the project.
- Produce scientific and lay reports on the outcomes and potential future steps.

Relevance to Defra

The number of cattle herd bTB breakdowns has been increasing in recent years. The government is increasingly relying on evidence-based policies. For some policies, such as badger and cattle management, it is very difficult, politically complex, expensive and time consuming to obtain field-based evidence. This proposal seeks to model a variety of different possible badger and cattle management strategies and determine their overall cost or benefit to society, in relation to the consequent change in cattle herd breakdown. These outputs, along with their intrinsic uncertainties and assumptions, can be used to help inform policy decisions on methods of badger and cattle management to be employed, potential spatial configuration for mixed strategies and the costs and benefits associated with these strategies. This will include, but is not limited to, badger culling, changes in cattle testing protocols and methods, and badger and cattle vaccination.

Methods

The CSL badger model has evolved over a ten-year period and has been subject to peer review through repeated scientific publication (Smith, Cheeseman & Clifton-Hadley, 1997; Smith *et al* 2001a; Smith *et al* 2001b; Smith *et al* 1995). It is a spatial mechanistic model of badger biology (births, deaths and movement) and TB epidemiology, based primarily on analysis of data from the Woodchester Park badger study (Delahay *et al* 2000; Rogers *et al* 1998; Wilkinson *et al* 2000), which has been running since the late 1970s. Various model structures have been analysed (Smith *et al* 1995) and concluded that the current structure gives the best fit with field data. The model runs using a six-month time step and is stochastic. In Phase II changes to the time-step are being investigated.

For this work, the model used a square grid of 100 x 100 cells, with each cell representing 0.04 km^2 (200m x 200m), constructed as a torus, so that the population is closed. The lack of immigration and emigration is acceptable as no control policy encompasses the whole simulation area, so that there are always large numbers of badger social groups to which immigration and emigration will occur. Badger main setts are distributed at random on the grid at a density of 0.75 km^{-2} (300 territories), an average density for the south-west (Wilson, Harris & McLaren, 1997). All empty cells are assigned to the nearest main sett to form a territory, allowing territories to have different numbers of neighbours.

Each territory is initially populated with the same number of male and female, adult and yearling healthy badgers. Each territory has a predefined maximum number of litters that may be produced, and this constrains the population size to an emergent carrying capacity. Assuming females of breeding age are present, the number of breeding females is determined probabilistically, and the probability of the second, third or forth litter being produced is less than that of the first.

The simulation was run for 20 years to stabilise the badger population, TB was introduced at random and the model run for a further 100 years to stabilise and allow a heterogeneous distribution of disease to emerge, similar to the Woodchester Park study site (Delahay *et al* 2000).

Disease transmission rates are set at the beginning of the simulation to produce a particular prevalence level. For Phase I, values were chosen to simulate a disease prevalence level of either 5% or 20% to represent a low or high prevalence site.

Badger social perturbation has been associated with a disruption in territoriality lasting several years (Cheeseman *et al* 1993; Tuyttens *et al* 2000; Woodroffe *et al* 2006). To simulate this in Phase II we set between-group transmission probabilities equal to within-group rates, for culled groups. This increase in transmission was simulated for three years after culling ceased.

A complete cattle simulation is being produced in Phase II, but is not yet available. In Phase I, the model assumed that each badger social group mapped to one cattle herd, and that each herd is equally likely to become infected by the occurrence of a 'super-excretor' badger. The transmission rate from badgers to cattle was assumed to be 2.5% per 'super-excretor' per six-months (Smith *et al* 2001b), although refined estimates are being produced in Phase II. Every cattle herd breakdown (CHB) is detected at the end of each year, ie assuming annual testing. Output from the CSL Phase I model indicated the number of CHBs, but not the number of individual cattle infected. In Phase I, it was assumed that cattle herd management did not affect transmission. In Phase II, the amount of variation around this value may be estimated.

Cost-benefit analysis (CBA) is the most favoured method of assessing animal diseases and their impacts at both farm and national levels. CBA is the identification, measurement and weighing-up of the costs and benefits of a project in order to decide whether it should go ahead. The basic CBA rule is that expenditure is judged potentially worthwhile if its benefits exceed its costs, where benefits and costs are defined to include any welfare gain and loss that occurs because of the expenditure on the project. However, cost also has to be thought of as opportunity cost, that is, the benefits forgone because of the project in question. After the impacts of a control strategy have been defined, they must be translated into economic terms. Future costs and benefits must be discounted for comparability. A discount rate of 3.5% is used throughout, in line with Treasury guidance (H M Treasury, 2003). The final output, a net present value (NPV), is the mathematical difference between the present value and flow of future returns and the present value and flow of costs: reducing the analysis to single monetary value. The control strategy then becomes economically viable if the NPV is greater than 0: ie the return is greater than the opportunity cost.

Two strategies were modelled in Phase I: an "immediate" trigger where the simulated CHB triggered a control strategy and an "historic" trigger where larger predefined areas were monitored for twenty years, and the worst 20% of the area (in terms of CHBs per km^2) were culled.

Three scales of control were modelled for the immediate-trigger: R1 (the area of the one badger social group that caused the CHB), R2 (the area of the territory central to the CHB plus all the neighbouring territories) and R3 (R2 plus all their neighbours). Thus, R1, R2 and R3 are increasing rings of neighbouring badger territories centred on the CHB.

So far, only one scale of control for the historic-trigger has been investigated (P1). These areas are created probabilistically, defined at the start of each simulation and tessellated. The mean size of the P1 areas was about 13 km^2: equivalent to the size of a single parish. The worst six P1 areas represented 20% of the total grid area. Badgers are culled probabilistically according to the proportion of each territory that is included in the P1 cull area.

For immediate-trigger strategies, every CHB triggered annual culling from year 120 onwards. Culling locally (R1, R2 or R3) was done annually for one, three or five years giving, for example, the strategies R1T1, R1T3 and R1T5 respectively. If a new CHB occurs after culling has started, the local culling period is reset to extend the number of years culled.

For historic-trigger strategies, culling started in year 120 and applied for a period of one, three or five continuous years giving, for example, the strategies P1T1, P1T3, and P1T5 respectively.

Culling was applied probabilistically to every badger in a group, independent of age, sex, or disease status. It was assumed in Phase I that full compliance and access to land is given. The Defra costs associated with each method were estimated. Trapping success rates was assumed to be 80% and gassing 80%, 90% or 95%.

VLA staff assessed herd breakdowns that could have been caused by the transfer of cattle by using the Cattle Tracing Scheme (CTS) movement data and VetNet. It is possible that some bought-in cattle were healthy, and infection was never-the-less caused by local wildlife. Thus, the proportion 'caused' by buying in cattle is likely to be a slight over-estimate.

Results

The CTS/VetNet results indicated that between 26% and 85% of all CHBs in 2002/3 could have been caused by badgers. This would represent between 400 and 1400 CHBs that may have been caused by badgers in high incidence areas. Thus the breakdown rate attributed to badgers is between 0.01 and 0.03 CHB km^{-2}. These rates are close to those from the CSL model, so we did not amended the transmission rate in the model to correct for this estimate.

Cost data were broken down to a cost per km^2 for a team of men with a 4 x 4 vehicle, in order to be compatible with model output (Table 1). To determine the effect of uncertainty in the costs we examined a 50% change in the cost of culling.

The benefit is the value of the reduction in herd breakdowns accrued over the duration of the project. Over the last ten years, there was an average of 9.068 cattle slaughtered per confirmed TB breakdown in England and Wales, and the total on-farm cost per slaughtered animal is £1,405. This included the cost of the individual animal, cattle testing, isolation and other associated costs. When the veterinary cost of breakdown testing is added this gives a total of £13,981 per breakdown. The benefit of an increased testing interval has not been included in phase I. It is also acknowledged that the costs of a breakdown can vary widely, so following the above approach we have examined the effect of a 50% change in average cost per breakdown.

Table 2 shows the total badgers culled per km^2 over a 20-year period for each of the control strategies. This shows that the area culled is a more important factor in determining the total badgers culled than is the number of years of campaign. Hence, in the model almost twice as many badgers are culled under an R2T1 strategy than under an R1T5 strategy.

The inclusion of social perturbation reduces the effect of culling on the CHB rate, and may in some circumstances increase the CHB rate. Given the limited time in Phase I to perform these simulations only 'historic-trigger' P1T1 scenario with a higher level of control was simulated with social perturbation. This indicated that any benefit of culling is quickly removed by an increase in cattle herd breakdowns. Part of this increase is due to an edge effect – if the area of control were significantly larger then an overall benefit (in terms of reduced CHBs) may occur. This prediction has been supported by the recent results of the RBCT trial (Donnelly *et al* 2005).

Figure 1 shows the cumulative costs compared to benefits over time of an 80% effective R1T1 trapping policy. This also demonstrates that the majority of the costs are borne in the early years, while the benefits accumulate slowly in the early years. The mean time for this policy to breakeven is 19 years. If the costs can be reduced by 50% then the mean breakeven time is 8 years. However, if the costs are 50% greater, or the benefits 50% less then this strategy does not breakeven within 30 years.

The overall cumulative discounted costs and benefits of each 'immediate trigger' trapping strategy are given in Table 3 and Table 4 for background prevalence in the badger of 5% and 20% respectively. This demonstrates that the NPV is only positive when the total effort is lowest (the R1T1 strategies). When the NPV is positive, a greater net benefit occurs when the prevalence of TB in the badger is higher (20% c.f. 5%). It should also be noted that in simulations where TB was eradicated from the badger population (eg R3T5, R3T3) the NPV is highly negative. The plus and minus 50% costs and benefits were calculated, and resulted in more strategies giving an overall economic benefit, particularly where there was a higher prevalence of TB in the badger.

All these control strategies were simulated assuming an 80% efficacy of control. If culling efficacy were reduced slightly it is likely that all trap and shoot strategies would give an overall negative NPV. The effect of less than full compliance by landowners, and the potential of social perturbation to affect these results also need to be assessed.

For a comparison of these 'immediate trigger' trapping strategies with 'historic-trigger' strategies see Table 5. No strategies simulated gave an economic benefit for trapping. However, gassing gave a positive NPV where there was a high prevalence of TB in the badger. The P1T1 policy reached an economic breakeven point in year 11, and the P1T3 policy in year 14. If the costs were reduced by 50% and the benefits increased by 50% then all but one strategy resulted in a positive NPV in this ideal world. The results show that the overall cost is slightly less when the badger TB prevalence is higher. This is a reverse of the 'immediate-trigger' simulations. Again further work is required to determine the effect of less than full compliance by landowners, and the effect of badger social perturbation.

Conclusions

Phase I successfully integrated the simulation model of badger TB, with cost-benefit analysis to produce a 'TB CBA' meta-model. This approach did not permit uncertainty and variability to be investigated, but concluded that trapping and shooting of badgers, as carried out using the methodology of the RBCT, is not an economically viable option in response to each herd breakdown, or when used in a more proactive manner, even in the absence of social perturbation. In the absence of social perturbation, gassing as a means of badger control only appears to be economic when highly effective in high prevalence areas.

In Phase II, the cattle layer is nearing completion and the cost-benefit economics has been included within the simulation model. Social perturbation functions have been improved from the Phase I analysis and initial analysis should be performed soon.

Future research

Many of the recommendations in the Phase I final report are currently being performed within Phase II. With sufficient access to the RBCT data the revised models should be able to predict the consequences of badger and cattle management decisions. The reliability of these predictions could be improved with further field data (which parameters can be improved cannot be ascertained at this stage). This model could also be used to predict the effect of different vaccines and different vaccination strategies.

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Publications generated from the project

Smith GC, Bennett R, Wilkinson D & Cooke R (2006) A cost-benefit analysis of culling badgers to control bovine tuberculosis. *The Veterinary Journal*, doi:10.1016/j.tvjl.2005.11.017.

Review comment

This is an ambitious project that aims to combine the badger/cattle model with the economic model to provide a useful and objective tool for supporting policy decisions. The model is well worth further development (through phase II of the project) so that its decision support functionality and value can be fully realised. The models have been developed with considerable thought given to the complexities intrinsic in multi-species disease transmission systems. Efforts have been made to investigate the sensitivity of model predictions – which will be very useful at both identifying the key parameters and highlighting the parameters that if estimated better would most reduce prediction uncertainty. However, a relative weakness of the project, as reported for Phase 1, is the treatment of the cost-benefit analysis. Benefit is defined as “the value of the reduction in herd breakdowns accrued over the duration of the project” or “the number of Cattle Herd Breakdowns” from the CSL model. But the discussion focuses on costs. The benefit components need more explicit elaboration, e.g. potential for reduced losses of cattle products, reduced losses in livestock as a capital resource, and thus savings in associated inputs. There were some concerns about the degree of “integration” of the economic and badger/cattle models. It is however, a worthwhile project in itself to simulate the distribution of economic outcomes associated with specified control actions.

Scores

Conclusions based on sound evidence: 2.0
Quality of science: 2.5
Overall rating: 2.3

Table 1 Summarised costs for trapping and gassing.

Salary cost per field worker/week[a]	£554
Total costs per team of 2 field workers/week[b]	£2,381
Initial capital outlay	£88,425
Initial survey cost/km^2	£1,358
Repeat survey cost/km^2	£540
Total cost of trapping/km^2	£2,163
Total cost of gassing/km^2	£1,031

[a]This includes the costs for all support and management staff.
[b]This includes travel, overheads and personal equipment.

Table 2 Total number of badgers culled per km^2 for the different "immediate trigger" control strategies over the first 20 years of culling using a cull rate of 80%.

Strategy	Badgers culled per km^2 (5% badger-TB prevalence)	Badgers culled per km^2 (20% badger-TB prevalence)
R1T1	0.37	1.30
R1T3	0.61	2.07
R1T5	0.84	2.68
R2T1	1.60	4.37
R2T3	2.30	5.41
R2T5	3.03	6.20
R3T1	3.06	6.30
R3T3	4.02	6.34
R3T5	4.99	6.63

Fig 1 The cumulative discounted costs and benefits over time of the R1T1 trapping and shooting badger-culling strategy. The cull is performed on a single badger social group once only, during the year after infection of cattle occurs. For both the costs and benefits the limit of plus and minus 50% are also presented.

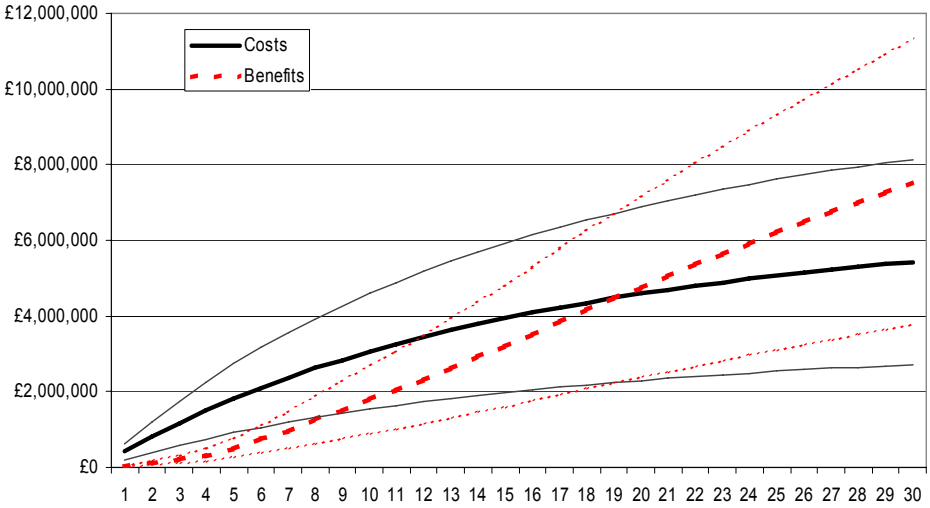


Table 3 The discounted costs and benefits, and the overall Net Present Value, associated with each 'immediate trigger trapping strategy (see Table 2) for a prevalence of 5% in the badger population and an 80% badger cull.

Strategy	Cost: Discounted PV over 30 years (£ million)	Benefit: Discounted PV over 30 years (£ million)	**Mean NPV (£ million)**	Cost per badger culled (£)	Cost /badger needed for positive NPV (£)
R1 T1[2]	5.4	7.6	**+2.1**	572	797[1]
R2 T1[2]	24.0	12.0	**-12.0**	671	335
R3 T1	49.4	14.7	**-34.7**	753	224
R1 T3[2]	10.6	9.2	**-1.4**	723	626
R2 T3[2]	42.2	14.6	**-27.6**	881	304
R3 T3	83.4	16.9	**-66.5**	1029	208
R1 T5[2]	15.0	9.1	**-5.9**	767	465
R2 T5	58.9	15.0	**-43.8**	946	242
R3 T5	119.6	18.0	**-101.6**	1209	182
Gassing R1T1[2]	3.5	7.6	**+4.0**	377	796

[1]If the NPV is positive then this figure is the maximum cost per badger for a positive net benefit.
[2]These strategies all return a positive NPV is the costs are reduced by 50% *and* the benefits are increased by 50%.

Table 4 The discounted costs and benefits, and the overall Net Present Value, associated with each 'immediate trigger trapping strategy (see Table 2) for a prevalence of 20% in the badger population and an 80% badger cull.

Strategy	Cost: Discounted PV over 30 years (£ million)	Benefit: Discounted PV over 30 years (£ million)	**Mean NPV (£ million)**	Cost per badger culled (£)	Cost /badger needed for positive NPV (£)
R1 T1[2]	19.5	22.7	**+3.1**	576	635
R2 T1[2]	71.9	50.4	**-21.5**	727	509
R3 T1[2]	119.7	58.6	**-61.1**	891	436
R1 T3[2]	36.5	34.8	**-1.7**	727	692
R2 T3[2]	110.7	60.0	**-50.7**	984	533
R3 T3[2]	174.4	65.5	**-108.9**	1380	518
R1 T5[2]	48.9	40.9	**-8.0**	780	651
R2 T5[2]	142.7	60.4	**-82.3**	1143	483
R3 T5	232.6	65.5	**-167.0**	1794	505
Gassing R1T1[2]	12.9	22.7	**+9.7**	381	667

[1]If the NPV is positive then this figure is the maximum cost per badger for a positive net benefit.
[2]These strategies all return a positive NPV is the costs are reduced by 50% *and* the benefits are increased by 50%.

Table 5 The discounted costs and benefits, and the overall Net Present Value, associated with different trapping and gassing strategies (see Tables 1 and 6) for a prevalence of 5% in the badger population.

Strategy	Cost: Discounted PV over 30 years (£ million)	Benefit: Discounted PV over 30 years (£ million)	**Mean NPV (£ million)**	Cost per badger culled (£)	Cost /badger needed for positive NPV (£)
Trapping					
80% culling rate, 5% prevalence					
P1T1[2]	13.5	7.2	**-6.3**	715	382
P1T3[2]	31.8	12.4	**-19.3**	877	343
P1T5	49.8	12.3	**-37.6**	974	239
80% culling rate, 20% prevalence					
P1T1[2]	12.4	11.4	**-1.0**	701	644
P1T3[2]	30.5	25.5	**-5.0**	851	711
P1T5[2]	46.4	35.6	**-10.7**	938	740
Gassing					
90% culling rate, 5% prevalence					
P1T1[2]	9.4	7.9	**-1.4**	439	322
P1T3[2]	20.6	13.9	**-6.6**	531	359
P1T5[2]	31.8	14.7	**-17.0**	582	270
95% culling rate, 5% prevalence					
P1T1[2]	9.3	9.7	**+0.4**	417	436[1]
P1T3[2]	21.0	14.0	**-7.0**	513	341
P1T5[2]	31.2	14.5	**-16.7**	570	265
95% culling rate, 20% prevalence					
P1T1[2]	8.6	17.3	**+8.7**	408	818
P1T3[2]	19.4	33.2	**+13.8**	495	846

[1]If the NPV is positive then this figure is the maximum cost per badger for a positive net benefit.

[2]These strategies all return a positive NPV is the costs are reduced by 50% *and* the benefits are increased by 50%.

SE3026 Bovine TB transmission in restocked herds: risk factors and dynamics

Organisation Department of Biological Sciences
University of Warwick

Start date 01/06/2002
End date 31/03/2006

Total cost £1.1m

Abstract
The FMD epidemic perturbed bTB transmission dynamics in that testing was temporarily reduced, cattle movement was reduced and new herds were formed through restocking. We have used this perturbation to test the hypothesis that cattle to cattle transmission of bTB occurs, and collected and assimilated data to quantify this effect. We have undertaken a study of bTB in GB cattle using 1) a cohort study of 148 herds in the south west of England where we collected prospective and retrospective data, 2) a population study of restocked herds using data from FMD VetNet and BCMS databases and 3) data-driven mathematical models of bTB transmission dynamics. The key results are that there are 1) risks of introduction of bTB associated with cattle sourced from herds with a history of bTB, 2) persistence that can be attributed to between cattle transmission and 3) a stationary farm risk, present when cattle are removed from the farm.

Aims
- To identify risk factors associated with the introduction of and transmission of bTB in restocked farms following the recent foot and mouth epidemic and associated culling.
- Study 1: to use a cohort study to investigate the survival time to bTB breakdown between restocked and non-restocked herds and the cattle and environment risk factors that increases the risk of bTB breakdown. The cohort was selected from farms within the RBCT study areas to test for interaction with impact of badger control.
- Study 2: to conduct a population study on restocked farms in England and Wales. [Note - this was originally proposed as a case control study].

Relevance to Defra
Policy should be underpinned by a breadth of science. Much previous scientific investigation of bTB has been focussed on potential reservoir hosts or small-scale experimental studies. This study is the most recent large independent field epidemiology study to investigate the role of cattle in the transmission and persistence of bTB, and complements the other disciplines and current research used to improve understanding of bTB.

The overall outcome will be a greatly enhanced quantitative understanding of bTB epidemiology and transmission dynamics, and the development of tools to be used in design of cost-effective control strategies. The combination of field and theoretical work within the same project helps to fulfill the aim highlighted by Krebs that “there

should be a better co-ordination of modelling and data collection to ensure that the appropriate data are collected and that best use is made of them in analyses" [1].

Methods

Study 1: Cohort

Approximately 450 farmers located within the RBCT were contacted. From these 150 farmers were recruited. Approximately 25% had stock restocked after FMD (exposed) and 75% had stock restricted during FMD (unexposed). The exposed and unexposed farms were non- contiguous but were matched within triplets of the RBCT. Over 90% of farms remained in the study for the full 3 years (Annex 1).

Each farm was visited on at least 3 occasions. A sample of blood was collected from all adult cattle at each visit. The farmer was interviewed once using a detailed questionnaire and all buildings were surveyed once. Cattle movements were obtained from cattle passports on the farm and from the BCMS electronic records of cattle movements. 30,000 serums are stored in a walk-in freezer at the University of Warwick. These are currently being analysed with funding from BBSRC.

The analyses of farmer purchasing practices, farm management, presence of other potential bTB reservoir hosts, RBCT triplet and cattle movements take into account time and repeated measures.

Study 2: Population Study

Initially this was proposed as a case control study of restocked vs non-restocked herds. However, we identified approximately 90% of all restocked cattle farms (using the FMD databases) and a population study, investigating bTB herd breakdown among restocked farms was possible. BCMS data and VetNet data were used to establish movements and locations and history of bTB before FMD.

Two key analytical approaches were the risk of herds breaking down with bTB after FMD and the risk of cattle within herds reacting to the bTB skin test.

Quantitative Models

A simple farm-level mathematical framework was developed which included the process of diagnosis, clearance and restriction of infected farms. The model was used to investigate the interactions between testing (surveillance) effort and control.
This model has been expanded to include within farm transmission, and compared to the observed distribution of number of reactors at the first test after FMD on bTB clear herds that were not culled during FMD.

The results indicated that testing frequency is critical to both surveillance and control of bTB, and consequently a study was undertaken in collaboration with VLA to investigate exposure of cattle to bTB testing by age, sex and location.

The models continue to be developed – in particular the development of a framework that includes both within farm transmission and testing programme design.

Results

Study 1

These data are being analysed in phases, which will ultimately be combined. The association between herd breakdown and farm management practices alone are: purchasing steers, purchasing cattle from markets (vs. private sales), herd size, storage of manure in enclosed spaces, not feeding supplementary minerals and vitamins. There was no statistical association between farmer reporting of presence of and direct contact with badgers, deer or cats (Annex 2).

Analysis of cattle movements between farms (including bTB history of source farms), cattle grouping within farms and impact of RBCT triplet are on-going.

Study 2

Farm-level risks for breakdown in restocked herds included both a risk from purchasing cattle from farms that previously had bTB and a risk from the restocked farm having a history of bTB. The increased risk of HBD decays exponentially with time since last HBD (Annex 3).

Animal-level risks for positive reaction to bTB skin test include coming from a herd with history of bTB, increasing age and not having been tested before being on the restocked farm.

Quantitative Models

The success of the bTB control programme is dependent on the frequency of testing, and not on the prevalence of infection. Reducing the frequency of testing results in increased prevalence but reduced ability to detect it. Increasing testing decreases prevalence and increases detection. Ability to control infection is constrained by the sensitivity of the test, the effectiveness of clearance of infected herds and the effectiveness of movement restrictions [2]. See Annex 4 for example figure showing the expected trends in numbers of infections and disclosed infections (restricted herds) as a consequence of changing testing interval.

The majority (>80%) of cattle are not tested during their lifetime. The majority of the testing effort is concentrated in a small minority of herds [3]. See Annex 5 for table of numbers of animals tested from a birth cohort.

From the combined VetNet / BCMS data, 7014 herds were identified that had been tested within 3m prior to FMD, and were unrestricted at the start of FMD. The distribution of numbers of reactors at the first test post-FMD is highly skewed. Although there are a number of different explanations for this pattern, it is consistent with the pattern expected if the risk of infection per animal is dependent on the number of other animals infected. Further, the degree of heterogeneity is challenging to explanations other than that infection is transmitted between cattle (Annex 6).

Conclusions

The results of this study to date indicate that:

1. There is an associated risk of HBD from the purchase of cattle from herds with a history of bTB;
2. There is an associated (stationary) risk of HBD from persistence of bTB on farm, outside of cattle, and this risk decays with time since HBD;

3. The distribution of numbers of reactors after a period without testing is highly suggestive of on-farm transmission between cattle;
4. Farm management practices associated with the risk of HBD included purchasing practices, management of manure and diet; and
5. The frequency of testing for bTB determines the success of both control and surveillance. We hypothesise that increasing testing coverage / effort will result in disclosure of more infection and greater control.

Future research

The development of strategic models that can be used to determine the risk of individual herds harbouring infection based on cattle movements and farm bTB history.

References cited in text

[1] Bovine Tuberculosis in Cattle and Badgers. Report by the Independent Scientific Review Group (Chairman: Professor J Krebs FRS). MAFF Publications, 1997.
[2] Medley, G.F. (2003) The design of test and clearance programmes. SVEPM, April 2003.
[3] Mitchell, A. Green, L.E., Clifton-Hadley, R., Mawdsley, J., Sayers, R.& Medley, G.F. An analysis of intradermal comparative cervical test (SICCT) coverage in the GB cattle population. SVEPM, April 2006.

Publications generated from the project

Medley, G.F. (2003) The design of test and clearance programmes. SVEPM, April 2003.
Mitchell, A. Green, L.E., Clifton-Hadley, R., Mawdsley, J., Sayers, R.& Medley, G.F. An analysis of intradermal comparative cervical test (SICCT) coverage in the GB cattle population. SVEPM, April 2006.
Carrique-Mas, J., Medley, G.F. & Green, L.E. Risk of bovine tuberculosis breakdowns in post-foot-and-mouth disease restocked cattle herds in Great Britain. SVEPM, April 2005.
Green, L. (2005) Modelling disease – a guide for the practitioner. *Cattle Practice* **13**, 243-248.
J. Carrique-Mas and A. Ramirez have presented their work orally and on posters at a number of different conferences.

Review Comment

The project was well conceived and complementary to other studies on establishing the relevance of cattle to cattle spread. The final report confirmed the risk of a herd TB breakdown as a result of cattle purchase, and more specifically in the context of FMD restocking. As a result of FMD, periods without testing appear to result in reactor patterns indicative of within-herd spread. Biologically this is sensible and supports the appropriate testing intervals in high risk areas. Increased testing frequency should increase the sensitivity of the surveillance system, but it is questionable whether this will be cost-effective. However, there are various potential sources of selection bias in this study; one of them being the 148 herds included in the cohort study, as they are too small a sample to be representative of either post-FMD restocked or continuously stocked herds. At the herd level this results in limited statistical power. The animal-level analysis has higher statistical power, but is affected by lack of independence problems. The use of multi-level models does

satisfy the statistical requirements but not the biological issues. Overall, the study provided some support for new policy initiatives such as the need to control the movement of cattle and the need to review the frequency of herd testing, however, the need for these initiatives was already clear. The study's overall goals were "a greatly enhanced quantitative understanding of TB epidemiology and transmission dynamics, and the development of tools to be used in design and cost-effective control strategies", disappointingly, the study did not achieve this and as a result the potential was lost and little new information was generated.

Scores
Conclusions based on sound evidence: 2.5
Quality of science: 2.4
Overall rating: 2.4

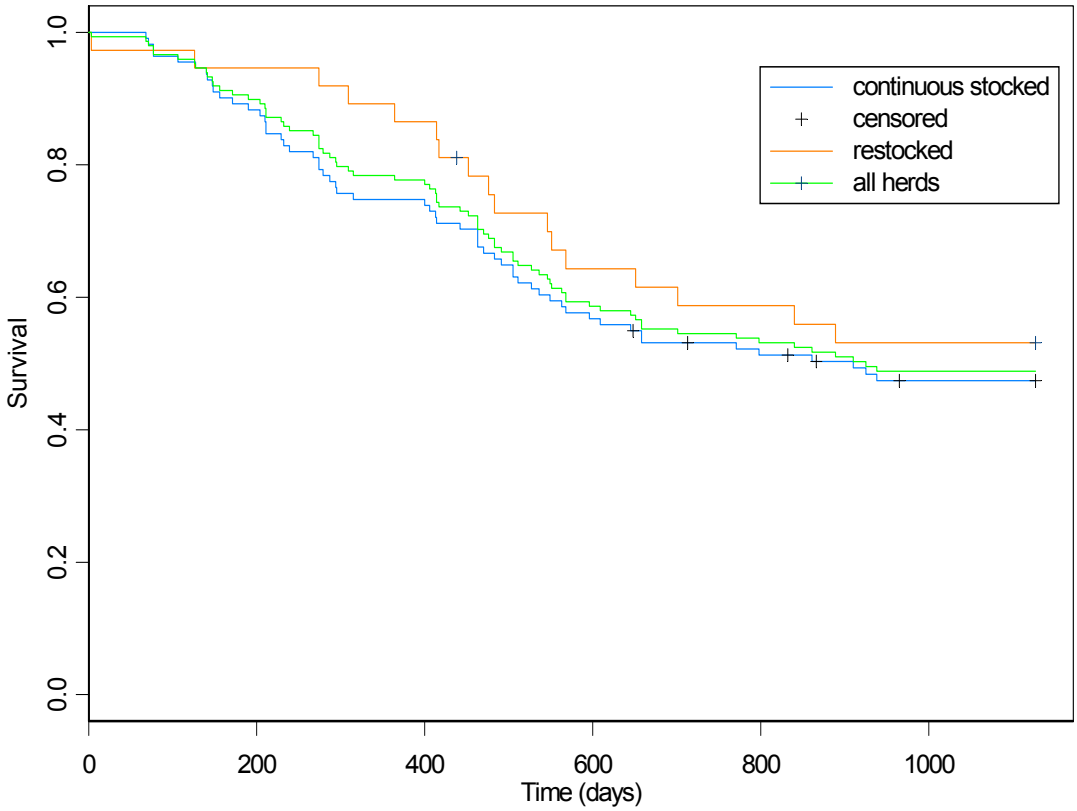
Annex. Interim summary data for SE3026

Below are the analyses completed on aspects of the data and the final results may differ.

1. Number of unrestricted cohort farms that had broken down at least once by November 2004.

A total of 75 (50.6%) of the 148 herds had had at least one HBD by November 2004. There were 59/112= 52.6% of the continuously stocked herds and 16/36= 44.4% of the restocked herds with at least one HBD. There were 56 confirmed HBD; 52 were culture positive. A total of forty herds have had at least two HBD in this time.

Fig 1 Kaplan – Meier survival curve for restocking status after FMD



2. Cox proportional regression multivariable model of management factors associated with HBD in 148 cattle farms.

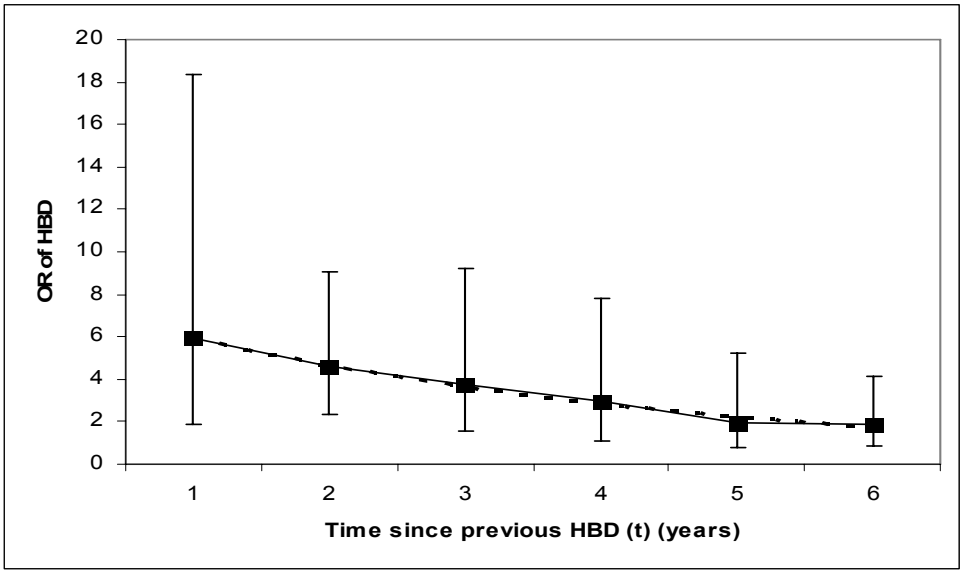
Variable	n	HR	Coef.	se	P	95% CI
Restocked after FMD	36	0.92	-0.09	0.34	0.8	0.47, 1.79
log number herd tests prior to first HBD	148	2.33	0.85	0.3	0.005	1.30, 4.20
Herd has dairy cattle	57	1.86	0.62	0.31	0.042	1.02, 3.39
log herd size	148	1.38	0.32	0.17	0.062	0.98, 1.93
Manure stored in enclosed space	83	1.98	0.69	0.26	0.009	1.19, 3.31
minerals lick and vitamins provided	72	0.42	-0.87	0.26	0.001	0.25, 0.69
Big bale wheat straw used on farm	22	1.56	0.45	0.33	0.17	0.82, 2.98
Bulls hired in	40	0.48	-0.73	0.29	0.014	0.27, 0.86
Farmer stated abortions occurred in cattle	16	1.97	0.68	0.37	0.064	0.96, 4.02
Purchase cattle from market	65	1.77	0.57	0.25	0.022	1.08, 2.88
Purchase steers	40	1.96	0.68	0.32	0.033	1.05, 3.65

3. Multivariable logistic regression analyses of associated risks with herd breakdown with bTB in GB from 2002 – 2004 in 2941 restocked herds.

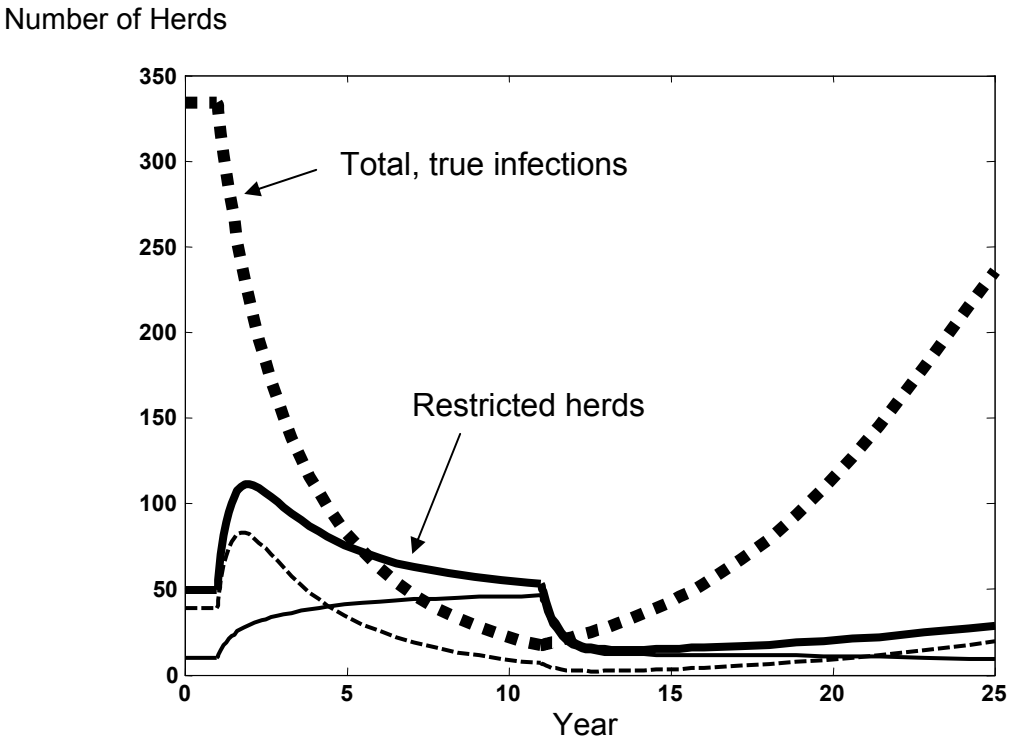
Explanatory variable	Number of herds	Coefficient	s.e.	OR	Lower 95% CI	Upper 95% CI
before June 2001	179	-4.652	0.484			
July - Dec 2001	1559	-0.106	0.303	0.899	0.491	1.649
Jan - June 2002	970	-0.57	0.34	0.566	0.287	1.116
July - Dec 2002	170	-0.264	0.527	0.768	0.268	2.203
After Dec 2002	63	-0.907	1.066	0.404	0.048	3.404
Log number of cattle sourced from high frequency testing area never had HBD with bTB		0.299	0.049	1.349	1.223	1.487
Last HBD in 2000		1.779	0.565	5.924	1.914	18.338
Last HBD in 1999		1.532	0.336	4.627	2.363	9.061
Last HBD in 1998		1.327	0.449	3.770	1.536	9.253
Last HBD in 1997		1.079	0.486	2.942	1.113	7.776
Last HBD in 1996		0.681	0.487	1.976	0.746	5.233
Last HBD in 1995		0.617	0.402	1.853	0.829	4.141
Log number of cattle tested		0.323	0.086	1.381	1.163	1.640
proportion of herd over 3 years of age at purchase		0.009	0.003	1.009	1.003	1.015

OR = odds ratio, CI = confidence intervals, s.e. = standard error.

The decay of risk of breakdown with time since previous breakdown (solid line) and confidence 95% intervals. Dashed line is fitted exponential decay (=$7.6e^{-0.25t}$).



4. The expected (model) outcome in numbers of herds infected and restricted as a result of changing testing frequency (from Medley, SVEPM 2003).



Explanation: Model is started at equilibrium with testing interval every 3 years in a population of 1000 herds: approximately 1/3 are infected (heavy dotted line), 50 are restricted (heavy solid line) of which 45 are true positive restrictions (dashed line) and 5 are false positives (solid line). At 1 year, the testing interval is reduced to annual, with a consequent increase in the number of diagnoses and greatly improved infection control. At 11 years, the testing frequency is reduced to every 4 years, with a consequent reduction in number of restrictions and control effectiveness.

5. Frequency Distributions of Total Number of Tests per Animal per Premise for a cohort of 96,862 animals born in 2000 (from Mitchell *et al* SVEPM, 2006).

No. Tests	*Number of locations on which cattle were tested*[1]					*Number of Animals*	*% of Animals*
	0	*1*	*2*	*3*	*4*	*Total*	
0	82682					82682	85.4
1		7424	0	0	0	7424	7.7
2		2477	529	0	0	3006	3.1
3		1239	263	19	0	1521	1.6
4		733	168	9	0	910	0.9
5+		1048	227	33	11	1319	1.4
Total	95603		1187	61	11	96862	

[1] Cattle locations are defined as County-Parish-Holding-Herd (CPHH) identifier.

6. Frequency Distribution of Number of Reactors in First post-FMD test among 7014 Herds "Uninfected" immediate prior to FMD.

Prevalence of Infection post-FMD	*All Herds*		*Herd Size <100*		*Herd Size ≥ 100*	
	Frequency	*%*	*Frequency*	*%*	*Frequency*	*%*
0	5781	82.4	3455	88.9	2326	74.4
>0, <0.1	1029	14.7	322	8.3	707	22.6
<0.2	121	1.7	58	1.5	63	2.0
<0.3	51	0.7	29	0.7	22	0.7
<0.4	10	0.1	7	0.2	3	0.1
<0.5	7	0.1	4	0.1	3	0.1
<0.6	7	0.1	5	0.1	2	0.1
<0.7	2	0.0	2	0.1	0	0.0
<0.8	1	0.0	0	0.0	1	0.0
<0.9	1	0.0	1	0.0	0	0.0
<1.0	0	0.0	0	0.0	0	0.0
1	4	0.1	4	0.1	0	0.0
Total	7014		3887		3127	

Herd-level infection prevalence in the first test post-FMD for all herds was 15% (1233/7014), whereas animal-level prevalence was 1.1% (9255 / 821216), and in four herds, all tested animals were positive. Smaller herds (<100 cattle) were less likely to have a HBD, but tended to have more reactors on HBD.

SE3112 Assessment of the economic impacts of TB and alternative control policies

Organisation Department of Agricultural and Food Economics
The University of Reading

Start date 01/05/01
End date 31/01/04

Total cost £156,959

Abstract
The economic assessment of bTB and its control at farm level involved (i) a survey of TB breakdown farms to estimate the costs associated with a TB breakdown and attitudes to TB policies (ii) Break-even analyses of the costs and benefits of alternative TB control strategies (iii) Exploration of the impact of a farmer levy to cover TB compensation payments. The research found a wide variation in farm breakdown costs ranging from £229 per case to £103,817 with the variation largely due to the number of reactors per breakdown. After compensation payments, an estimated 79% of dairy farms and 65% of beef farms experienced a net loss. Seven alternative TB control policies were assessed using break-even analysis (the reduction in incidence of TB in cattle which would result in a net benefit of control). Results show the 'proactive badger culling' strategy to be most costly requiring a large reduction in TB incidence to be worthwhile, followed by cattle vaccination, then reactive badger culling and badger vaccination, then zoning, with 'no routine TB testing' potentially the least cost. The strategies considered represent examples of possible strategies. More analyses of these and/or of other policies could be undertaken with exploration of different specifications and assumptions. Levies on milk sales and slaughtered cattle to fund TB compensation payments would be relatively high if only farms in TB 'hotspot' areas are included (especially for beef producers) but a levy at national level would have relatively little regional or national impact.

Aims
- Identify relevant negative effects of TB at farm level and for the UK economy, together with relevant costs and benefits associated with selected control strategies.
- Establish a database of relevant qualitative and quantitative information necessary for an economic assessment of TB and alternative control policies at farm and national levels.
- Develop a framework within which relevant costs and benefits can be considered.

Relevance to Defra
This research has high policy relevance for Defra providing data and a framework for cost-benefit assessment of alternative TB control policies.

Methods

1. Farm survey of 151 cattle farms involving visits to farms by trained investigational officers.
2. Literature review and consultation on possible alternative TB control strategies.
3. Construction of a farm level model to model the costs associated with a TB breakdown.
4. Economic assessment using spreadsheet model and break-even analysis of the costs and benefits (in terms of reduced TB incidence in cattle) of alternative policies at national level.
5. Analysis of impact of TB levy using spreadsheet model and Reading Land-Use Allocation Model.

Results

1. The main farm-level costs of a breakdown were identified as slaughter costs (the largest cost element) and costs associated with testing, isolation, and movement restrictions. The survey found a large variation in the costs of a breakdown ranging from £229 per farm to £103,817 per farm depending on the number of reactors and their valuation, the length of the breakdown etc. In only 10% of cases was the cost of a breakdown above £18,513 for dairy and £11,462 for beef farms. After compensation payments have been taken into account, 20% of dairy farms and 35% of beef farms appear to have a net gain associated with their breakdown. In a few cases these net gains appear to be substantial (mostly 'pedigree' herds). However, 79% of dairy farms and 65% of beef farms experienced an estimated net loss (ranging from £37 to £17,021).

2. Seven alternative TB control policy scenarios were chosen for assessment: (i) the current 'test and slaughter' policy (ii) no on-farm routine TB testing (iii) zoning (cattle moving from TB hotspot areas must be tested) (iv) reactive badger culling (culling of badgers on/near TB breakdown farms) (v) proactive badger culling (culling of badgers in TB hotspot areas) (vi) cattle vaccination and (vii) badger vaccination. Costs associated with the current 'test and slaughter' TB control strategy are estimated. Given the lack of information to estimate the benefits associated with each control strategy, the levels of disease reduction (in cattle) necessary to cover the costs associated with implementing each strategy have been estimated. Results show the 'proactive badger culling' strategy to be most costly, followed by cattle vaccination, then reactive badger culling and badger vaccination, then zoning, with 'no routine TB testing' potentially the least cost. Choice of preferred strategy will depend on the likely impact on the incidence of TB in cattle together with other considerations (such as the preferences of society in terms of concerns about badger culling etc).

3. The likely impact on cattle farmers of farmers paying for testing under the current programme and for farmers paying a levy to cover TB compensation payments was explored. Testing costs average around £3.51 per animal per year in TB hotspot (annual testing) areas. Levies on milk sales and finished slaughter cattle are estimated at £0.005 per litre and £39.36 per head respectively if only cattle farms in hotspot areas pay the levy in England (£0.0055 per litre and £63.80 per head respectively for Wales) compared to levies of £0.00187 per litre and £7.21 per head respectively if applied to all milk and finished cattle output in England and Wales. These levies are what would be required to fund the 2002 TB

compensation payments. The levy for beef producers in hotspot areas could, in some cases, outweigh current gross margins of those fattening cattle for slaughter. Using a simple beef equilibrium model, a levy of £7.21 per finished animal in England and Wales (around 1.4% of average finished cattle price) is estimated to result in a drop of around 0.6% in UK beef output, 0.5% increase in the beef price and a fall of 0.4% in beef consumption. Regional impacts on agriculture of farmers paying testing costs and of a levy in TB hotspot areas were found to be low with no significant change in dairy cow numbers and only 1% fall in beef cattle numbers (with a corresponding increase in sheep numbers).

Conclusions
The main conclusions and recommendations of the report are that: (i) The survey of farm-level costs found that most producers suffered a net loss from a TB breakdown even after compensation but that a few producers appeared to be getting a substantial net gain from compensation. (ii) Proactive badger culling is costly and would require a large reduction in the incidence of TB in cattle to have a positive net benefit. The strategies considered represent examples of possible strategies. More analyses of these and/or of other policies could be undertaken with exploration of different specifications and assumptions. (iii) There is a clear need for more information and research on the benefits of different TB control strategies and a review of research should be undertaken. (iv) If a levy on cattle production to fund TB compensation payments only included TB 'hotspot' areas, then the cost, especially to those fattening beef cattle for slaughter, may be relatively high for producers in those areas. A levy at national level would have less regional impact.

Future research
Economic appraisal (cost-benefit assessment and break-even analysis) of further defined alternative TB control strategies.

Stakeholder preference survey of alternative TB control strategies.

Publications generated from the project
Bennett, R. and Cooke, R. 92005) Control of bovine TB: preferences of farmers who have suffered a TB breakdown. *The Veterinary Record,* **156**, 143-145.
Bennett, R. M. and Cooke, R. J. (2006) Costs to farmers of a tuberculosis breakdown. *The Veterinary Record*, **158** (in press).

Review comment
The project aimed to fill significant gaps in understanding the costs and benefits of bTB and approaches to its control across a broad spectrum. In practice, attention focused on farm level effects and possible control strategies. The authors were careful to draw attention to the limitations of their research outcomes but this does not undermine the substantive worth of the work. A case for further modelling of farm situations is persuasively made. This was a good example of how established economics research methods can be applied to an animal disease problem to provide crucial information to enable formal policy evaluation e.g. the farm survey has been extremely useful, it was well-conducted and remains the primary source for this information. The breakeven assessment of control methods is much-needed and provides some useful insights. Although this was a stride forward, it does not provide a comprehensive framework for economic assessment of control policies. The gaps

in knowledge and data are now more easily discernable and future work will help provide an economic framework for assessment of control policies. In particular there is a need for more information on the benefits of different TB control strategies, their implications for human health, and trade, as well as livestock production. If these claims are reinforced by further evidence, it follows that the main beneficiaries from bovine TB control are cattle farmers and that under present circumstances bTB is not a significant threat to human health or trade.

Scores
Conclusions based on sound evidence: 3.1
Quality of science: 2.9
Overall rating: 3.1

SE3116 The economic value of changes in badger populations

Organisation Department of Agricultural and Food Economics
The University of Reading

Start date 01/06/03
End date 30/11/04

Total cost £75,330

Abstract
The aim of the research was to derive an implicit valuation for the costs and benefits associated with changes in badger populations in specific locations affected by bTB and alternative control policies. A stated preference 'choice experiment' survey approach, involving four attributes (size of badger population, cattle slaughtered due to bTB, badger management strategy and household tax) at four levels with eight choice sets of two alternatives presented to respondents, was used to elicit people's attitudes and values concerning badger populations and bTB in cattle. This involved a telephone survey of over 400 citizens randomly chosen in England and Wales. Results show that although 92% of respondents agreed that controlling bTB in cattle is important, 38% thought that it should be controlled by management of badger populations and 36% disagreed (the remainder neither agreed nor disagreed). The study estimates a willingness to pay (WTP) of £0.13 per household per year per 100,000 badgers (within population limits of 100,000 to 4000,000) and £1.73 per household per year per 10,000 cattle slaughtered due to bTB, which aggregates to £28 per badger and £3,750 per bovine slaughtered due to bTB respectively for all households in England and Wales. Management strategy toward badgers had a very high valuation, highlighting the importance of government policy towards badgers for respondents.

Aims
- Undertake a literature and information review.
- Identify the important valuation characteristics and contexts associated with changes in badger populations.
- Design a valuation method to elicit appropriate valuations across the range of characteristics and contexts.
- Undertake 'focus groups' to explore people's underlying attitudes towards badgers, cattle farming and bTB.
- Design and undertake a 'nationally-sampled' (England and Wales) survey from which can be derived valuations of changes in badger populations.
- Provide an assessment of the validity, reliability and robustness of the valuation estimates derived.
- Provide appropriate 'final' valuations for incorporation into a cost-benefit framework for the economic assessment of bTB and alternative policies.

Relevance to Defra
Need to understand public attitudes, preferences and values concerning badgers and bTB for policy decisions concerning bTB control.

Methods

An Advisory Panel was set up to help steer the research project. A literature and information review of relevant aspects affecting badger populations in England and Wales was undertaken and consultations with scientists with a particular knowledge of badgers were carried out. A workshop was undertaken to assess valuation methodology and to identify and define the important valuation characteristics and contexts associated with the valuation of changes in badger populations. Three focus group discussions were held to better understand people's attitudes to badgers, bTB in cattle and badger culling and to test the survey questionnaire. A choice experiment questionnaire was designed, tested and piloted. In the choice experiment respondents were presented with sets of alternative combinations of attributes (the principle dimensions of the bTB and badger issue) and asked to choose their most preferred alternative. Repeated choices by respondents from sets of alternatives reveals the trade-offs respondents are willing to make between attributes. The attributes used in the choice experiment were: the size of the badger population; badger management policy; cattle slaughtered due to bTB; and price (increase in tax) to the respondent household with four levels of each attribute specified. A questionnaire was designed which (i) elicited factual information about respondents' experience of badgers (ii) elicited respondents' attitudes to badgers (iii) presented information to respondents about badgers, badger populations and bTB in cattle, followed by a series of eight choice card sets each comprising two alternatives from which they were asked to choose their most preferred alternative (iv) asked respondents to explain their choices (v) elicited respondents' attitudes to badger culling and bTB and (vi) ascertained socio-economic information on respondents. The questionnaire was administered in a combined mail and telephone survey of a stratified random sample of 401 citizens in England and Wales undertaken by a national market research company. Results of analyses were presented at a final workshop to critically appraise the research and its findings.

Results

Attitudes

Eighty-three per cent of respondents thought badgers to be an important wildlife species in Britain, 71% thought that the management of wildlife such as badgers is sometimes necessary, 54% thought that we should actively manage the badger population (22% disagreed) and 51% thought that there can be fewer badgers as long as they do not become an endangered species (27% disagreed). Ninety-two per cent of respondents agreed that controlling bTB in cattle is important, but 38% thought that bTB should be controlled by the management of badger populations and 36% disagreed. Seventy-three per cent of respondents objected to badgers being intentionally killed but 87% agreed that controlling badger populations is acceptable if it can be done without killing badgers.

Valuation estimates

A conditional logit (CL) model was used to estimate the relative values or willingness to pay (WTP) that respondents implicitly gave to changes in badger populations, cattle killed due to bTB and taxes based on the choices that they made from the paired alternative choices (see Table 1 below). This was the preferred model from a number of alternative model specifications. All attributes have the correct signs and are highly significant in the model and the model had a reasonable goodness of fit. Further analyses revealed a significant difference in valuations depending on

whether respondents were in bTB 'hotspot' areas or not. Estimates of people's WTP for England and Wales (adjusted for sample bias) were £0.13 per household per year per additional 100,000 badgers and £1.73 per household per year per 10,000 reduction in cattle slaughtered due to bTB. These estimates can be aggregated for all households in England and Wales to give valuations of £2.8 million per year per 100,000 badgers and £37.5 million per year per 10,000 cattle slaughtered. A number of criteria are used to show the validity of the estimation method and of the estimates derived. The coefficient for the 'policy' attribute in the model was very high (compared to those for either size of badger population or number of cattle slaughtered) suggesting that respondents placed great importance and a high value on the management strategy towards badgers (moving away from culling). However, the choice experiment survey was not designed to estimate WTP for different badger management strategies and so it was not possible to derive meaningful, reliable estimates of the value that people place on particular badger management policies.

Conclusions
The estimate of the value of changes in badger populations is considered a reasonable one to use within a cost-benefit framework (within the population limits included in the study), given the likely importance of this aspect and the fact that no alternative measure currently exists. However, it should be stressed that this value takes no account of society's preferences regarding badger management policies (ie whether badgers are culled and the method of culling used), which our research suggests is of great importance to people. The estimate of the value of cattle slaughtered due to bTB could be used as an alternative valuation to those based on the loss of production and costs of veterinary intervention.

Future research
Economic appraisal (cost-benefit assessment and break-even analysis) of further defined alternative TB control strategies.

Stakeholder preference survey of alternative TB control strategies.

Publications generated from the project
Paper under review in Journal of Agricultural Economics.

Review comment
Defra recognises that trade-offs between farming and wildlife interests are of great concern to a wide range of stakeholders. Projects such as this are therefore important to establish methods to bring these wider interests into the policy evaluation and development arena. The project addresses some of the considerable difficulties involved and makes some useful suggestions for further work. Thus this project provided a different and useful perspective on badgers and TB, and was a sound and useful project. The project utilised well established techniques from environmental economics and provided unique information and was excellent value for money. Interpretation of the empirical results is nevertheless challenging. For example, Appendix 2 notes that the total number of badgers in Great Britain is estimated at around 300,000. Thus estimates for England and Wales of a willingness to pay of only £0.13 per household per year per 100,000 badgers provokes obvious questions about the real strength of concern (i.e. valuation) society puts on doing the 'right' things by the badger population. Either this outcome is in

some sense a ‘true’ estimate, or else it may indicate methodological limitations in the analysis. It is a positive attribute of the project that its outcomes point to such important issues for further consideration. This project highlights the fact that measuring crucial externality values is a major research task, but demonstrates the possibility of doing so in a constructive and practical manner.

Scores
Conclusions based on sound evidence: 2.9
Quality of science: 2.8
Overall rating: 3.1

Table 1 Results for CL model.

Attribute	Coefficient	Std error	Pr > | t |
Badger	0.001257	0.000268	<0.0001
Policy	0.429100	0.027000	<0.0001
Cattle	-0.023300	0.002093	<0.0001
Price	-0.011800	0.000864	<0.0001

N=3216; Log likelihood = -1953; Likelihood Ratio = 552.12;
McFadden’s LRI = 0.1238; Veall-Zimmermann = 0.2522;

SE3036 A quantitative risk assessment on the role of wild deer in the perpetuation of TB in cattle

Organisation Central Science Laboratory
Woodchester Park

Start date 04/07/05
End date 31/10/06

Total cost £146,656

Abstract
Bovine TB is a serious disease in cattle in Britain. While the badger is considered the primary wildlife reservoir, recent research at CSL has identified four species of wild deer infected with the disease. Two of these (red and fallow deer) were qualified as posing a potentially high risk to cattle, based on the prevalence of the disease amongst carcasses sampled and the behavioural ecology of these species. This project aims to produce a quantified risk assessment of the role of wild deer in perpetuating TB in cattle. We are using a comparative approach, with risks posed by badgers as a benchmark against which to assess the role of deer. This is considered essential due to the large number of knowledge gaps and high degree of uncertainty surrounding transmission of TB from deer to cattle.

A thorough literature review will underpin the project and provide data for the risk model. Direct and indirect contact between cattle and deer and their excretory products is being measured in the field. Excretory products are alleged to provide a key source of TB transmission between badgers and cattle. In-depth histopathological examination of deer lymph nodes and lung lesions from CSL and VLA collections is being undertaken to assess the loads of bacilli and likelihood of shedding in each deer species. Similar data will be obtained for badgers. A sensitive PCR method will be used to assess true prevalence and species of mycobacterial infection since standard, routine methods have proved unreliable in the past. All these data will be used to populate the risk assessment model.

Knowledge gaps will be identified and prioritised for future research. Information from the project will improve our understanding of the likely role of deer in perpetuating TB in cattle and will thus aid Defra to further develop informed policy options for controlling TB in cattle.

Aims

The project aims to quantify the risk of TB transmission posed to cattle by five species of wild deer known to have been infected with bTB. The following core objectives will be addressed:

1. Empirical quantification of deer behaviour contributing to direct and indirect deer-cattle contact rates in space and time;
2. Qualification of the likelihood of *Mycobacterium bovis* excretion by deer and empirical estimation of the amount of bacilli that deer may excrete into the environment;
3. To determine the presence, prevalence, true species and genetic type of *Mycobacterium* in infected deer lymph nodes and lung lesion samples;
4. Development of a generic model to quantify the overall risk of *M. bovis* infection in cattle posed by each species of deer and badgers independently and together;
5. Quantification of the overall risk of *M. bovis* infection in cattle posed by each species of deer and badgers, independently and together, via each potential transmission route; and
6. Identification of knowledge gaps limiting the precision of risk assessment.

Relevance to Defra

Despite the potential disease risks to cattle posed by wild deer, information is currently lacking on aspects of pathology including routes of excretion, behavioural ecology and contact rates with cattle. However, quantification of the risks posed by each deer species during the current project will improve our understanding of the likely role of deer in perpetuating TB in cattle and will thus aid Defra to further develop informed policy options for controlling TB in cattle.

Methods

To address objective 1 direct contact between deer and cattle is being quantified in two ways. Firstly, pasture containing cattle is being monitored for deer activity by direct observation between 20.00 and 06.00 hours for one night on each of 30 farms in the west of England where roe deer and fallow deer are known to be present. The second method involved circulating data recording sheets to over 600 deer stalkers in the west and southwest of England to record their observations on deer on grazing lands. To estimate indirect contact rates between deer and cattle, dung from each species is being mapped in a single pasture on each of the 30 farms. Defecation rates for each species and dung decay rates are being applied to these to calculate the spatio-temporal overlap in pasture occupancy between deer and cattle.

Histological examination of 220 samples of deer tissues is being undertaken at the VLA, Weybridge to address objective 2. Pathology will be scored and bacterial loads estimated to assess the likelihood and loads of bacterial excretion into the environment by each deer species. The same data will be produced for samples of badger material in order to provide a direct comparison.

A sub-sample (n = c.200) of the material examined histologically is being examined by polymerase chain reaction and genotyping at University College London to address objective 3.

To address objective 4 three steps are being undertaken. Data on disease prevalence, loads and the pathology of TB in deer and badgers are being

summarised from the laboratory work detailed above and these are being augmented by similar data from the literature. Data on deer, badger and cattle distribution and abundance are being collated and evaluated to produce species abundance maps. Preliminary predictive models of prevalence and loads of *M. bovis* in each species of deer and badgers across the southwest of England are being constructed, with variability parameterised by species and locality. Uncertainty in the data are being represented by statistical distributions based on collated data ranges supplemented by expert opinion.

Preliminary models to estimate cattle exposure to infected deer and badgers and their excretory products will be constructed to model direct and indirect contact rates across the southwest of England. These models will be linked with the TB prevalence and load model and contact rate model and with species abundance maps to assess the risk posed by each species of deer and badgers to cattle. This will address objective 5.

To address objective 6 a simple list of assumptions and dummy values used during model construction will identify knowledge gaps. Input-output scatter plots, correlation statistics and other sensitivity analyses as appropriate will be used to assess the relative effects of uncertainties in the different model inputs on the key model outputs.

Results

A thorough literature search and review has been undertaken to underpin the project. This has taken account of international and unpublished literature, where available.

Surveys of direct and indirect contact rates between deer and cattle have been undertaken on 15 of the 30 farms. Preliminary observations revealed three incursions by roe deer onto cattle pasture, five incursions by fallow deer and three by muntjac. In contrast, fifteen observations were made on roe incursions onto other fields, more than 10 incursions by fallow deer and one by a muntjac. In all cases, deer avoided cattle and moved away from them in response to their investigation. The maximum time deer were observed spending at cattle pasture was 30 minutes by a group of five fallow deer.

Very few deer faeces were found at pasture.

Samples from 179 deer have been processed by the VLA for histopathological examination. Data sheets listing species, age class, bodyweight and notes on gross pathology have been collated for all of these.

The subcontract with University College London on TB genomics started in January 2006. Primers to deer mitochondrial DNA have been designed ready to assess recovery from samples stored in paraffin blocks and primers for the *M. bovis* and MOTT PCR studies have been ordered. Discussions are underway with the VLA to identify whether it is possible to extract samples from source material directly without the need to process into paraffin. However, it appears that some samples have been stored in formalin and it will be important to assess what damage this has done to the DNA.

Conceptual models have been constructed following the literature review and lengthy discussions between CSL and VLA staff.

Conclusions
The literature suggests that, in other countries, wild deer may pose a risk to cattle when they achieve unusually high densities, for example, resulting from winter supplementary feeding. At very high densities, deer may suffer a high prevalence of disease and make more contacts with cattle. Observations on deer at cattle pasture and mapping of their dung suggests that direct and indirect contact between deer and cattle are likely to be rare events throughout the west of England. However, we cannot, at this stage, rule out the possibility of particular problem locations existing in areas of high deer densities.

It is too early to conclude anything from the histopathology and genomics work.

Future research
Empirical work undertaken to support the risk model during this project uses fairly course tools. In order to improve on the likely high degrees of uncertainty and variability within the data used, improved studies on TB pathology in deer, to determine routes and loads of bacterial excretion for each species and improved diagnostic tests, to obtain a more accurate estimate of disease prevalence would be required. In addition, more reliable data on deer abundance and distribution and more detailed and wide ranging studies on deer behavioural ecology and contact rates with cattle would contribute to the construction of more reliable models.

Publications generated from the project
Pending.

Review comment
A potentially useful but ambitious project which should provide detailed data on the epidemiology of bTB in several species of wild deer, and on the interactions and possible contacts between (infected) deer and cattle. As such this project should be a valuable contribution to the question of wildlife bTB reservoirs. Setting the project within a risk assessment framework should ensure that the findings are presented in such a way as to have direct policy relevance. Additionally the study appears to follow a standard risk assessment format, as defined by OIE. There is however a possibility that the investigations specified will not provide sufficient data to parameterize the model. The final model may therefore have to be relatively simple, which is not necessarily a disadvantage. This project reports later than SE3037 and will provide a useful comparison in approaches and recommendations.

Scores
Conclusions based on sound evidence: 3.0
Quality of science: 2.5
Overall rating: 3.0

SE3037 A quantitative risk assessment of the role of wild deer in the perpetuation of TB in cattle

Organisation Risk Solutions
London

Start date 01/07/2005
End date 21/03/2006

Total cost £49,718

Abstract
Recent research has identified that several species of deer may pose a potential TB transmission threat to cattle. However, current knowledge does not support an informed judgement about the role that wild deer may play in either acting as a spillover host, or as a reservoir of infection to cattle. Defra therefore wishes to perform a quantitative risk assessment to improve understanding of the role of wild deer in the perpetuation of TB in cattle. This research project therefore aims to:

1. Identify, collate and review national and international literature to determine the current understanding of the role of wild deer in the perpetuation of TB in cattle, what data is available to support any quantitative risk assessment exercise, and where the current uncertainties and gaps in knowledge and data lie;
2. Determine the possible scope for a quantitative risk assessment (taking account of any uncertainties and gaps in current knowledge and data);
3. Develop a QRA model and carry out a risk assessment and appropriate sensitivity analyses to test the results; and
4. Present the results to Defra, along with recommendations for future research.

Because data in this area is sparse, the project combines available data and expert judgment in a model that can explicitly handle uncertainty. If some data gaps cannot be filled the model can be used to explore what values the data items would have to take for the risks from deer to be significant.

Aims
Scientific objectives for the project are:

- Objective 1 – Scoping: develop a qualitative understanding of the role of wild deer in the perpetuation of TB in cattle and determine the scope for a quantitative risk assessment (taking account of any uncertainties and/or gaps in current knowledge and/or data).
- Objective 2 – Develop QRA model: convert the qualitative representation developed in stage 1 into a quantitative model, and carry out a risk assessment and appropriate sensitivity analyses.
- Objective 3 – Report and recommendations: report the results and conclusions reached, summarise the potential magnitude of the TB risk due to wild deer and make recommendations for whether further research is needed, for example to improve the data estimates for the most important variables identified through the

sensitivity analyses, or to fill any material gaps in the knowledge base that the project had identified.

Relevance to Defra

Bovine TB is one of the most difficult animal health problems currently facing the farming industry in Great Britain. It is known to be endemic in badger (*Meles meles*) populations in the UK and has also been found in a wide range of other wildlife. Of these other species, wild deer are considered to present the largest additional risk[i]. The risk assessment model will support policy decision making in this important area by helping Defra establish:

- The likely role and significance of deer in the perpetuation of bTB, or
- If there is insufficient data available to reach robust conclusions, priorities for research.

Methods

Scoping

A quantitative representation of the issues has been constructed in the form of an influence diagram based on a literature review, consultation with the expert community, and an expert workshop. The review of the availability of data has been carried out for each key factor to determine:

1. What data does exist, and to what level of confidence;
2. Whether expert opinion could be used as a substitute for actual data; and
3. Where neither field data nor expert opinion is possible.

Modelling

We are building a 3 species disease transmission model, with differential equations solved using a fourth order Runge-Kutta approximation. The model will be populated with available data and expert judgement, using range estimates and a scenario approach to explore the impact of uncertainties. An expert workshop will inform the inputs and comment on the outputs.

Scenario Analysis and Reporting

We are carrying out a series of analyses and reporting the findings.

Results

To date we have completed the scoping stage, agreed the basis for modelling and are now constructing the model.

The model will estimate the potential number of new cases of bTB in cattle per year due to wild deer, and will subtract this from the background rate of new bTB cases in cattle overall. In this way it should be possible to determine to what extent deer are having (or may have) a material effect on the overall development of the disease.

The model should allow the following questions (identified in the original ITT) to be considered:

- What is the current prevalence of TB in the wild deer population (Q2);
- What is the deer-to-deer transmission rate for TB, and is it self sustaining, or is the observed prevalence the result of spill over from another species (Q3);
- What is the deer-to-cattle transmission rate for TB (Q4);

- What is the background incidence rate per year for TB in cattle, and therefore what proportion of the risk is potentially due to wild deer Q5); and
- What is the dependency of the above on regional risk factors, for example the regional density of deer, cattle, or both (Q6)?

The model could also be used to assess the impact that future population growth might have on the role of wild deer in the transmission of bTB. The model will be capable of accommodating the variability and uncertainty that exists in the input parameters, and of reflecting this in the output parameters. It will also be able to produce estimates of the likely range of values that an uncertain parameter may have given an expected outcome, and to demonstrate at what value an uncertain parameter becomes significant enough for the risk posed from wild deer to cattle became material.

The model is framed so that the relationship between the various input parameters can be assessed at a regional level. However there are currently very few regions for which there is adequate data available and are therefore focusing this initial implementation of the model on a region such as the South West of England where there is a high incidence of bTB in cattle and a high concentration of deer. However, some elements of the modelling are also considering other regions where particular interactions are not considered to be occurring in order to eliminate uncertainty and to allow some parameters to be estimated.

Where there is little or no data, model nodes are being designed to accommodate possible scenario states (eg High, Medium or Low contact rates). In addition, in order to minimise parameter numbers, we plan to frame the model as a comparison between the relative impacts of deer compared with badgers.

Parameter values will be established to calculate the “base case” proportion of bTB cattle risk potentially due to wild deer, and then the model used to perform sensitivity analyses to determine the most importance of each variable in the model.

Building the Model

Much of the modelling will comprise estimating parameters such as bTB prevalence and new cases based on a range of input parameters. This calculation element of the model is being developed as an Excel spreadsheet, allowing the underlying input data, parameter interactions and mechanistic assumptions to be clearly visible and documented.

Variability and uncertainty in parameters and mechanisms will be accommodated with the use of range data (either gained directly from the available data or elicited from experts). Additional tools such as Palisade’s @Risk add-in and PWC’s Decision Analysis software package DPL will be used to carry out the sensitivity analysis and consideration of scenario probabilities.

By building the model so that particular scenarios can easily be considered, it should be possible (using the known data for those particular scenarios) to narrow down the range of uncertainty for particular parameters. This will then allow us to consider the

probability that parameters have values in particular ranges given that particular outputs are being observed.

An initial series of model runs will be undertaken to identify the most significant data items and to test the influence of any underlying assumptions.

Conclusions
At this stage we can conclude that it is possible to build a useful quantitative risk model, that can be used to explore the extent to which deer are having (or may have) a material effect on the overall development of the disease and to prioritise areas for further research.

Future research
Future research priorities will be explored using the completed model.

References cited in text
[i] Delahay *et al* (2004), *The risk to cattle from wildlife species other than badgers in areas of high herd breakdown risk*. CSL Defra Research Project SE3010.

Web link reference:
http://www.defra.gov.uk/science/project_data/DocumentLibrary/SE3010/SE3010_1628_FRP.doc.

Review comment
Reviewers found the project difficult to assess given the short duration of the project and lack of an annual report. Also there was some confusion over how the project differed from SE3036. A major concern regarding the project was that a rather elaborate modelling system was being built on very limited understanding of the role of deer in bTB spread, and that the justification for the modelling approach was missing. For instance, the mean field approach for modelling disease spread may not be appropriate if there is spatial or temporal heterogeneity in any aspect of the disease epidemiology. In this area, disease heterogeneity is a known issue. There is also the issue of the lack of good data with which to model. On the positive side the Risk Solutions team have a good track record in animal disease risk assessment so it is expected that a useful initial QRA to assist policy development and highlight areas where additional information is needed will be delivered.

Scores
Conclusions based on sound evidence: 2.0
Quality of science: 1.9
Overall rating: 1.8

Tuesday 4th July 2006

Pathogenesis/Genomics

Tuesday 4th July 2006

Venue: The Diskus, Transport House, 128 Theobald's Road, London WC1X 8TN

09:00 – 09:30	Registration and Coffee		
09:30 – 09:40	Introduction		Chairman- *Alastair Macmillan*
	Code	**Title**	**Speaker(s)**
09:40 – 09:55	SE3226	Development of tools to study immunopathology in badger TB	VLA
09:55 – 10:10	SE3225	In depth histopathology characterisation of lymph node granulomas in natural and experimental bovine TB	VLA
10:10 – 10:45	SE3015	*Mycobacterium bovis* pathogenesis	IAH
10:45 – 11:05	Coffee		
11:05 – 11:30	SE3024	Low dose TB infection in cattle: disease dynamics and diagnostic strategies	VLA
11:30 – 11:55	SE3027	Pathogenesis and immunology of *Mycobacterium bovis* infection in cattle	IAH
11:55 – 12:20	SE3033	Housing of naturally infected cattle (field reactors) at VLA for immunological and bacteriological analysis	VLA
12:20 – 12:55	SE3013	Pathogenesis and diagnosis of tuberculosis in cattle - complementary field studies	VLA
12:55 – 13:40	Lunch		
13:40 – 14:05	SE3017	Development and evaluation of strain typing methods for *Mycobacterium bovis*	VLA
14:05 – 14:25	SE3206	Genome sequence analysis of *Mycobacterium bovis*	VLA
14:25 – 15:00	SE3030	Application of post genomics to reveal the basis of virulence, pathogenesis and transmissibility of *M. bovis*	VLA
15:00 – 15:20	SE3220	Molecular and epidemiological characterisation of the PPD diagnostic reagent	VLA
15:20 – 16:30	Coffee		
15:40 – 18:30	Closed session – confidential project specific issues. Proposed way forward of the research area		

SE3226 Development of tools to study immunopathology in badger tuberculosis

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/10/2005
End date 30/09/2006

Total cost £44,036

Abstract
Bovine TB is one of the most difficult animal health problems in Great Britain, and badgers have been identified as a likely reservoir for *Mycobacterium bovis*.

The introduction of a vaccine for badgers has the potential to reduce the risk of infection to cattle, and result in significant cost savings. BCG is being used in experimental studies with badgers in University College Dublin, and it is likely to be the first vaccine in the field. However, in order to help to determine the efficacy of a badger TB vaccine, we need to understand more about the development of the disease in this host.

In-depth analysis of granulomatous lesions, and of immune mediators (eg cell populations, cytokine and growth factors) will be used in an attempt to better understand the immunopathogenesis of this infection, and to evaluate possible indicators of protection. For this study we will be using experimental vaccination materials from University College Dublin, and will also be reviewing materials from badger postmortems at VLA. The *in situ* demonstration of immune mediators in lesions will provide vaccinate *vs*. naive comparisons of host-microbe interactions. With the information generated we hope to be able to better assess the immune components that contribute to lesion development and/or to protection. This should aid in the investigation of the efficacy of candidate vaccines.

Aims
To assess the immune mediators that contributes to lesion development, or containment of *M. bovis* in badger tuberculosis.

This will be achieved by studying the following:

- Granuloma immunopathology: *in situ* demonstration of cell markers, cytokines and other immune mediators in lesions of vaccinates vs. non-vaccinates; and
- Characterization of disease stage in samples from natural disease and from vaccine trials: to develop a grading system for lymph node granulomas, to evaluate the size and distribution of granulomas and the number of mycobacteria within lesions.

Relevance to Defra
This project addresses one of the 12 strategic goals set in the "Government strategic framework for the sustainable control of bovine tuberculosis in GB". Specifically: Government will continue to develop a sound scientific evidence base by supporting research to improve our understanding of the disease and generate new tools, particularly in relation to diagnostics and vaccines.

The tools we will develop could be applied to future Defra funded badger TB vaccination or pathogenesis studies.

Methods
Materials
Tissues from 15 badger lung and lymph nodes from vaccination /challenge experiments at University College Dublin.

Tissues from a selection of naturally infected animals.

Methods
Haematoxylin/Eosin staining (H&E) and Ziehl-Neelsen staining (Z&N).

Immunohistochemistry with an avidin-biotin-complex for a variety of cell markers and cytokines (eg CD68, CD3, CD79a, CC15, TGF-β, type 1 procollagen…).

Results
At the time of preparing this summary, all samples from vaccine experiments have been identified and retrieved from the histopathology archives. Also discussions with owners of tissues from naturally infected animals have taken place and numbers agreed. Appropriate sections have been cut and stored for subsequent histological and immunohistological staining.

Conclusions
Too early to derive any conclusions, since this project has only been running for 4 months. Examination of slides has not yet been completed.

Future research
Hopefully this project will allow determination of patterns of disease expression at the local tissue in badgers, which are currently unclear. This will improve the understanding of disease, and will help with the development/refinement of models of *M. bovis* infection for this host. The information generated will be of special interest for efficacy assessment of any future vaccine candidate.

Review comment
The project is well planned, not overly-ambitious, and could potentially provide a sound understanding of lesion development and immune mediators in badgers. However, the success will depend on how many badger reagents can be developed to facilitate the work. This will rely largely on antibody reagents raised against immune cell molecules in other species, but found to cross-react with the badger. Additionally, the pathological changes in *M. bovis*-infected badgers which have been BCG-vaccinated or non-vaccinated can be very subtle so histopathological examination of tissues is hoped to assist in differentiating protected animals. This

will help ensure that experimental animals match naturally infected ones. The potential of the work therefore, is that the results could be used to verify the efficacy of badger vaccines. It would be valuable if the histopathological findings could be compared with the bacterial load in the lymph nodes (bacterial counts or acid-fast bacilli).

Scores
Conclusions based on sound evidence: 2.3
Quality of science: 2.8
Overall rating: 2.6

SE3225 In-depth histopathology characterisation of lymph node granulomas in natural and experimental bovine tuberculosis

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/10/2005
End date 30/09/2006

Total cost £46,590

Abstract
In this project we have proposed to carry out in-depth histopathology characterization of lymph node granulomas from both natural and experimental *M. bovis* infections. We plan to describe the stages of granuloma development using immunohistochemical and/or molecular analysis to reveal the influx of $CD3^+$ (T-cells), $CD79^+$ (B-cells), $CD68^+$ (monocytes), $CC15^+$ ($\gamma\delta$-T cells) cells, and evidence of new collagen formation.

This proposed study will compare natural and experimental infection models by: (a) grading granulomas into 4 stages (initial, solid, minimal necrosis, and necrosis & mineralization), (b) providing an estimate of the area of lymph node affected by the granuloma, (c) assessing the number of acid fast mycobacteria present in granulomas, and (d) immunohistochemical and molecular documentation of cell types, cytokines and matrix proteins in different stages of lesion development.

From this work, patterns of disease expression at the local level will be identified and compared between natural and experimental infections. The results generated in the project will also help to enhance models to assess vaccine efficacy.

Aims

- Characterization of disease stage.
  - Classification of lymph node granulomas by stage of advancement.
  - Density and distribution of granulomas within organs.
  - Number of mycobacteria within lesions.

- Granuloma immunopathology.
  - Immunohistochemistry for cell markers to identify cellular components on a subset of samples.
  - Immunohistochemistry and molecular analyses for chemokine or cytokine expression, growth factors and matrix proteins on a subset of samples.

- Develop a temporal association with granuloma development in bovine lymph nodes.

Relevance to Defra
This project addresses one of the 12 strategic goals set in the "Government strategic framework for the sustainable control of bovine tuberculosis in GB". Specifically: Government will continue to develop a sound scientific evidence base by supporting research to improve our understanding of the disease and generate new tools, particularly in relation to diagnostics and vaccines.

Methods
Materials
500 tissues from naturally infected animals originating from Defra funded project SE3013: "Pathogenesis and diagnosis of tuberculosis in cattle-complimentary field studies".

88 tissues from experimentally infected animals originating from Defra funded project SE3024 "Low dose TB infection in cattle: disease dynamics and diagnostic strategies".

Up to 60 tissues from experimentally infected animals to complete a time course experiment.

Methods
Haematoxylin/Eosin staining (H&E) and Ziehl-Neelsen staining (Z&N).

Immunohistochemistry with an avidin-biotin-complex for CD68, CD3, CD79a, CC15, TGF-β, type 1 procollagen.

In-situ hybridisation for pro-collagen 1 m-RNA.

Results
At the time of preparing this summary, all samples from natural and experimental infections have been identified and retrieved from the histopathology archives. Appropriate sections have been cut and stored for subsequent histological and immunohistological staining.

All sections have been stained by H&E and Z&N for detection of mycobacteria.

Conclusions
To early to derive any conclusions, since this project has only been running for 4 months. Examination of slides has not yet been completed.

Future research
Hopefully this project will allow determination of patterns of disease expression at the target tissue, which is currently undetected through gross pathology or preliminary histopathology observations. This will improve the understanding of disease, and will help with the development/refinement of animal models for this disease. Also the information generated, will be invaluable for any future vaccine candidate studies.

Review comment
This project was generally well received, and makes use of material generated by other Defra-funded studies and so represents good value-for-money. The project follows on from a previous study which developed a classification system for tuberculous granulomas and undertook immunohistochemical staining of lymph node granulomas. The most valuable component of this study is the comparison of granulomas from experimentally and naturally-infected cattle as well as a time course of granuloma development in experimentally-infected animals. The data can be of use in efficacy assessments of future vaccine candidates and can be combined with the earlier work on a classification system for tuberculosis granulomas. However, it was felt that some of this work should have been conducted as part of the cattle pathogenesis programme projects (SE3013 and SE3015) and that not enough background literature was available to set the project in context. There is scope to do more with the statistics from this dataset.

Scores
Conclusions based on sound evidence: 2.2
Quality of science: 2.6
Overall rating: 2.7

SE3015 *Mycobacterium bovis* pathogenesis

Organisation Institute for Animal Health
Berkshire

Start date 01/07/00
End date 31/03/04

Total cost £2,440,159

Abstract
This work benefited from the combined experience and large animal facilities of the bTB groups of QUB/DARDNI, VLA and IAH.

A PCR test was developed for the detection of mycobacteria. The test detected as few as 1 colony forming unit. There were inconsistencies between the detection of bacteria by PCR and culture; probably due to environmental mycobacteria contamination, which caused specificity problems in the PCR method. Culture is still the most reliable method for detection of mycobacteria, but PCR, which is faster, should still be pursued.

Intranasal (IN), compared to intratracheal (IT), inoculation resulted in more nasal shedding of *M. bovis*. Shedding occurred in IFNγ^+ and tuberculin skin test positive (TT$^+$) animals with TB lesions, but it also occurred in animals that were IFNγ^+ with no visible lesions. One of these animals was also TT-negative (TT$^-$). The latter animal would not have been identified by TT and could act as a reservoir for *M. bovis*.

Repeated TT of naïve animals did not affect disease development or diagnosis of infection after challenge. Subtle changes on the immune response of repeatedly tested animals, compared to controls, were observed after challenge. When skin tested after infection the response in a second TT was reduced compared to the first TT one.

Mathematical modelling was used to further understanding of disease process.

Aims
1. Develop conventional and quantitative PCR based assays to enumerate *M. bovis* in nasal secretions taken from experimentally infected animals.
2. Determine how route of infection and dose affects nasal shedding of *M. bovis.*
3. Establish an in-contact transmission model for *M. bovis.*
4. Determine how physical contact and dose affects spread of infection.
5. Determine the effect of skin testing prior to exposure on the pathogenesis and diagnosis of disease.
6. Development of mathematical models.

Relevance to Defra
This project addresses R3 of the Animal Health Research Requirements 2001-2002 document on bTB, namely:

What additional information on the pathogenesis and transmission of TB within herds can be obtained from an epidemiological study on infected and at risk herds?

This research also addresses point 18 of the government's response to the Kebs report "Research strategy" with particular attention to points:

(a) statistical analysis and epidemiological modelling to assess local variation in risk (recommendation A(i));

(b) development of improved tests for detection of *M. bovis* in badger carcases and in environmental samples (recommendation A(iii)); and

(c) development of appropriate techniques for research into bovine TB transmission routes (recommendation A(iv)).

This project also provides information on whether the current activities for the diagnosis of bovine TB, ie the repeated tuberculin testing of naive animals (TT), may have an effect in cattle that become infected at a later point in their lives.

Methods

1. Develop assays to assess bacterial shedding

Conventional and quantitative PCR were used to develop assays to enumerate excreted *M. bovis* in secretions from experimentally-infected animals. The targets for PCR were IS6110 and IS1081. Growth of *M. bovis* was measured by growth in solid medium and in a BACTEC 460TB instrument, measuring ^{3}H-uracyl uptake by mycobacteria.

2. Determine how the route of infection and the dose affects shedding of *M. bovis*

Conventionally reared calves were challenged IN or IT with *M. bovis* AF2122/97. Immune responses were monitored in peripheral blood by measuring proliferative responses and production of IFNγ after stimulation with purified protein derivative (PPD) from *M. bovis* (PPD-B) and *M. avium* (PPD-A) and measuring antibody levels in serum.

Animals were monitored for clinical signs of disease and skin tested with PPD from *M. avium* and *M. bovis* no more than one week prior to post mortem. A full post mortem examination was carried out on all animals to confirm disease.

5. Effect of repeated skin testing on the sensitivity of the test and on disease

The comparative intradermal skin test was applied to one group of six calves five times at 8-week intervals. These and six control calves were subsequently inoculated IT with a dose of *M. bovis* that produced mild disease. The development of the delayed type hypersensitivity reaction, IFNγ, IL-10, proliferative responses and antibody levels in sera were compared in the two groups.

6. Development of Mathematical Models

The mathematical modelling was developed in two parts. – The first, involved analysis of data from the cattle-to-cattle pathogenesis experiments, and the second, analysis of field breakdown data.

Results

1. Develop assays to assess bacterial shedding

Assess in vitro assays (QUB)

QUB research aimed to develop conventional and quantitative PCR-based assays to enumerate excreted *M. bovis* in secretions from experimentally-infected animals. Growth of *M. bovis* in the BACTEC 460TB instrument was used throughout these experiments as the gold standard against which developments in PCR were evaluated. The preparation of bovine nasal mucus samples was crucial to ensure successful culture or PCR detection. A standard sample decontamination protocol was established, using N-acetyl-L-cysteine as mucolytic agent. Decontamination reduced the initial growth rate in BACTEC 460TB without compromising sensitivity. A BACTEC 460TB growth curve titration, for spiked sterile nasal mucus, was used to estimate cfu/ml. Aliquots of nasal mucus samples from all experiments and outbreak investigations have been stored appropriately, most have been cultured. The existing sequence-capture protocol, involving lysozyme/proteinase K digestion and extensive disintegration using the FastPrep120 instrument, was appropriate for nasal mucus samples but was modified to reduce hybridisation times. The Qiagen DNA minikit was also used to prepare PCR-ready DNA templates for analysis. Experiments showed that PCR detection sensitivity was comparable between these two methods.

For conventional PCR, IS*6110* was the target for amplification. Due to the likely paucibacillary nature of nasal mucus samples from experimentally-infected animals a nested-PCR protocol was required. First-round amplification produced a 123bp product. Second-round amplification produced a nested 92bp product. Single cell suspensions of *M. bovis* AF2122/97 were produced in 7H10 broth and PCR-ready DNA was extracted using both sequence-capture and Qiagen extraction protocols. The nested conventional PCR had a sensitivity of ~1cfu per reaction from both templates, which is equivalent to the sensitivity of BACTEC 460TB. Single cell suspensions of *M. bovis* AF2122/97 were also diluted into sterile bovine nasal mucus and PCR-ready DNA was extracted using both sequence-capture and Qiagen extraction protocols. Again the nested conventional PCR had a sensitivity of ~1cfu per reaction, equivalent to the sensitivity of BACTEC 460TB.

Conventional nested PCR was applied to samples for which an aliquot was cultured in experiment TB16 only. PCR and culture both detected 14 of 26 samples, 26 of 26 samples were positive by either PCR or culture, but only in 2 of 26 samples did the PCR and culture results agree. Reasons for this remain unclear. Culture was used throughout the rest of the experiments.

The use of Real-Time PCR for quantitation of M. bovis DNA in nasal secretions validated using spiked samples (QUB)

A real-time nested PCR assay was developed using SYBR Green 1 as intercalating dye for detection on the Corbett thermocycler. Experiments showed that this assay had a sensitivity ~1 cfu per reaction with the *M. bovis* AF2122/97 single cell suspension in both 7H10 broth and sterile bovine nasal mucus. In order to ensure improved specificity of PCR product detection a TaqMan-based probe has also been designed and synthesised. Preliminary experiments have been attempted. Aspects of technology transfer have been recently facilitated by the visit of Adam Whelan (VLA) to Tommy McCorry (QUB). QUB approaches to *M. bovis* culture, conventional and real-time PCR and sequence-capture were the subject of this transfer.

Real-time PCR for quantitation of M. bovis DNA applied to nasal secretions from all experimentally infected calves and nasal mucus secretions from reactor animals from two multiple reactor herds (QUB)

Bacterial culture was applied to aliquots of all nasal mucus collected for all experimental infections and for 2 multiple reactor herds. Excretion matrices were prepared from culture data. Conventional PCR was compared to culture data for experiment TB16 only. There was poor correlation between culture and conventional PCR data for aliquots of the same sample. Reasons for this remain unclear. Every effort was made in pursuit of this milestone, but results to date have not been promising.

Development of IS1081 real-time PCR quantitation of M. bovis in bovine nasal secretions (Imperial College/VLA)

A method for the quantitation of *M. bovis* in bovine nasal secretions using a rt-PCR method based on the amplification of the *M. tuberculosis* complex specific insertion element IS1081, was developed at Imperial College, London. Unlike the insertion element IS6110, multiple copies of IS1081 are present on the *M. bovis* genome. The conventional IS1081 PCR resulted in a product size of 135bp. The rt-PCR method was developed using SYBR-green as an intercalating fluorescent marker for detection on Corbett Rotogene thermocycler. The PCR methodologies developed at QUB and Imperial College were transferred to, and evaluated at, VLA using bovine nasal samples spiked with known amounts of *M. bovis*. Cfu detection sensitivity was confirmed retrospectively by culturing spiked nasal samples on Middlebrook 7H11 agar. Whilst the nested IS6110 (QUB) and IS1081 (Imperial College) PCR's were sensitive to a theoretical single genome copy sensitivity, their sensitivity was determined experimentally to be ca 10cfu following analysis of DNA extracted from spiked samples. Whilst equivalent in sensitivity, the IS1081 PCR method was considered to be more practical than the nested IS6110 method since only a single PCR step was required. In summary, whilst PCR methodologies may provide rapid confirmation of *M. bovis* in infected tissues or mucus secretions, culture still provided the most reliable results with robust sensitivity and specificity.

2. Determine how the route of infection and dose affects shedding of *M. bovis*

Compared to low-dose (10^4 cfu), more severe disease was observed following high-dose (10^6 cfu) inoculations. 23/24 and 21/24 of the inoculated animals developed PPD-B specific IFNγ and skin test responses, respectively. *M. bovis* nasal shedding was observed in 15/24 animals and a dose-response was observed between the level of inoculum and shedding frequency. Shedding was detected following low IN, high IN and high IT inoculations. A distinct shedding pattern was observed in calves infected IN, with an initial phase at <30 days post-infection (PI), followed by a second phase around 80-100 days PI. The median cfu of *M. bovis* shed was 8 (range 4-312). 15/15 animals that shed bacilli were PPD-B IFNγ^+ and 14/15 were skin test positive. 2/15 animals that shed *M. bovis* had no-visible lesions (NVL) at necropsy, and one of these was skin test negative, however both were PPD-B IFNγ^+.

In summary, the findings of this study could have significant implications for the current skin testing control programme. In particular, skin test negative, (IFNγ^+) NVL animals have the potential to excrete *M. bovis*. Also skin test and IFNγ test positive

NVL animals are also capable of shedding *M. bovis* and therefore such animals should not *a priori* be classified as false-positive for bovine tuberculosis.

5. Effect of repeated skin testing on the sensitivity of the test and on disease

No differences in IFNγ, IL-10 and proliferative responses were seen between calves that had been repeatedly skin tested compared to calves that had not been skin tested prior to challenge. After infection with *M. bovis* no differences in the development of the DTH and IFNγ responses to PPD were noted as a consequence of the repeated skin testing prior to challenge. No differences between the groups were evident when ESAT-6 was used as antigen and IFNγ was assayed, although two animals that responded to PPD did not respond with ESAT-6. However, there did appear to be subtle effects of repeated skin testing on the immune response post challenge that did not affect the diagnostic tests. After challenge control animals showed greater proliferative responses than animals given repeated skin tests prior to challenge, indicating that the procedure did have consequences for immune responses following infection. In both groups a marked reduction in the intensity of the skin test and in the number of animals that would be recognized as reactors was evident when animals were tested 15 weeks post infection compared to their responses 8 weeks earlier that could have consequences for diagnosis of TB. An antibody response was not evident as a result of repeat skin testing prior to infection but was seen in both groups of calves following skin testing performed 7 weeks after infection.

6. Development of Mathematical Models

Time series data of bacteria shed by experimentally infected cattle at QUB and at the VLA, showed that excretion of bacteria is episodic. The data are consistent with a hypothesis that experimentally infected cattle can be differentiated into some that produce bacteria at most on an intermittent basis, and those the are much more consistent, despite the fact that both groups show a positive immunological response. Should this pattern also be consistent with natural infection, this would suggest that only a proportion of those cattle testing positive for TB would be important contributors to cattle-to-cattle TB transmission. The epidemiological implications for this are currently being investigated, using a stochastic mathematical model. Quantitative shedding data from 12 cattle experimentally infected at IAH Compton are consistent with the hypothesis that, while the episodic shedding pattern differs from animal to animal (as above), the data do not support significant differences in the quantity of bacteria shed per episode. This provides a hypothesis for the mechanism of bacterial shedding, and emphasizes the importance of determining the dose-response relationship for TB transmission – ie transmission may be the result from a single exposure to a single large dose of bacteria, or it may be the result from prolonged, low level exposure.

Conclusions

1. Culture is at present the most sensitive and reliable method for detecting *M. bovis* but PCR, being faster, should be pursued.
2. Shedding of mycobacteria in IN inoculated animals was noted in two phases, one at less than 30 days and one at around 80-100 days post inoculation. Some inoculated animals that were skin test non-responders and had no visible lesions were $IFN\gamma^+$ and shed mycobacteria. This type of animal in the field may be involved in the transmission of disease but remain undetected by the skin test.

4. After repeated skin testing, no significant effect on the development of disease or the TT or the IFNγ assay that might affect diagnosis in the field was noted.
5. Analysis of shedding data was consistent with the hypothesis that cattle are either intermittent shedders or more consistent shedders despite both groups showing positive immune responses. If this is the case in natural infections only a proportion of TT^+ animals would be important in the cattle-cattle transmission of TB. The number of bacteria shed at any one time is similar for both persistent and intermittent shedders. It may be possible that the extent of shedding on any one occasion would be sufficient to transmit disease from cattle to cattle and this would make intermittent shedders important in disease transmission.

Future research

As indicated above, culture remains the most sensitive and reliable technique for the detection of *M. bovis*, however, this technique is lengthy and therefore hampers more practical applications in the field. The use of PCR as a diagnostic technique for the presence of *M. bovis* in suspected samples should continue to be explored.

Under experimental conditions, it was found that the transmissibility of disease from cattle to cattle, as evaluated by the presence of TB lesions in cattle housed together with infected animals, is an infrequent occurrence – although in using these data to quantitatively estimate transmission frequency may be difficult due to the high numbers of air changes needed to maintain negative pressure, which may significantly reduce the available infective particles and thus transmission. Moreover, the conversion from $IFN\gamma^-$ to $IFN\gamma^+$ status of recipient animals after a period housing with infected animals, indicates that transmission of *M. bovis* from cattle to cattle may be a more frequent event that transmission of disease. Whether these animals may become reservoirs for *M. bovis* that would not be detected by the tuberculin skin test (TT) needs to be addressed. Indeed, some of the donors, had no visible lesions, and in one case no positive TT, but were $IFN\gamma^+$ indicating that there is a need to develop improved techniques for the detection of *M. bovis* colonized animals. It also indicates that there is a need to explore further the carrier state in bovine TB.

It is known that the skin test has an effect on the immune response of infected cattle to mycobacteria, namely the boosting of IFNγ and antibody responses. Further research should be carried out to determine whether these responses could be used as supplements to the skin test to help in the detection of animals that although infected are TT^-, as were some experimentally infected animals in the present project.

The use of mathematical models will help to focus the efforts on diagnosis and possible vaccination. Accordingly, further development of mathematical modelling of *M. bovis* transmission should be carried out; this should include animals that have been vaccinated and then infected with BCG, to determine whether the proposal of vaccination belts is a workable proposal.

Publications generated from the project

1. McCorry, T., A. O. Whelan, M. D. Welsh, J. McNair, E. Walton, D. G. Bryson, R. G. Hewinson, H. M. Vordermeier, and J. M. Pollock. 2005. Shedding of *Mycobacterium bovis* in the nasal mucus of cattle infected experimentally with tuberculosis by the intranasal and intratracheal routes. Vet Rec **157:**613-618.

2. McCorry, T. P., C. M. McCormick, M. S. Hughes, J. M. Pollock, and S. D. Neill. 2004. Mycobacterium nonchromogenicum in nasal mucus from cattle in a herd infected with bovine tuberculosis. Vet Microbiol **99:**281-285.

3. Kao, R.R., Charleston, B., Hope,J.C., Martin, M. and Howard, C.J. *Mycobacterium bovis* Shedding Patterns from Experimentally Inoculated Cattle, and impact of BVDV Coinfection. Submitted to Proc. Roy. Soc. Lond. B (under revision following referees comment).

Review comment

This project received a very mixed response and was described as both good and poor value for money. It was felt that the in-contact transmission experiments should have either been better designed or not performed under the conditions H&S required. This work was a large contributor to the project costs but did not yield the information it was designed to and did not clarify transmission further e.g. the dynamics of nasal shedding are still unresolved. The mathematical model of shedding added little and the conclusions were felt to be obvious from the raw data. Positively, the work confirmed that culture is gold standard for detection of *M. bovis* (though PCR may be useful in conjunction to culture), and the demonstration that IFNγ test +ve animals can be infected and shed bacteria, even if TT-ve is important for the development of control protocols. In contradiction to previous findings, there was no significant effect on the development of disease or the skin test when animals were repeatedly skin tested. Many of the project results were confirmation of what was suspected. In terms of balance of the results in the final report, some felt that too much relevance was given to the finding that one (out of 15) experimentally inoculated calves failed to react to the skin test despite being IFNγ test positive and an *M. bovis* excretor. As the SICCT test is not 100% sensitive, this finding confirmed that some of the infected cattle missed by the SICCT could be detected by the IFNγ test (and vice-versa). This is borne out by other projects also. It was useful to have developed this experimental infection model. However there was concern that the number and prosaic quality of the publications was poor and that a grant of that size should have produced many more papers and of higher quality. Overall, the study provides evidence that transmission from infected animals is low during the early stages of the infection. It also showed that a transitory immune response is found early but that this may not be associated with disease development. The study highlights that further work in this area requires more natural transmission studies. The occurrence of animals that appeared to be infected (IFNγ +), but had no disease is also of considerable interest. It would be interesting to see whether these animals could develop disease at a later time point.

Scores

Conclusions based on sound evidence: 2.9
Quality of science: 2.7
Overall rating: 2.7

SE3024 Low dose TB infection in cattle: disease dynamics and diagnostic strategies

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/10/02
End date 31/09/06

Total cost £2,613,900

Abstract
In the majority of cattle (ca. 60 %) presenting with bovine tuberculosis in GB, pathology is restricted to the lower respiratory tract (lung and/or lymph nodes draining the lung). The model best reproducing this pathology presentation is the intratracheal challenge model, which is being used in these studies. An additional aim is to establish and validate an aerosol challenge system that is also likely to target the lower respiratory tract. Applying both models side-by-side could synergistically allow us to define factors relevant to transmission and pathogenesis. We firstly determined the minimum infective dose of *Mycobacterium bovis* necessary to stimulate specific immune responses and generate pathology in cattle. Calves were infected by the intratracheal route with 1,000, 100, 10 and 1 cfu of *M. bovis*. Results showed that half of the animals infected with 1 cfu of *M. bovis* developed pulmonary pathology typical of bovine tuberculosis. No difference in the severity of pathology was observed for the different doses of *M. bovis*. All animals that developed pathology were skin test positive and produced specific IFNγ and IL-4. There was no difference in the size of the skin test reaction, the time taken to achieve a positive IFNγ result, or in the levels of IFNγ and IL-4 between animals infected with the different doses of *M. bovis*, suggesting that current diagnostic tests (skin test and IFNγ test) can detect cattle soon after *M. bovis* infection regardless of dose.

In order to study immune responses in cattle containing the infection with low, or undetectable bacillary loads, we generated a “memory cow” model that we adapted from a drug-assisted model of protective immunity developed originally for mice. We have shown thus far that cattle infected with *M. bovis* (spoligotype 9) and then treated with isoniazid (INH) from week 4 post-infection for 10 weeks harbour minimal pathology compared to untreated animals, with far fewer and calcified lesions, yet still presented with strong cellular immune responses (IFNγ, DTH). We have shown that INH is safe, in that treatment did not impair either the growth of the animals nor their liver function, and was rapidly cleared from the circulation. An experiment is now underway to determine protective immunity in infected and treated animals by re-challenge with *M. bovis* of a different, but equally pathogenic spoligotype (spoligotype 35).

An aerosol challenge model for cattle was established by titrating infective *M. bovis* doses over a 4 log range. The aerosols were delivered using a Maddison chamber system, and the results so far demonstrated that even high doses delivered by aerosols (ca. $>10^4$ cfu) resulted in visible pathology confined to the lower respiratory

tract although bacilli could also be detected in some animals in the upper respiratory tract.

Aims

- To determine the minimum infective dose of *M. bovis* in cattle.
- To generate a "memory cow" model of protective immunity, re-infection/re-exposure and potential latency of *M. bovis* in cattle.
- To establish and validate an aerosol challenge model for *M. bovis* infection in cattle.

Relevance to Defra

The study of disease dynamics, infective dose and routes in relation to diagnostic strategies during this work is relevant to assess the risk of *M. bovis* transmission between cattle and within the environment. In addition, we will assess whether immunity generated as a result of one *M. bovis* strain results in protection against different strains. These assessments will help Defra and ISG to refine TB control strategies and initiatives. Effective refined control strategies will be of significant economic benefit to the industry and the consumers whose confidence in the safety of meat products needs reassurance. The development of the 'memory cow' model may facilitate the identification of protective antigens for use in vaccine development, as well as a true marker of protective immunity. It will also assess the development of diagnostically relevant immune parameters in cattle that contain the disease (latency) or that are repeatedly exposed to subsequent *M. bovis* infections. To appraise different challenge models in a comparative manner also further helps the scientific community to choose the appropriate infection model to address different scientific questions.

Methods

Bacterial strains. Infection inocula were prepared from mid log-phase frozen seed stocks of *M. bovis* strain AF61/2122/97 (spoligotype 9) or strain AF61/1307/01 (spoligotype 35).

Cattle. Friesian Holstein heifers and bullocks >4 months old from a TB-free herd were randomly sorted into experimental groups. The disease-free status of the animals was further confirmed before recruitment using the IFNγ test. Calves were inoculated intratracheally using established protocols (Buddle *et al* 1995).

Skin Test. The single comparative intradermal tuberculin skin test was performed in accordance with standard protocol as published in the European Commission Regulations.

Post Mortem. Calves were euthanized by intravenous injection of sodium pentabarbitone, and a detailed post mortem carried out as described earlier (Vordermeier *et al* 2002). Samples of lesion material and apparently uninfected tissues were used for histopathological and microbiological examination and confirmation of *M. bovis*. Individual tissues were assigned a Pathology Score depending upon number, size and character of the lesions observed (Vordermeier *et al* 2002).

Isoniazid treatment of cattle. Cattle were dosed with an oral suspension of isoniazid (UCB Pharma Ltd UK.) prepared by grinding 100mg tablets, resuspending in water and applying using a Phillips Drencher (Cox Surgical UK). Animals were weighed weekly, and the isoniazid adjusted to give a dose of 25mg/kg per day following the protocol of Leite *et al* (2000).

Cytokine assays. Supernatants from antigen-stimulated whole blood cultures were assayed for IFNγ using a commercially available kit (BOVIGAM: Biocore, USA) and following the manufacturer's instructions. The Il-4 bioassay was carried out as previously described (Rhodes *et al* 2000) to determine IL-4 in whole blood culture supernatantws.

Liver Function Tests. Serum samples were assessed for bilirubin, aspartate aminotransferase, and gamma glutamyl transferase using kits obtained from Olympus Life and Materials Science Europa GmbH.

Aerosol infection using a Maddison chamber system customised to allow defined delivery of *M. bovis* doses to cattle.

Results

Project Aim 1: To determine a minimum infective dose of *M. bovis* in cattle. Groups of cattle were infected with 1, 10, 100, and 1000 cfu, respectively. Post-mortem examinations conducted between 24 and 26 weeks post-infection demonstrated that 3/6, 5/6, 5/6, and 5/6 of the calves infected with 1, 10, 100, and 1000 cfu, respectively, had gross pathological lesions typical of bTB. All cattle that developed visible pathology were skin test and *M. bovis* culture-positive. There was no difference in the size of the skin test reaction relative to *M. bovis* dose (Table 1), nor was there any significant difference in the degree of pathology observed relative to *M. bovis* dose (Table 2). Skin test negative animals contained no visible pathology, were *M. bovis* culture-negative, and histo-pathologically normal.

Strong specific IFNγ responses were observed in all 14 skin test-positive cattle from between 3-5 weeks post-infection. Thereafter all 14 animals maintained positive IFNγ responses throughout the experiment. The time taken to generate a positive IFNγ response and the intensity of the response did not vary with *M. bovis* dose. 13/14 skin test-positive calves also produced specific IL-4 responses. The IL-4 responses were transient and were observed between 5-7 weeks post-infection for most animals. There was no difference in the strength of the IL-4 response relative to *M. bovis* dose.

Project Aim 2: To generate a "memory cow" model in cattle. 16 calves were infected with *M. bovis* (UK spoligotype 9). 8 of these were then treated with the anti-mycobacterial drug isoniazid (INH) from week 4 post-infection for 10 weeks. All animals developed sustained positive specific IFNγ responses from week 4 post-infection and were tuberculin skin test positive. At post mortem however, INH-treated calves showed no or only minimal visible pathology (Table 3), with fewer, small (typically 1mm) lesions that were calcified, compared with a larger number of caseous, coalesced lesions found in the untreated animals (data not shown), suggesting an involvement of the host immune response in containing these residual bacilli left after chemotherapy. INH was given following a recommended dose (Leite

et al 2000) of 25mg/kg/day, and this regimen was shown in our experiment not to affect either growth (weight gain/week) of individual animals or liver function (serum tests for bilirubin, γ-glutamyl transferase and aspartate aminotransferase showed no deflection from normal values throughout). Furthermore, INH was shown by HPLC to be rapidly cleared from individual serial plasma samples (applying the method by Grasso *et al* 2000, Seifart *et al* 1995).

An experiment is now underway to assess the potential protective immunity in animals that have been infected with *M. bovis* (spoligotype 9), treated with INH (from 3 weeks post-infection), rested for 4 weeks and then re-challenged with *M. bovis* of a different, but equally pathogenic (AO Whelan, VLA, pers comm) spoligotype (spoligotype 35). By using a different spoligotype, the origins of any *M. bovis* recovered from post mortem tissues (ie primary or secondary *M. bovis* infection) can be established.

Project aim 3: To establish and develop *M. bovis* aerosol challenge model for cattle
A series of three experiments are being undertaken as part of this project to titrate the infective doses required for *M. bovis* infection via the aerosol route. The results of the first two experiments can be summarized as follows.

Experiment 1: Aerosol infection with 10^4 cfu, synopsis. This experiment initiated the titration series to establish low dose infection delivered by aerosol. 5 calves were infected with 7 x 10^4 cfu *M. bovis* (AF2122/97) and IFNγ responses were measured in all calves at 15 days post-infection (PI). Three calves were skin tested at 24 weeks PI and were found to be positive to PPDB. At post mortem examination lesions typical of bovine TB were disclosed in all cattle, and were confined to the lower respiratory tract with the exception of one lesion, which was found in the palatine tonsil (not confirmed by bacterial culture). Lesions were present in the lungs of all cattle and also in the caudal, cranial and mediastinal lymph nodes. Bacterial culture of all upper respiratory tract tissues identified the presence of *M. bovis* in 3 sites from 4 cattle.

Experiment 2: For this experiment, 5 calves were given 10^2 cfu by aerosol. Immunological responses were detected between days 16 and 23 PI (PPDB) and by day 23 PI three calves were responding to ESAT-6 and CFP10. The onset of these responses were very similar to the 10^4 cfu experiment, however, the magnitude of the response was much lower. This also applies to the proliferation response. The lower infectious dose (10^2 cfu) has induced a more discriminative immune response with one calf unresponsive to ESAT-6 while another does not respond to CFP10. Individually, responses showed a much greater degree of variation compared to 10^4 cfu exposure. In some calves responses were barely positive, while in others, responses were strong and consistent. Antibody responses were detected in 3 calves at day 27 (*M. bovis* sonic extract) while 4 calves responded to rMPB83 (days 16-28 PI). Immune responses will be monitored further, up to March 2006, when skin test reactivity using PPDB will be measured. Following skin test, a detailed post mortem examination will be carried out to record lesions (if present) and to generate a post mortem score. All parameters measured will be used to compare 10^4 with 10^2 cfu infection.

Experiment 3: will be initiated by the end of January 2006 with a scheduled completion date of September 2006. For this experiment five calves will be given

between 10 and 50 cfu with immune responses monitored over a 9 month period. As with the previous experiments in this series of experiments, immunological data along with post mortem scores will be recorded and used for comparison.

Conclusions

1. One cfu *M. bovis* is sufficient to cause established tuberculous pathology in cattle. This pathology is identical to that resulting from significantly higher experimental doses (up to 1000 cfu in this study) and reflects the pathology seen in the majority of naturally infected field reactor cattle.
2. Cattle infected with 1 cfu that developed pathology exhibited strong positive responses to the diagnostic skin test. Furthermore, the infectious dose of *M. bovis* had no bearing upon the time taken to obtain a positive IFNγ response in those animals that went on to develop pathology. These data are in accord with very low numbers of bacilli transmitted aerogenously between cattle. However those animals that do go on to develop pathology and therefore become a likely source of infection within a herd can be detected at an early stage with the IFNγ test, and later with the tuberculin skin test.
3. Isoniazid treatment of cattle experimentally infected with *M. bovis* significantly reduced the resulting pathology. Treated animals contained no or fewer, small and calcified lesions compared with untreated animals, indicative also of host-mediated containment of bacilli in addition to drug action, a prerequisite to study immune responses of cattle with low bacillary loads.
4. It can also be concluded from this experiment that, as in humans, cattle that have contained *M. bovis* infection at undetectable or very low bacillary loads (latently infected), still present with potent cellular immune responses, and can therefore be detected using tuberculin skin tests or the *in vitro* IFNγ assay. The removal of such animals is desirable (see paragraph 6).
5. However, it has to be stressed forcefully that at present the degree of latency, or if it indeed occurs in cattle at a significant degree, is not known (see section on future research).
6. Importantly, latently infected animals should not be viewed as self-healed or assume that they will not develop clinical symptoms and thus transmissible tuberculosis. It is therefore highly relevant to remove such animals (should they occur) as possible future sources of infection.
7. Our finding that even at very low bacterial loads, or when bacteria where undetectable, both skin testing and IFNγ testing were able to detect these animals is encouraging.
8. Challenge by the aerosol route demonstrated that even high doses delivered by aerosols (ca $>10^4$ cfu) resulted in visible pathology confined to the lower respiratory tract (although bacilli could also be detected in some animals in the upper respiratory tract).
9. From the results of using both aerosol and intratracheal infection routes, one can therefore conclude that the lower respiratory tract is exquisitely susceptible to infection with very low numbers of tubercle bacilli, and that delivery directly into the lung via aerosol will result in visible pathology mainly confined to the lung and associated lymph nodes. Therefore, as expected from the pathology seen in naturally infected animals, the predominant infection route in GB is via aerosol delivery of very few bacilli directly into the lung.

Future research
It would be interesting to assess whether and to what degree true latency develops in cattle infected with *M. bovis*.

It could also be of interest to establish whether distinct infection routes result in different, diagnostically relevant, immune responses (respiratory tract versus alimentary tract).

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Publications generated from the project
Published/submitted papers:

Dean, G.S., Rhodes, S.G., Coad, M., Whelan, A.O., Cockle, P.J., Clifford, D.J., Hewinson, R.G. and H.M. Vordermeier. 2005. Minimum infective dose of *Mycobacterium bovis* in cattle. Infection and Immunity, **73**(10): 6467-6471.

Pollock, J.M., Rodgers, J.D., Welsh, M.D., McNair, J. Pathogenesis of bovine tuberculosis: The role of experimental models of infection. Vet Microbiol. 2005 Dec **26**; [Epub ahead of print].

Johnson, L., Dean, G., Rhodes, S., Hewinson, G., Vordermeier, M and A. Wangoo. Low dose *Mycobacterium bovis* infection in cattle results in pathology indistinguishable from that of high dose infection. Manuscript in preparation.

Conference presentations:

Dean, G.S., Rhodes, S.G., Coad, M., Villarreal-Ramos, B., Mead, E., Clifford, D.J., Hewinson, R.G. and H.M. Vordermeier. Isoniazid treatment of bovine tuberculosis: development of a memory model. Presentation at the British Society for Immunology Annual Congress, Harrogate 2005.

McNair, J., Rodgers, J., Connery, N., Welsh, M., Bryson, D., McMurray, D., Pollock, J. Bovine tuberculosis vaccine-challenge strategy: An improved challenge model based on aerosolised delivery of *M. bovis*. IV International Conference on *Mycobacterium bovis*. Dublin, 22-26th August 2005

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Review comment
This was a well co-ordinated but expensive project that answered most of the points originally made. The questions asked seem relatively minor but are useful to the overall understanding of pathogenesis, diagnosis and epidemiology of bTB. More output papers are expected and the memory cow experiments are still to conclude. The dose experiments using the intra-tracheal model fit well with idea of very low dose aerosol transmission between cattle (essentially a stochastic event) and that the lower respiratory tract is very susceptible to low numbers of bacilli. They also show that both the skin and IFNγ tests last beyond reduction of infection (and not affected by dose). The results have complemented those of parallel studies in naturally infected field reactors. The aerosol model is difficult but producing reproducible results. The results raise experimental evidence to support the long held belief that latently infected animals can occur but that they should be detectable using the skin or IFNγ tests. Further work to investigate the latency issue is desirable. Other future areas would be to define the minimum infective aerosol dose, and whether the route of vaccine administration is important to vaccine immunity.

Scores
Conclusions based on sound evidence: 3.1
Quality of science: 3.4
Overall rating: 3.3

Table 1 Comparative tuberculin skin test results for individual animals 12 weeks after infection with *M. bovis.*

Infective dose of *M. bovis*	Change in skin test response (mm)		
	PPDA	**PPDB**	**ΔSCITT[a]**
1 cfu			
2805	0	0	0
2858	7	50	43
2865	2	36	34
2871	0	0	0
2877	0	0	0
2878	1	18	17
10 cfu			
2806	0	38	38
2861	3	30	27
2863	3	42	39
2866	0	0	0
2869	0	33	33
2876	3	22	19
100 cfu			
2802	6	28	22
2859	0	0	0
2927	3	23	20
2928	3	33	30
1000 cfu			
2923	0	22	22
2924	0	2	2
2925	0	22	22
2926	6	44	38

[a] ΔSCITT (change in response to PPDB – change in response to PPDA) equal to or greater than 10mm was considered positive.

Table 2 Pathology scores for skin test positive calves infected with *M. bovis.*

Infective dose of *M. bovis*	Pathology Scores[a]		
	Lymph Nodes	Lung	Total
1 cfu			
2858	0	4	4
2865	10	8	18
2878	2	2	4
10 cfu			
2806	6	6	12
2861	2	0	2
2863	6	0	6
2869	1	0	1
2876	4	6	10
100 cfu			
2802	3	3	6
2927	6	6	12
2928	2	2	4
1000 cfu			
2923	2	7	9
2925	11	5	16
2926	7	6	13

[a]Scores for individual animals were calculated as previously described (27) for lung and lymph nodes. No significant difference (by Kruskall-Wallis test) in degree of pathology between the infected groups.

Table 3 Pathology and Culture Scores INH treated and untreated cattle.

Animal	**Lymph Node**	**Lung**	**Total pathology score**	**Total Culture Score**
M. bovis + INH				
CW3195	0	2	2	1
CW3196	3	0	3	2
CW3197	0	0	0	0
CW3198	2	3	5	2
101721	0	0	0	0
201708	0	0	0	2
601726	2	3	5	7
601740	0	2	2	4
Mean	0.9	1.2	2.1	2
M. bovis				
CW3199	9	7	16	16
CW3200	2	3	5	4
CW3201	3	4	7	11
CW3202	3	5	8	7
101714	3	4	7	3
401724	6	3	9	17
501718	15	4	19	18
701734	7	3	10	22
Mean	6.0	4.1	10.1	12.3
	(0.0032)*	(0.0027)*	(0.0014)*	(0.0039)*

***, p-values (Mann-Whitney)**
Semi-quantitative scoring of pathology taken from Vordermeier *et al* (2002), Infect. Immun. 70(6):3026-3032
Semi-quantitative scoring of cfu from cultured tissues courtesy of A.O.Whelan: Individual lymph node tissue samples were plated out and growth scored as follows for each lymph node: 0, no growth; 1, 1-10 cfu/plate, 2, 10-100 cfu/plate; 3, 100-1000 cfu/plate. Individual lymph node culture scores from one animal were added up to calculate the total culture score.

SE3027 Pathogenesis and immunology of *Mycobacterium bovis* infection in cattle

Organisation Institute for Animal Health
Berkshire

Start date 01/04/02
End date 31/03/05

Total cost £1,506,135

Abstract
The aim was to analyze the mechanisms of immunity to *M. bovis* in cattle. Cellular immune responses to *M. bovis*, BCG and effects of prior exposure to environmental mycobacteria were analysed.

The effects of infection with *M. bovis* or BCG on antigen presenting cell (APC) function were assessed. *M. bovis* survived better inside dendritic cells (DC) compared to macrophages (mθ), and the cytokines secreted differed. Initial interactions of *M. bovis* with APC could be pivotal in determining the outcome of infection. Within lymph nodes from infected animals down-regulation of pro and anti-inflammatory cytokine expression was observed.

$CD4^+$ and $CD8^+$ T lymphocytes secreted IFNγ in response to *M. bovis* infection. NK cells reacted to BCG infected DC and could be involved in responses *in vivo* by secreting IFNγ.

Prior exposure to *M. avium* gave partial protection against *M. bovis* infection. However, diagnosis by the skin test or IFNγ test was compromised. In animals exposed to *M. avium* prior to BCG followed by *M. bovis* challenge no additional, or less, protection compared to BCG alone was observed and diagnosis was compromised. Use of ESAT-6 and CFP-10 was more effective for detecting infection following pre-exposure to *M. avium,* but was not effective in all cases. Responses to DNA vaccination were not affected by inclusion of IL-12, even after BCG boosting. BCG vaccination at different ages indicated that neonatal vaccination is an effective strategy significantly reducing pathology and bacterial load. Neonatal vaccination in the field prior to environmental mycobacteria exposure would maximize the protective efficacy of BCG.

Aims
- Investigate the interaction of APC and *M. bovis*.
- Assess the T cell responses of cattle following vaccination and infection with virulent *M. bovis*.
- Determine how prior exposure to environmental mycobacteria interferes with the immune response to *M. bovis*.
- Investigate alternative means of effecting a more efficacious response to vaccination.

Relevance to Defra

The objective of this programme was to dissect the mechanisms of immunity to *M. bovis* in cattle. This falls within the framework of work called for under the Animal Health and Welfare Portfolio. Specifically it answers the call for research on bTB that was proposed by the Krebs report and by the ISG: Research requirement R24 'Further development and refinement of novel reagents for diagnosis and immunological control of bTB in animals and studies of disease pathogenesis' (2002-2003).

Methods

Cattle were exposed (challenged) to *M. bovis* via the IN route, this causes disease mainly in the head lymph nodes, typical of approximately 1/3 of field bovine TB cases. Both BCG and *M. avium* were inoculated subcutaneously (SC). These routes and the doses of mycobacteria used have previously been determined as optimal. Groups of young adult calves were exposed to *M. avium* then *M. bovis*, or *M. avium* followed by BCG then *M. bovis*. The efficacy of BCG vaccination was assessed in neonatal calves vaccinated when aged less than 28 days old, then subsequently challenged with *M. bovis*. In one experiment the effect of DNA vaccination with plasmids encoding Ag85A in conjunction with IL-12 or IL-18 was assessed. The animals were skin tested using the comparative skin test with PPD-A and PPD-B according to EU guidelines. The extent of disease was assessed by post mortem examination and evaluation of bacterial counts in tissue samples. *In vitro* assays included the IFNγ whole blood test. Whole blood was stimulated with PPD and serum removed at 24h. This was analysed for IFNγ, IL-10, IL-4 and TNFα levels by ELISA. Numbers of cells expressing IFNγ were assessed by ELISPOT. Antigen presenting cells were derived from blood monocytes by culture either alone or with the addition of the cytokines GMCSF and IL-4 to derive dendritic cells. These were infected *in vitro* with optimal numbers of bacteria (that gave minimal cell death but maximum number of cells infected). Cytokine secretion was assessed by ELISA and cell surface phenotype by flow cytometry. In addition, the ability of infected antigen presenting cells to stimulate T cell responses was assessed by measuring T cell proliferation and IFNγ secretion. Cytokine expression was also assessed by real time quantitative PCR in lymph nodes taken from *M. bovis* infected, BCG vaccinated or control animals.

Results

Analysis of the effect of infection with M. Bovis on APC function.

Survival of *M. bovis* within APC is a central event in the pathogenesis of tuberculosis. Different APC types are proposed to have different roles in immune responses. thus, monocyte/macrophages are critical in the removal of bacteria from host tissue. Dendritic cells (DC) are pivotal in the induction of immune responses in naïve animals, they are also the most effective cell in inducing CD4 and CD8 T cell responses *in vitro* as well as *in vivo*. The maturity of APC affects their ability to take up antigens and stimulate T cells. Maturity is affected by a variety of stimuli (including bacteria) and cytokines.

Objectives

1. Examine how the activation and maturation of APC affects the manner in which they respond to infection with mycobacteria by assaying bacterial survival and APC viability.
2. Determine how the cytokines synthesized by the APC vary following infection *in vitro* with virulent and avirulent mycobacteria and how activation and maturation affect this.
3. Examine how different APC vary in their ability to stimulate T cell responses following infection with avirulent or virulent *M. bovis*.

1. ***Bacterial survival in DC and macrophages***
   mθ and monocyte-derived DC were infected with virulent *M. bovis* AF2122/97 or BCG Pasteur and the survival of the APC and of the intracellular bacteria monitored. Varying infection doses of *M. bovis* and BCG were compared for infectivity levels and induction of host cell death. Colony forming unit (cfu): cell ratios of 1:1 for *M. bovis* and 10:1 of BCG gave minimal cell death and equivalent levels of infection (~80% cells infected at 24h). No difference in APC survival was evident over a 48 hour incubation period using these cfu: cell ratios. Survival of *M. bovis* was greater than that of BCG in both macrophages and DC. The survival of both strains was greater in DC than in macrophages. Addition of IFNγ to DC cultures resulted in reduced survival of *M. bovis* and BCG but had no significant effect on survival within macrophages.

2. ***Cytokine synthesis by DC and macrophages infected with virulent and avirulent M. bovis***
   Bovine macrophages and monocyte-derived DC infected with virulent *M. bovis* or avirulent BCG responded to infection by producing different cytokines. We found differences in expression of transcripts for the pro-inflammatory cytokines, interleukin (IL-1β) and IL-6. Numbers of transcripts for both cytokines were up-regulated in both APC types and for macrophages, expression of IL-1β and IL-6 was higher following infection with BCG compared to *M. bovis*. Expression of immunoregulatory cytokines, IL-10, IL-12 and TNF-α protein, were higher following *M. bovis* infection compared to BCG (Figure 1). Differences in cytokine production by DC or macrophages was evident, which may have important consequences for the type of immune response that develops. Macrophages primarily secreted IL-10 and TNF-α with little or no IL-12. DC responded to *M. bovis* infection by up-regulating production of IL-12 and TNF-α and infected DC secreted more TNF-α than macrophages. No differences in IL-18 mRNA expression were detected following mycobacterial infection in either APC type. These data suggest initial infection of DC would induce a Th1 response, while infection of macrophages can result in the production of cytokines that would be expected to modulate or slow the immune response. Such down-regulation of the host immune response could benefit bacterial survival but may also be a mechanism to limit pathological damage to host tissue.

3. ***Stimulation of T cells by macrophages and DC***
   Dendritic cells and monocyte/macrophages infected with BCG or *M. bovis* or pulsed with PPD were compared for their ability to stimulate specific CD4 and CD8 T cell responses using protocols described by Hope *et al* (2000). For these experiments APC were used at day 3 of culture and both DC and macrophages

were actively phagocytic at this time. A comparison of DC or monocyte/macrophages pulsed with BCG is shown in Figure 2. The capacity of BCG infected, or control (unpulsed) APC to induce proliferative responses of purified CD4 or CD8 T cells from a calf vaccinated with BCG was assessed. In this experiment the BCG-infected APC were from an MHC matched animal. Similar results were observed with autologous APC. The DC were clearly more effective than macrophages and the CD4 response more extensive than the CD8 response.

Characterize the cd4 and cd8 T cell responses in immunity to, and pathogenesis of, tuberculosis

Although an immune response is vital for controlling *M. bovis* infection it is also a component of the pathology of the disease. The cytokines produced by T cells affect the outcome of mycobacterial infections. For example IFNγ and IL-12 are essential for immunity and TNFα may contribute to both immunity and pathology. Understanding the immune response is a necessary component of understanding the pathogenesis of infection and establishing correlates with immunity that will lead to the development of novel diagnostic tests and eventually to improved vaccine design and vaccination strategies.

Objectives

4. Analyse the cytokines produced by CD4 and CD8 T cells in BCG-vaccinated calves and in calves challenged with virulent *M. bovis*. Determine any correlates with immunity and whether assays for cytokines other than IFNγ could be useful in diagnosis of infection.
5. Analyse the cytokines produced in lesions in the respiratory tract and associated lymph nodes in cattle with TB and determine the relation between severity/type of lesion and types of cytokine.
6. Assess how the CD8 T cell responses relate to immunity and pathogenesis. Determine the capacity of CD8+ T cells to kill *M. bovis* infected APC and the bacteria within the APC, and investigate the mechanisms.

4. ***Cytokine production by CD4 and CD8 T cells.***

IFNγ, IL-10, IL-4 and TNF responses have been monitored by ELISA in calves vaccinated with BCG and challenged with *M. bovis* and in calves exposed to *M. avium* followed by BCG vaccination or *M. bovis* infection. Details of the calf experiments and results are noted below in the context of the particular experiments. IFNγ responses were evident post vaccination with BCG, after exposure to *M. avium* and after infection with virulent *M. bovis*. IL-10 responses were evident post *M. bovis* challenge with a time course similar to that seen with IFNγ. No IL-4 responses were evident in any of the experiments. TNF responses were occasionally evident in animals challenged with *M. bovis*.

A clear correlation of IFNγ with extent of lesions at necropsy was evident. Recombinant antigens tested included ESAT-6, CFP-10, Ag85B, Rv3873, Rv3879, Rv1979c, Rv1769 (Cockle *et al* 2002). Of these ESAT-6 and CFP-10 were the most useful in detecting responses in animals infected with *M. bovis* although not all animals were responsive to these antigens as previously described (Vordermeier *et al* 2002; van Pinxteren *et al* 2000).

5. ***Cytokine synthesis in infected lymph nodes***
Lymph nodes (parotid, retropharyngeal, submandibular) were taken from (a) non-infected control calves, from (b) animals infected intranasally with *M. bovis*, from (c) animals vaccinated with BCG and challenged with *M. bovis* and from (d) animals vaccinated with BCG. All samples were taken 3-4 months after challenge or in animals of a similar age. Tissue was taken into guanidine thiocyanate (GTC) and RNA isolated to assay transcripts for a range of pro-inflammatory and anti-inflammatory cytokines by real-time PCR (Figure 3 and Table 1).

For each cytokine, differences between the four animal groups were assessed using a two-way factorial analysis of variance using a model that included the interaction term (vaccinated: challenged). The effects of each factor were tested independently using type III sums of squares. Where the interaction term was not significant, the significance of each factor was taken from the analysis of variance (ANOVA). Where the interaction term was significant, within group two-sample t-tests were used to assess the significance of each factor. Differences between groups were analyzed to determine whether infection, vaccination or an interaction between infection and vaccination had a significant effect on the cytokine expression level (Table 1).

This quantitative assay showed that levels of transcripts for IL-6, IL-10, IL-4 and TNF were significantly reduced in lymph nodes of infected animals compared to controls. Levels of transcripts for IL-12 and IFNγ were not significantly different. The observations indicate that *M. bovis* infection is producing a suppressive effect on several pro- and anti- inflammatory cytokines, possibly due to persistence of IL-12 and IFNγ responses and this effect on the local immune response may be a component of the ability of the bacteria to survive over long periods in the host. Vaccination had effects on IL-10 and IFNγ expression.

6. ***The CD8 T cell response in bovine TB***
The adaptive CD8 T cell response has been investigated in animals infected with *M. bovis* and animals vaccinated with BCG. A CD8 T cell response was evident when DC or mθ were used as the APC. Stimulation of bovine $CD8^+$ T-cells following vaccination with BCG (A) or infection with *M. bovis* (B) is shown in Figure 4. mθ and DC cultured for 5 days *in vitro* before pulsing with BCG were tested for their ability to stimulate $CD8^+TCR1^-$ T-cells expressing, or not expressing, CD45RO. Dendritic cells cultured for this length of time are more mature than the 3 day cultured DC used above and shown in Figures 1 & 2. These DC were found not to phagocytose BCG as effectively and 5-day mθ were better at stimulating antigen specific responses of CD8+ T cells than 5-day DC.

CD8 T-cells were subdivided into $TCR1^+$ and $TCR1^-$ in order to differentiate between γ/δ and α/β T-cell receptor positive cells. The response of $CD8^+$ T-cells was found to be variable between animals and could be detected at different time points in different animals. The cells that responded to infected mθ expressed $CD45RO^+$, a phenotype which is associated with memory/activated cells.

To further characterize bovine CD8+ T-cells, subsets were evaluated for their ability to synthesize IFNγ following mitogenic stimulation or their steady state expression of perforin. These two effector molecules are both necessary for

immunity against mycobacteria. Over 40% of $CD8^+$ T-cells producing IFNγ or expressing perforin were found to be $CD45RO^+$ (Figure 4C) which is the phenotype of the T-cells responding to APC infected with mycobacteria.

Interference in immune responses to M. Bovis by environmental mycobacteria

Prior exposure to environmental mycobacteria affects the immune response that is produced by cattle following infection with virulent *M. bovis* or vaccination. Both the recognition of bacterial antigens and the bias of the cellular immune response may be affected. Both could have consequences for the pathogenesis of the disease and the development of immunity to vaccination. Immune responses other than the development of the delayed hypersensitivity reaction and synthesis of IFNγ by lymphocytes may be affected by environmental bacteria and these responses have not been well characterized. Prior exposure to environmental mycobacteria could also have consequences for diagnosis of infection, the tests for which are dependent on assaying the immune response that has been mounted.

Objectives

7. How does exposure to environmental bacteria (in this model *M. avium*) affect the immune response to infection with virulent *M. bovis* and the pathogenesis of infection?
8. Whether prior exposure to environmental mycobacteria (*M. avium* in this model) affects the immune response to vaccination (BCG in this model) and the immunity that is engendered to virulent *M. bovis* challenge.

7. ***The effect of exposure to environmental bacteria (M. avium model) on the immune response and pathogenesis of M. bovis in cattle***

   In this experiment there were 4 groups of calves. In one group 5 calves were inoculated with *M. avium* on week 0 followed by *M. bovis* on week 12 (*M. avium/M. bovis* group). In a second group 5 calves were inoculated with *M. avium* on week 0 (*M. avium* alone group). In a third group 5 calves were inoculated with *M. bovis* on week 12 (*M. bovis* alone group). A fourth group of 4 calves remained as non-exposed controls. All animals were skin tested at week 24 and subsequently necropsied.

   Exposure to *M. avium* induces partial protection against disease caused by *M. bovis* challenge

   One group of 5 calves inoculated with *M. avium* and another group inoculated with medium (control) were challenged intra-nasally 12 weeks later with 10^4 *M. bovis*. The effect of prior *M. avium* exposure on the development of disease was assessed by post mortem examinations performed 12 weeks post challenge (Table 2). Macroscopic and microscopic lesions typical of TB were observed in 4 of 5 animals that were exposed to *M. bovis* only and to *M. avium* plus *M. bovis.* In both groups of calves the lesions were confined largely to the lymph nodes in the head, with one animal in each group found to have TB lesions in a single bronchial lymph node. The extent of lesions varied between animals. Differences were observed in the lesion scores of the *M. avium-M. bovis* group compared to the *M. bovis* alone group but these differences were not significant. No lesions were found in calves exposed only to *M. avium* or in the mock control group of calves. Bacteriological examination of tissues indicated that *M. bovis* was present in numbers up to 5.2×10^4 cfu per gram from the majority of tissues with gross

lesions (17/23 tissues; Table 3), with a significant correlation between the number of isolated bacteria and lesion score ($p < 0.01$). Significantly fewer bacteria were present in tissues from the *M. avium-M. bovis* group compared to the *M. bovis* alone group ($p = 0.045$).

Effect of prior exposure to *M. avium* on development of skin test response to avian and bovine PPD

Reactions to avian and/or bovine PPD in the skin test were observed in 14/15 animals exposed to either *M. bovis* or *M. avium* but not in 4 control calves (Table 4). The standard interpretation of the comparative intradermal skin test for diagnosis of bTB states that, for an animal to be classed as an *M. bovis* reactor (implying infection with *M. bovis*), the increase in skin test thickness with PPD-B must be >4mm than that seen with PPD-A. All four of the 5 animals in the *M. bovis* alone group that had lesions at necropsy had responses to PPD-B that were > 4mm greater than those to PPD-A, compared with only one of five animals, three of which had lesions, in the *M. avium-M. bovis* group. The difference in response between PPD-B and PPD-A is important for the interpretation of the diagnostic skin test and was significantly higher in the *M. bovis* alone group compared to the *M. avium-M. bovis* group ($p = 0.02$). Animal number 282 which had the highest pathology score in the *M. avium-M. bovis* group (lesion score 9) was not detected as a reactor using the standard interpretation of the test (B-A = 0mm). Using the severe interpretation of the skin test, applicable to herds with previously confirmed TB cases, the majority of animals would be identified as, at least, inconclusive reactors (including number 282) and would be subject to repeat testing. However, this would not be the case for single animals on farms which could be missed. Skin test reactions were strongly PPD-A biased in the *M. avium* alone group; all 5 animals had responses to PPD-A that were greater than 4mm above those to PPD-B. Skin test reactivity in the mock control group was insignificant (Table 4).

Cytokine secretion in whole blood following *M. avium* exposure

Immune responses were monitored for 12 weeks in 2 groups of calves inoculated with *M. avium* (Figures 5a and b) and 2 groups of mock-inoculated animals (Figure 5c and d). Exposure to *M. avium* induced increases in IFNγ secretion in response to PPD-A. This response was evident as early as 2 weeks post *M. avium* exposure and peaked at 6 weeks. The PPD-A specific IFNγ remained detectable throughout the 12 week period but was reduced significantly by week 12. Inoculation of *M. avium* also induced IFNγ secretion in response to PPD-B. However, although the kinetics of PPD-A and PPD-B responses were similar, the PPD-B responses were lower than those to PPD-A at all time points. No significant secretion of IFNγ was observed in the control mock-inoculated calves.

Cytokine responses post *M. bovis* challenge

At 12 weeks two groups of calves were challenged with *M. bovis* (Figures 5b and c). The remaining calves were not challenged (Figure 5a and d). Lymphocytes from both groups of challenged calves secreted IFNγ post challenge (Figure 5b & c). In the *M. avium-M. bovis* group (Figure 5b), increased IFNγ secretion to PPD-A was observed by 2 weeks. The IFNγ secreted was biased towards PPD-A with lower amounts secreted in response to PPD-B. The ratio of PPD-B: PPD-A induced IFNγ was highest at week 22 but was consistently lower than 2 in all

animals. Interestingly, the highest PPD-B: PPD-A IFNγ ratio was noted for animal #282 at week 22 (ratio 1.6). This animal had the highest lesion score within the *M. avium-M. bovis* group. In the *M. bovis* alone group strong PPD-B biased IFNγ secretion was observed at 4 weeks post challenge. A lower level of IFNγ was secreted in response to PPD-A in these calves and this did not reach the levels observed in calves that were pre-exposed to *M. avium*. The ratio of PPD-B:A induced IFNγ was consistently >2 in all animals except #336 which was skin test negative and had no visible lesions at post mortem. Overall the ratio of PPD-B:A induced IFNγ was higher in the *M. bovis* alone animals compared to the *M. avium-M. bovis* calves.

Although PPD-B specific IL-10 was also observed in all of the calves infected with *M. bovis*, the levels were similar in both the *M. avium-M. bovis* and the *M. bovis* alone groups and there was no significant difference between the groups at any time post challenge (data not shown).

Comparison of responses to ESAT-6 and CFP-10 with those to PPD

There were significant differences between calves in reactivity to ESAT-6 and CFP-10 in both the *M. avium-M. bovis* group and the *M. bovis* alone group. The number of time points at which ESAT-6 and CFP-10 IFNγ responses were positive, and the magnitude of these responses, are indicated in Table 5. In this table supernatants taken from blood cultured with antigen have been compared at weeks 4, 6, 8, 10 and 12 post *M. bovis* challenge. By this time an immune response will have developed. A sample is considered positive when the number of pg/ml of IFNγ is > 250 pg than is seen in control samples incubated with medium (for ESAT-6 or CFP-10) or PPD-A (for PPD-B).

Two calves failed to give a positive response to any antigen. These were calves #270 and 336 that did not appear to have been productively infected with *M. bovis* as they displayed no skin test reactions and had no visible lesions and were *M. bovis* culture negative.

Two of the four animals in the *M. avium-M. bovis* group with confirmed disease escaped detection via the IFNγ test after the use of PPD at all 5 sampling points post *M. bovis* challenge. However, all four infected animals were correctly identified as infected with *M. bovis* at 2 or more of the 5 sampling points post challenge when ESAT-6 or CFP-10 were used as diagnostic antigens (Table 5). Furthermore, calf number 282 although detected by ESAT-6 on 2 occasions, failed to respond on other sampling points, did not have PPD-B biased skin test reactivity, but did have significant disease (lesion score 9). Animals inoculated with *M. bovis* alone that had lesions gave positive responses with PPD or ESAT-6 and CFP-10 on most, but not all occasions (Table 5). None of the calves in the mock control group or the *M. avium* alone group responded to these antigens (data not shown). The implication is that these recombinant antigens are useful for diagnosing infections but they do not detect all animals on all occasions and are less effective in *M. avium* primed animals. Thus, single time point diagnoses using these reagents would not be expected to be reliable.

IFNγ secretion by lymphocyte subsets

To determine the lymphocyte subset(s) producing IFNγ in response to mycobacterial antigens, intracellular IFNγ expression by $CD4^+$ and $CD8^+$ lymphocytes was assessed in cultures of PBMC which had been stimulated by *M. avium* or *M. bovis* infected DC (Figure 6).

Four weeks following inoculation of *M. avium*, significant ($p < 0.05$) stimulation of $CD4^+$ cells by *M. avium* infected DC was observed (Figure 6a; square symbols), this response had waned by week 12. This CD4 response was transiently boosted in the *M. avium-M. bovis* group 4 weeks post challenge (Figure 6a; open squares). In those animals which were inoculated only with *M. bovis*, no *M. avium* reactive $CD4^+$ cells were observed (Figure 6a; closed circles).
High non specific responses of $CD8^+$ T cells to *M. avium*-infected DC were observed at time 0 in the majority of animals (Figure 6b) and these did not appear to be altered by exposure of the animals to *M. avium* (Figure 6b).

Prior to *M. bovis* challenge no significant responses of either CD4 or $CD8^+$ cells were noted following stimulation with *M. bovis* infected DC. However, following *M. bovis* challenge increases in both $CD4^+$ and $CD8^+$ T cell responses were observed. Four weeks post challenge increased IFNγ secretion by $CD4^+$ T lymphocytes in response to *M. bovis* infected DC was observed (Figure 6c; $p < 0.05$). That was reduced significantly ($p < 0.05$) by week 8 post challenge (Figure 6c; open squares). By contrast, in the *M. bovis* alone group the response of the $CD4^+$ cells to *M. bovis* DC was still increasing at week 8 (Figure 6c; closed circles).

The CD8 response to *M. bovis* infected DC was slightly increased by 4 weeks post challenge and had waned by 8 weeks in the *M. avium-M. bovis* group (Figure 6d; open squares), but not in the *M. bovis* alone group, where responses were increased at week 8 post challenge (Figure 6d; closed circles)

The percentage of IFNγ $^+$ CD4 or CD8 cells at week 8 post-challenge was correlated with lesion score ($p < 0.05$). Each of the animals in a given group showed a similar response pattern except #336 which showed little or no stimulation of T cells by either *M. avium* or *M. bovis* infected DC. No significant responses were noted in the mock control group (Figure 6; open circles).

8. ***The effect of environmental mycobacterium (M. avium model) on immune response and protection induced by BCG***

This experiment comprised 4 groups of 6 calves. Group 1 was inoculated with *M. avium* at week 0, vaccinated with BCG at week 12 and challenged with *M. bovis* at week 24. Group 2 was inoculated with BCG at week 12 and challenged with *M. bovis* at week 24. Group 3 was challenged with *M. bovis* at week 24. Group 4 was inoculated with *M. avium* at week 0 and BCG at week 12. All animals were necropsied at week 36.

Animals exposed to *M. avium* before vaccination with BCG were similarly protected

The extent of lesions at necropsy for the animals challenged with *M. bovis* are recorded in Table 6. No lesions of TB were evident in animals in the group exposed to *M. avium* and then BCG but not challenged with *M. bovis*. Animals vaccinated with BCG were protected against disease produced by *M. bovis*, as judged by reduced extent of lesions ($p = 0.039$ Mann Whitney). Animals exposed to *M. avium* before vaccination with BCG were also protected against *M. bovis* ($p = 0.022$). There was no difference between the animals given *M. avium* before BCG and those that were not ($p = 0.775$).

Animals exposed to *M. avium* before BCG produce an immune response biased towards avian antigens

The IFNγ responses in the four groups of calves are shown in Figure 7. Exposure to *M. avium* produced a response biased towards avian antigens. This avian bias was boosted by vaccination with BCG and contrasts with the response to BCG alone which did not have this avian bias. The response to *M. bovis* alone was strongly biased towards bovine antigens but in animals previously exposed to *M. avium* and BCG was biased towards avian antigens. These observations are consistent with those published previously (Howard *et al* 2002) in which *M. avium* primed for an avian response on vaccination with BCG. It also extends the observations above and shows that even following BCG the prior exposure to *M. avium* masks the bovine bias seen with PPD after *M. bovis* infection. Of importance is the finding that the BCG response was boosted, not inhibited by *M. avium* and protection was not compromised.

The diagnosis of TB by the skin test is masked by prior exposure to *M. avium*

The responses in the comparative intradermal skin test of the calves in the four groups are compared in Table 7. All six animals inoculated with *M. bovis* alone developed TT responses indicative of infection. All six animals inoculated with BCG and challenged with *M. bovis* had TT that were biased towards the bovine antigen but less extensive than seen in the non-vaccinates. The animals inoculated with *M. avium* before BCG had *M. avium* biased responses in 4/6 cases and a bovine biased response in 1/6 cases. Animals exposed to *M. avium* followed by BCG and challenged with *M. bovis* had responses biased towards PPD-B of > 4 mm in 2/6 cases and these were animals with lesions. None of the other animals had lesions although two of these would be classed as IR using the severe TT interpretation.

Induction of effective immune responses to vaccination

Older cattle may not respond well to vaccination because of prior exposure to environmental mycobacteria. Vaccination of neonates may be an effective way of immunising cattle to circumvent this problem. An appropriate cytokine bias in the immune response may be essential for effective vaccination. The use of Th1 biasing components could be the most effective way of vaccinating.

Objectives

9. Determine whether the immune response of conventionally reared neonatal calves differs from that seen in older animals using BCG as the model vaccine.
10. Test the hypothesis that inclusion of a cytokine that biases a Th1 response in a DNA vaccine enhances the effectiveness of vaccination.

9. ***Comparison of immune responses of calves aged 1day to 3 weeks with those of older animals***

   Groups of 5 calves aged 1 day, 1 week, 3 weeks and about 9 months were vaccinated subcutaneously with 10^6 cfu BCG Pasteur. Five calves aged 3 weeks and 4 calves aged 1 day remained as non-vaccinated controls. The immune response compared in the IFNγ test is shown in Figure 8. The response of all animals was similar with the younger animals responding as intensely as the older ones. The younger animals were challenged with 10^4 cfu *M. bovis* intranasally 12 weeks after vaccination and the extent of lesions at necropsy 12 weeks later shown in Table 8. Vaccinates had less extensive lesions compared to controls and there was no difference for animals vaccinated at 1 day, 1 week or 3 weeks.

10. ***Effect of including IL-12 with DNA vaccination***

    Cattle were inoculated at weeks 0 and 3 with nothing; plasmid encoding Ag85; plasmid encoding Ag85 and IL-12; plasmid encoding Ag85 and IL-18; and plasmid control ie encoding IL-12 and IL-18 (not shown, results were similar to those in animals inoculated with nothing). After six weeks all animals were inoculated subcutaneously with approximately 10^6 BCG Copenhagen. Blood taken at regular intervals was stimulated in culture with medium alone (N.C.), PPD-B, Modified Vaccinia Ankara (MVA) virus wild type (MVA-WT) or MVA expressing Ag85A (MVA-Ag85) or purified mycobacterial Ag85 complex. Supernatants were collected after 24 hr and assayed for the presence of IFNγ using a standard IFNγ ELISA. Following immunization with recombinant plasmid, no Ag85 specific responses were detected in any of the groups and no boosting of the response to plasmid or to heterologous boost with BCG noted (Figure 9). Thus, no evidence was obtained for an adjuvant effect of IL-12 and or IL-18 on priming with DNA.

Conclusions

Infection of DC increased secretion of IL-12, whereas infection of mθ induced secretion of IL-10, an immunoregulatory cytokine. Survival of *M. bovis* was significantly higher in DC than mθ. Differential infection of APC could influence the outcome of infection. Lymph nodes from infected animals showed altered cytokine profiles suggesting modulation of the immune response by *M. bovis*. A CD8 response was evident in *M. bovis* infected cattle. This was restricted to the $\alpha\beta$TCR$^+$CD45RO$^+$ subset expressing IFNγ and perforin. *M. avium* conferred a degree of protection against *M. bovis* but interfered with diagnosis of infection by the skin test and IFNγ test. Use of ESAT-6 and CFP-10 improved diagnosis of *M. avium* exposed animals with disease. DNA vaccination was not enhanced by the addition of IL-12 and IL-18. Vaccination of animals aged 1 day to 3 weeks with BCG induced significant protection against *M. bovis,* with reduced severity of disease and bacterial load.

Future research

- Neonatal vaccination with BCG is a promising strategy that requires further research. The duration of the immune response induced by BCG should be addressed to assess the requirement for boosting the immunity of animals in the field.
- Strategies for enhancing the protective efficacy of BCG need to be examined for example heterologous prime boosting using viral vectors.
- There is a requirement for improved diagnostic tests capable of discriminating BCG vaccinated from infected individuals. This is essential if BCG is ever to be used as a field vaccine.
- Marked differences in responses to infection were noted. Genetic differences between animals that could contribute to resistance should be explored.

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Publications generated from the project

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Clin. Exp. Immunol. **141**, 432-439.

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Hope JC, Thom ML, McCormick PA, Howard CJ (2004).
Interaction of antigen presenting cells with mycobacteria.
Vet. Immunol. Immunopathol. **100**, *187-195.*

Widdison S, Schreuder L, Villarreal-Ramos B, Howard CJ, Watson M, Coffey TJ (2006).
Cytokine Expression Profiles of Bovine Lymph Nodes: Effects of *Mycobacterium bovis* Infection and BCG Vaccination.
Clin. Exp. Immunol. in press.

Thom ML, Widdison S, Coffey T, Howard CJ, Hope JC.
Bovine macrophages and monocyte derived dendritic cells produce different cytokine profiles in response to infection with virulent and avirulent *Mycobacterium bovis.*
Submitted for publication.

Review comment

This was a well received immunology/pathogenesis study with some very applied outcomes relating to vaccine development. The technologies concerned with defining the bovine immune system, developed at IAH over the past decade, have been exploited well and this project has led to some nice publications, although the variations in cytokines can be difficult to interpret in terms of vaccine designs and diagnostics. Here it was felt that there was a lack of a crystallised unifying hypothesis on role of the various molecules and assays measured, in terms of possible applications towards novel vaccines and/or diagnostic procedures. That said, the finding that BCG given to young calves might be protective should be followed up further. Another valuable finding was that relating to the interaction between *M. avium* and *M. bovis* infection/ vaccination, although in the field situation most strains of *M. avium* would likely infect via a mucosal route so it is uncertain how representative were the strain of *M. avium* used and the method of inoculation in this study. As a final note, the final report could have been more accessible had greater emphasis been placed on the implications of the results and how they support, complement, further, replicate or contradict the existing body of scientific evidence.

Scores

Conclusions based on sound evidence: 3.1
Quality of science: 3.1
Overall rating: 2.9

Figure 1
Differences in IL10, IL12 and TNFα synthesis by macrophages and dendritic cells (DC) infected in vitro with BCG or *M.bovis*
Four animals compared

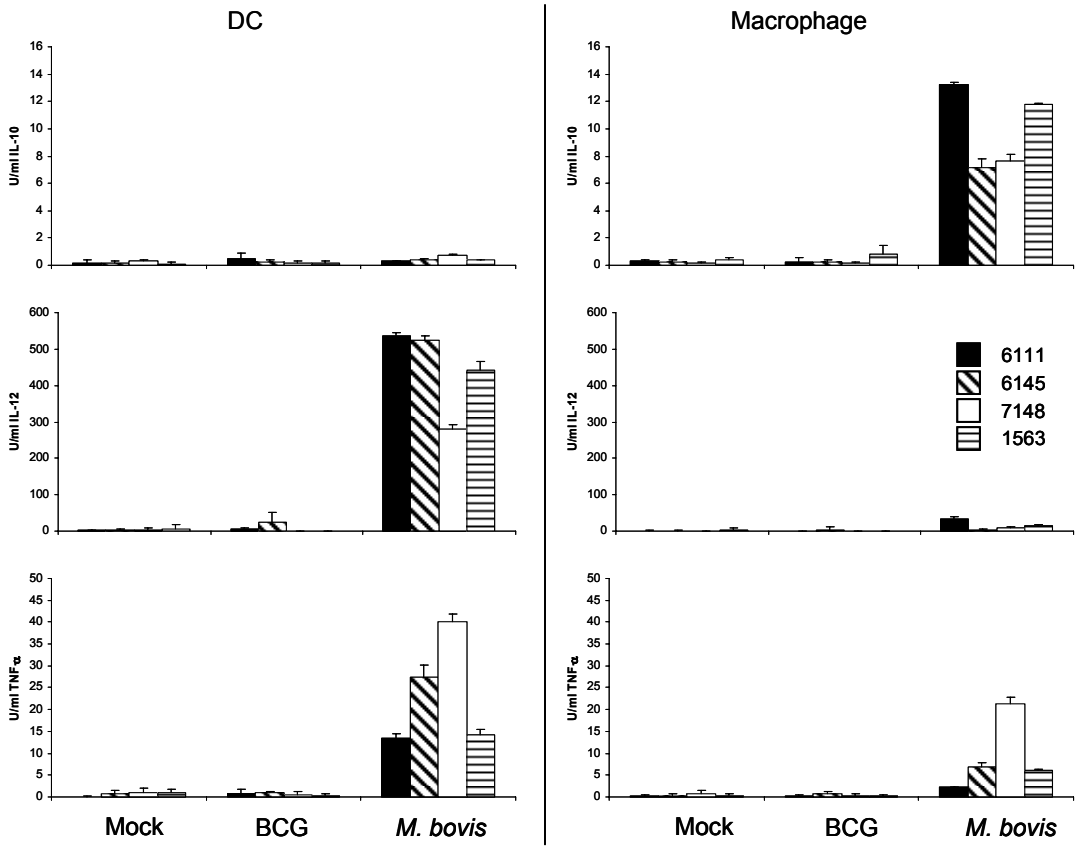


Figure 2.
Comparison of dendritic cells and monocytes for ability to stimulate CD4 and CD8 T cells.
DC = dendritic cells. Mo = monocytes.
Proliferative response after 5 days shown.

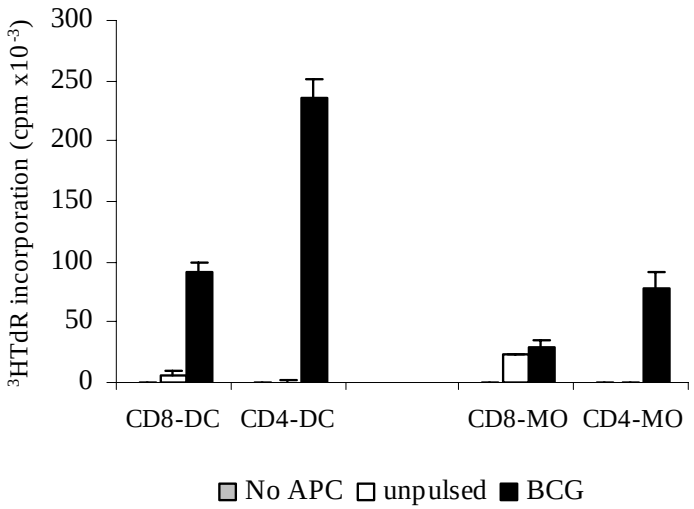


Figure 3.
Real-time PCR analysis of cytokine production by bovine lymph nodes.
IL-4 (A), IL-6 (B), IL-10 (C), TNF (D), IL-12 p40 (E) and IFN-γ (F)
Log(2) copy number per 100ng lymph node cDNA starting template.
○– Unchallenged, unvaccinated animals, ● – unchallenged, vaccinated animals,
□ –challenged, unvaccinated animals, ■ – challenged, vaccinated animals.
Results are averaged triplicate values for each lymph node sample.
Error bars indicate SD of each sample.

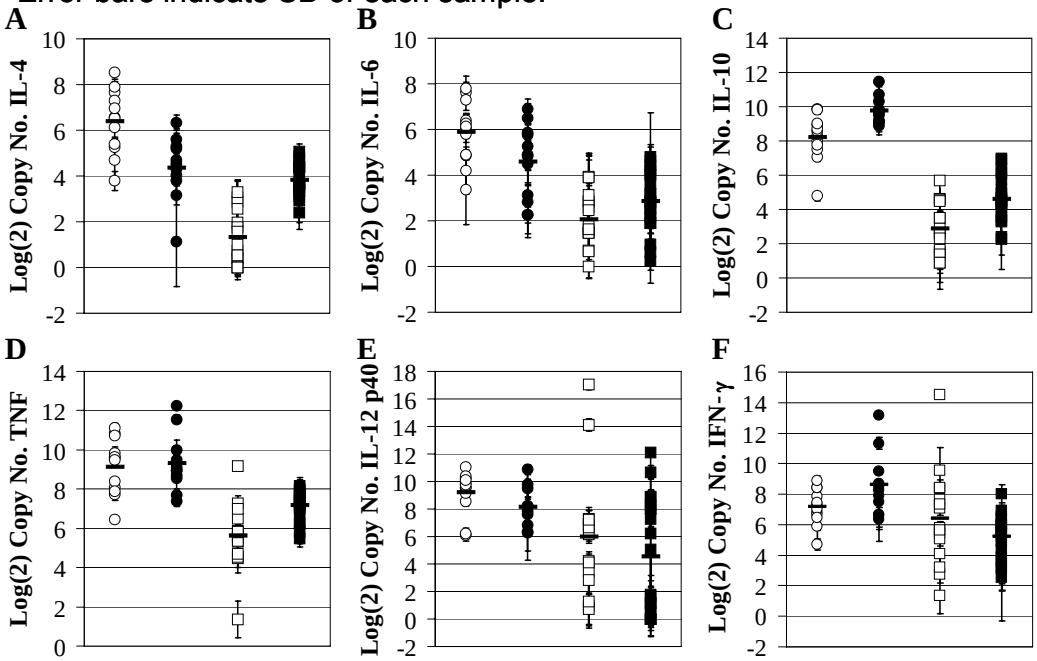


Figure 4.
Stimulation of bovine CD8+ T cells following vaccination with BCG (A) or infection with *M. bovis* (B) and perforin expression by CD8 T cells (C)

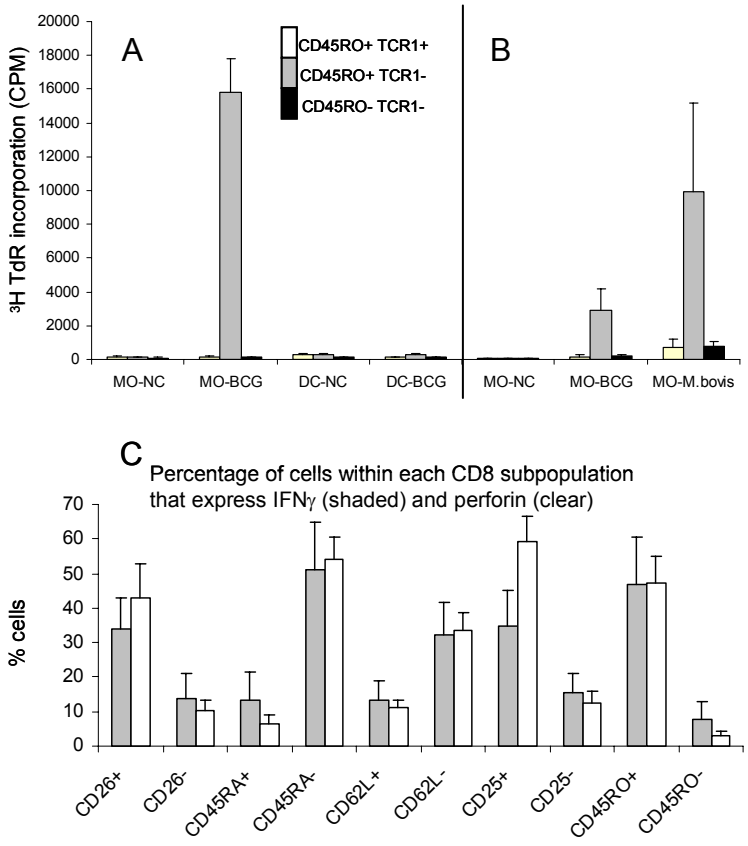


Table 1 Effect of infection and vaccination on cytokine expression levels.

Cytokine	Infection effect	Vaccination effect	Interaction effect
IL-4	**<0.001**	0.484	**<0.001**
IL-6	**<0.001**	0.633	**0.048**
IL-10	**<0.001**	**0.004**	0.917
TNF	**<0.001**	0.105	0.193
IL-12 p40	0.059	0.308	0.847
IFNγ	**0.007**	0.942	**0.040**

Figures indicate P-values for the effect of infection, vaccination or an interaction between the two factors on the cytokine expression level per animal. Bold type indicates significance (P<0.05)

Table 2 Lesions at necropsy 12 weeks post *M. bovis* challenge in *M. avium* exposed and control calves.
No lesions were evident in tonsils, diaphragmatic, apical, medial or intermediate lobes of the lung, or in 3 other bronchus associated lymph nodes. L and R = left and right. Twenty two tissues were examined for each animal. Score 0 – 4[a] indicates increasing severity of macroscopic lesions.

Tissue ⇓	***M. avium* + *M. bovis* group**						***M. bovis* only group**					
Animal Number ⇒	270	291	356	349	282	Median	336	286	277	353	343	Median
Parotid R	0[a]	0	0	0	1		0	3	0	3	2	
Parotid L	0	0	0	0	0		0	0	0	0	3	
Submandibular R	0	0	0	3	1		0	0	3	3	1	
Submandibular L	0	0	0	0	3		0	0	0	0	2	
Retropharyngeal R	0	1	3	0	0		0	0	3	2	4	
Retropharyngeal L	0	0	1	3	1		0	0	3	4	3	
Mediastinal	0	0	0	0	0		0	0	0	0	0	
Bronchial 1	0	0	0	0	3		0	2	0	0	0	
Total Tissues Affected	**0**	**1**	**2**	**2**	**5**	**1.5**	**0**	**2**	**3**	**4**	**6**	**3**
Total Lesion Score	**0**	**1**	**4**	**6**	**9**	**4**	**0**	**5**	**9**	**12**	**15**	**9**

Table 3 *M. bovis* viable counts in tissues taken post-mortem.
[a]Viable count (log_{10} cfu per g tissue). The limit of detection using the isolation technique is 5 colonies. Six lymph nodes examined for each animal.

Tissue ⇓	*M. avium* + *M. bovis* group					*M. bovis* only group				
Animal Number ⇒	**270**	**291**	**356**	**349**	**282**	**336**	**286**	**277**	**353**	**343**
Parotid R	0[a]	0	0	0	0	0	4.6	0	3.61	3.32
Parotid L	0	0	0	0	2.98	0	1.69	0	0	3.86
Submandibular R	0	0	2.78	4.15	0	0	0	4.19	4.08	2.65
Submandibular L	0	0	0	0	4.48	0	0	4.22	4.23	3.51
Retropharyngeal R	0	0	3.7	3.52	3	0	0	4.72	4.22	3.97
Retropharyngeal L	0	0	0	4.16	0	0	0	0	0	4.05
Total Number of Tissues Affected	**0**	**0**	**2**	**3**	**3**	**0**	**2**	**3**	**4**	**6**

Table 4 Skin test responses 12 weeks post challenge with *M. bovis.*
[a]Animal number. [b]Increase in skin thickness from day 0 to day 3. med = median. [c]Interpretation of tuberculin skin test (according to EC regulation 1226/2002). R = reactor; I = inconclusive reactor; N= negative.
***** Statistically significant difference between these groups p = 0.03

Group	*M. avium* / *M. bovis*						*M. bovis* alone						*M. avium* alone						Control				
	270[a]	291	356	349	282	med	336	286	277	353	343	med	279	283	304	339	329	med	273	289	333	368	med
PPD-A[b]	17	13	14	14	3	13	1	7	7	9	9	7	13	7	11	4	8	8	0	0	-1	0	0
PPD-B[b]	2	11	18	21	3	11	0	23	25	21	37	23	4	1	5	0	3	3	0	1	-1	-1	0
B-A[b]	-15	-2	4	7	0	0*	-1	16	18	12	28	16*	-9	-6	-6	-4	-5	-5.5	0	1	0	1	0.5
Skin Test Interpretation[c]																							
Standard	N	N	I	R	N		N	R	R	R	R												
Severe	N	I	R	R	I		N	R	R	R	R												

Figure 5.
IFNγ response in calves exposed to M. avium before infection with *M. bovis*
(a) *M. avium* alone **(b)** *M. bovis* alone (week12) **(c)** *M. avium* followed by *M. bovis* (week 12)
(d) Mock inoculated controls

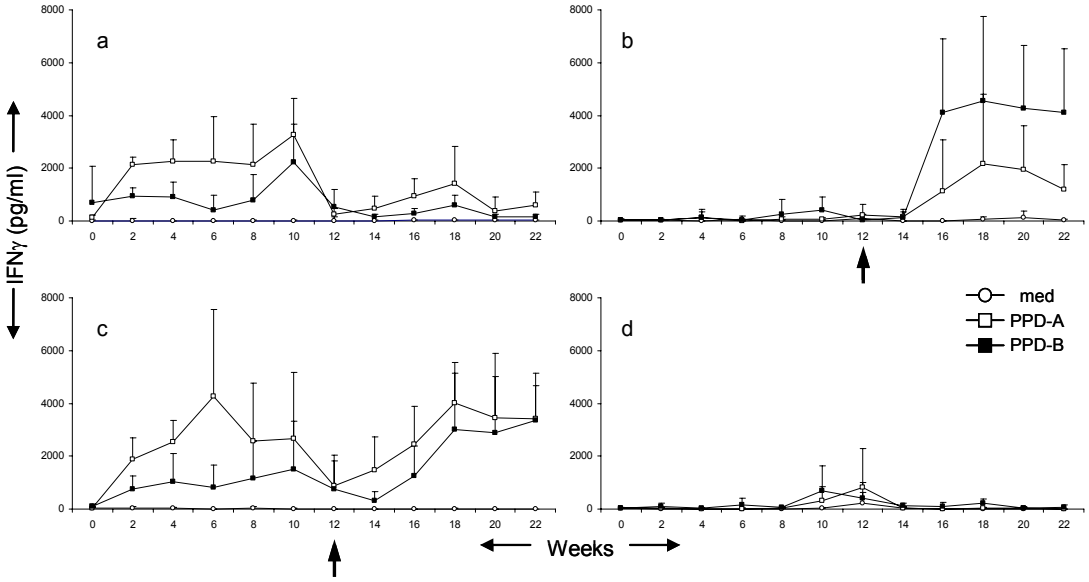


Table 5 Summary of time points when a positive diagnosis of infection was obtained by IFNγ test using PPD or recombinant antigens.
[a]Animal number
[b]A>B etc indicates response with PPD-A or PPD-B was 250 pg greater than with the other antigen.
* Number of time positive out of 5 possible time points, weeks 4,6,8,10 and 12 post *M. bovis* challenge.
** At these time points the test was negative (ie < 250 pg per ml IFNγ more than in controls)
Range of IFNγ pg/ml indicated in parentheses.

Group	*M. avium* / *M. bovis*					*M. bovis* alone				
	270[a]	282	291	349	356	277	286	336	343	353
Lesions	-	+	+	+	+	+	+	+	+	+
	No. positive time points*									
E6 (range: pg/ml IFNγ)	0	0	0	3 (3333-3979	3 (358-639)	0	0	0	4 (4760-3962)	2 (586-636)
CFP-10 (range: pg/ml IFN γ)	0	2 (353-375)	2 (328-2016)	0	2 (281-303)	3 (967-1566)	3 (331-678)	0	4 (1341-3975)	4 (712-3711)
CFP-10 or ESAT-6	0	2	2	3	3	3	3	0	4	4
A>B	5	3	4	1	2	0	0	0	0	2
B>A	0	2	0	1	0	4	4	0	4	3
B=A	0	0	1	3	3	1**	1**	5**	1**	0

Figure 6
IFNγ synthesis by CD4 or CD8 T cells incubated with dendritic cells infected with *M. avium* or *M. bovis*

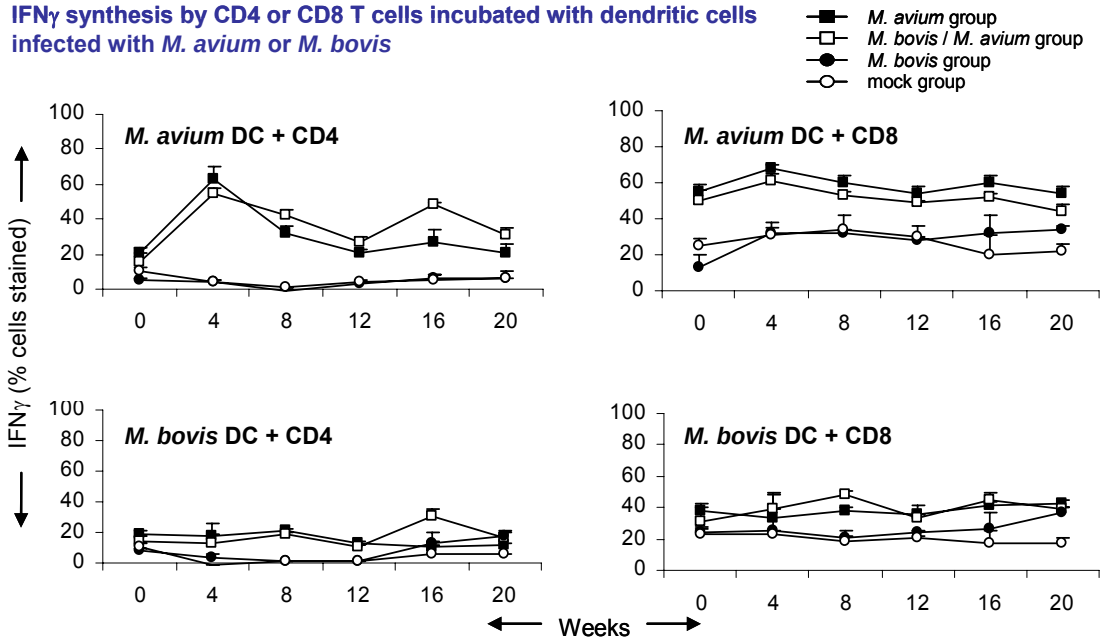


Table 6 Extent of lesions at necropsy for animals exposed to *M. avium* before BCG.
No lesions were present in animals exposed to *M. avium* and then BCG but not challenged.

Group ⇒	***M. avium* / BCG / *M. bovis***						**BCG / *M. bovis***						***M. bovis***					
Parameter ⇓	493	497	522	527	541	559	494	498	502	523	542	568	496	500	505	524	529	575
Head nodes total pathology score	0	0	2	0	3	0	0	5	0	0	8	0	14	1	8	13	2	10
Chest total pathology score	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total pathology score	0	0	2	0	3	0	0	5	0	0	8	0	14	1	8	13	2	10
Head no. tissues affected	0	0	2	0	2	0	0	2	0	0	4	0	5	1	3	4	1	5
Chest no. tissues affected	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total no. tissues affected	0	0	2	0	2	0	0	2	0	0	4	0	5	1	3	4	1	5

Figure 7.
IFNγ responses in calves inoculated with *M avium* (week 0) prior to BCG vaccination (week 12) and challenge with *M bovis* (week 24)

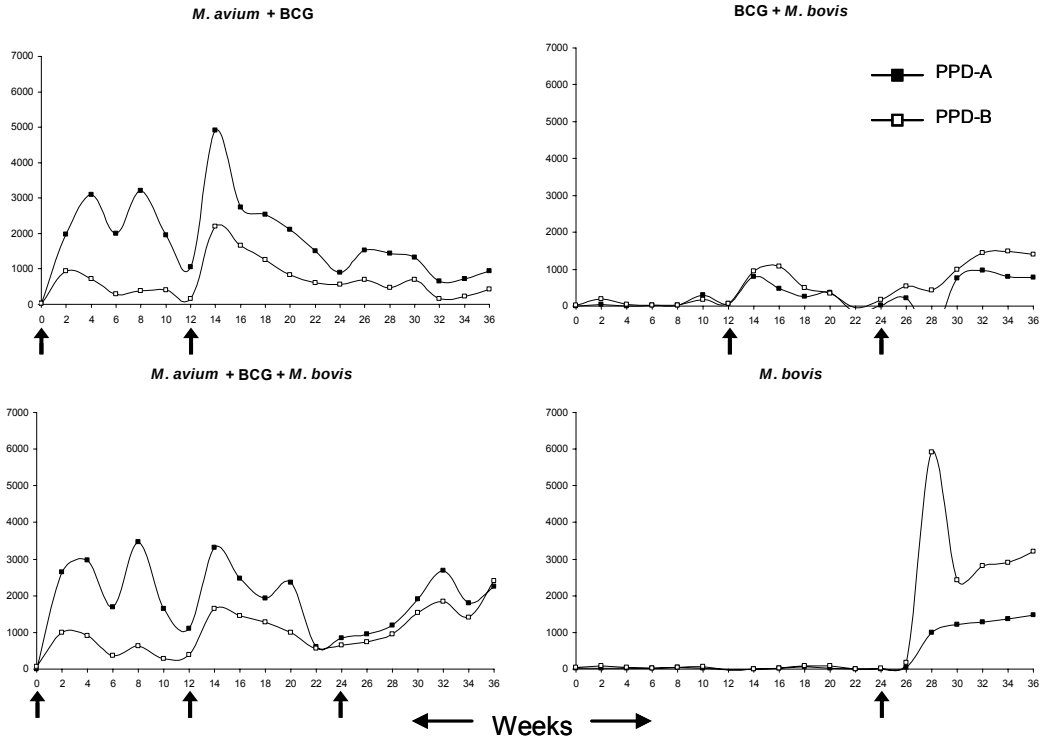


Table 7 Responses in the comparative intradermal skin test of animals exposed to *M. avium* before BCG and *M. bovis* challenge.
[a]Animal No. [b]Increase in skin thickness in mm

M. avium / BCG group				BCG / *M. bovis* group			
	PPD-A	PPD-B	B-A		PPD-A	PPD-B	B-A
301492[a]	10[b]	6	-4	501494	9	24	15
501501	9	10	1	201498	9	32	23
501508	11	5	-6	601502	6	14	8
101525	11	4	-7	601523	6	9	3
101532	12	8	-4	401542	10	21	11
601551	9	4	-5	201568	5	14	9
M.avium / BCG / *M. bovis* group				*M. bovis* group			
	PPD-A	PPD-B	B-A		PPD-A	PPD-B	B-A
401493	26	16	-10	701496	2	25	23
101497	15	17	2	401500	10	35	25
501522	12	19	7	201505	8	33	25
301527	13	8	-5	701524	2	27	25
301541	8	16	8	501529	12	53	41
701559	18	19	1	201575	5	18	13

Figure 8
Response of calves of different ages to BCG Pasteur

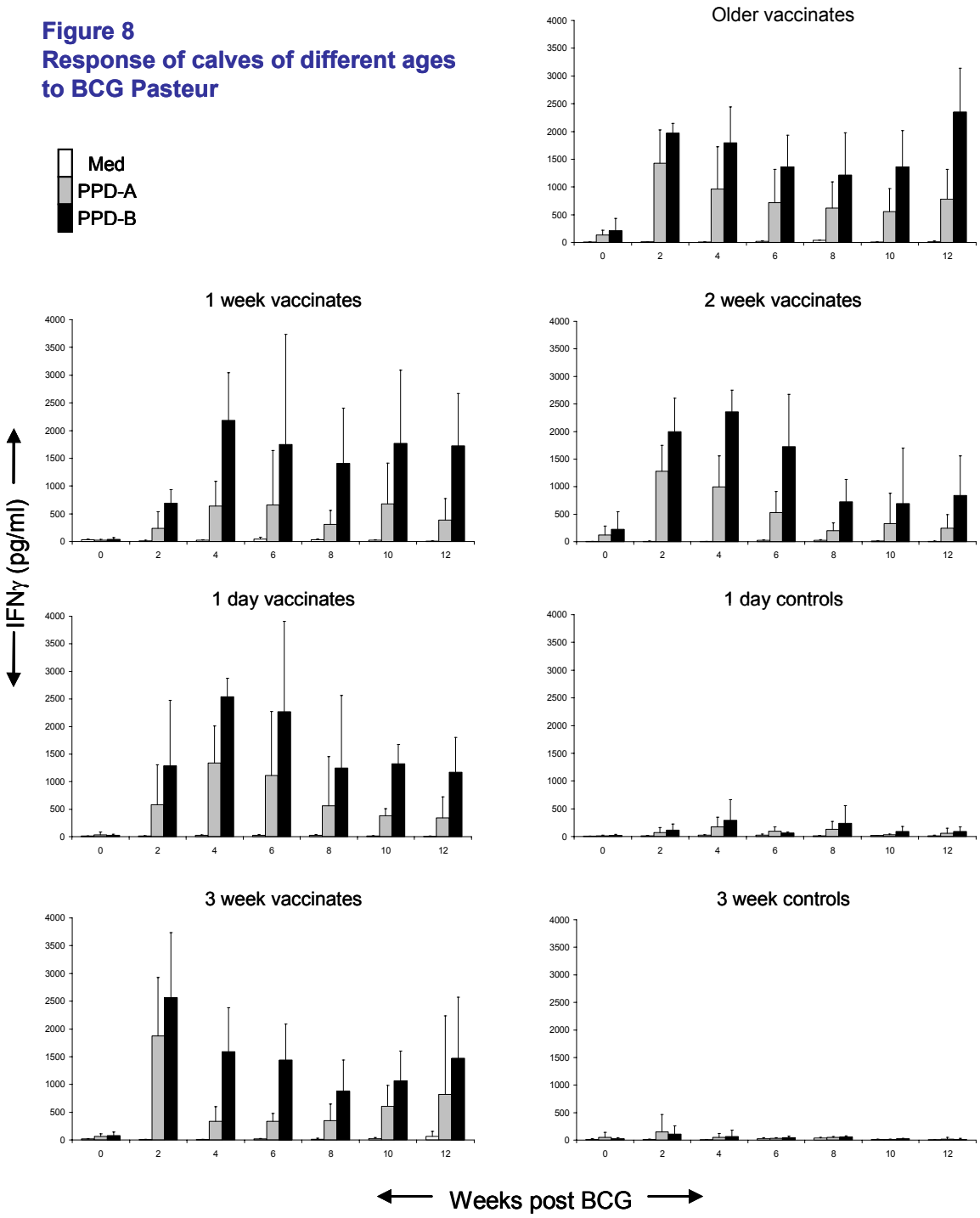


Table 8 Lesions at necropsy of animals vaccinated aged 1 day to 3 weeks compared to unvaccinated controls.

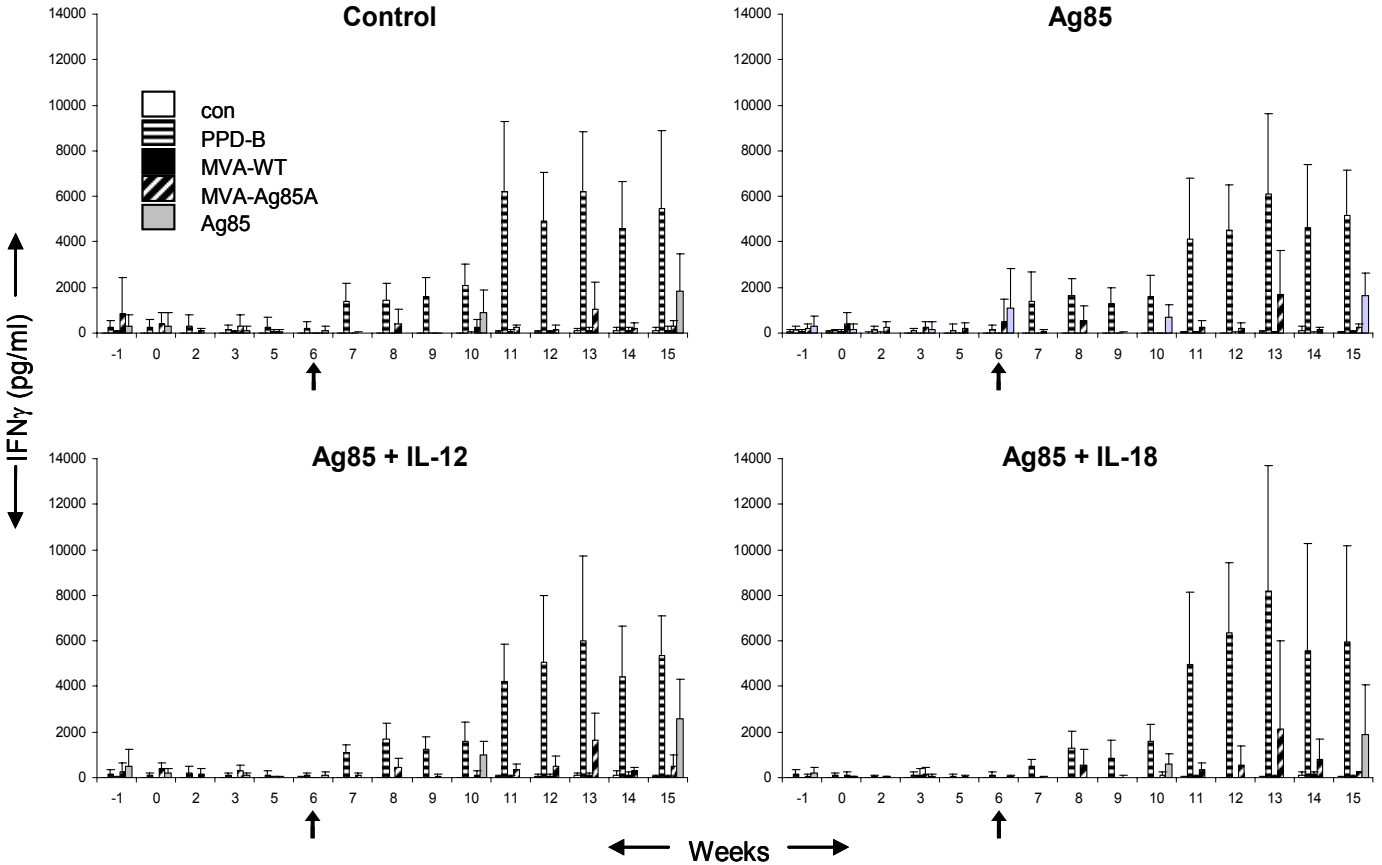
[a]Animal No.

Group ⇒	**1 week vaccinates**					**1 day vaccinates**					**1 day control**			
	[a]520	526	530	545	546	540	548	554	633	642	549	555	558	564
Head nodes total pathology score	10	4	0	9	1	3	0	8	0	4	9	13	13	8
Chest pathology score	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Total pathology score	11	4	0	9	1	3	0	8	0	4	9	13	13	8
Head number tissues affected	6	3	3	5	1	3	0	6	0	2	4	5	5	4
Chest number tissues affected	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Total number tissues affected	**7**	**3**	**0**	**5**	**1**	**3**	**0**	**6**	**0**	**2**	**4**	**5**	**5**	**4**

Group ⇒	**3 week vaccinates**					**3 week control**				
	[a]582	583	600	601	604	623	624	628	629	630
Head nodes total pathology score	3	2	0	0	5	13	12	8	11	0
Chest pathology score	0	0	0	0	0	0	0	0	5	0
Total pathology score	3	2	0	0	5	13	12	8	16	0
Head number tissues affected	2	1	0	0	3	4	4	2	4	0
Chest number tissues affected	0	0	0	0	0	0	0	0	2	0
Total number tissues affected	**2**	**1**	**0**	**0**	**3**	**4**	**4**	**2**	**6**	**0**

Figure 9.
Production of IFNγ in whole blood assays

Cattle were inoculated at 0 and 3 weeks with either plasmid encoding Ag85; plasmid encoding Ag85 and IL-12; plasmid encoding Ag85 and IL-18 or plasmid control (encoding IL-12 & IL18, data not shown as it was essentially similar to the control group shown). All animals were inoculated subcutaneously with approximately 10^6 BCG Copenhagen at Week 6. The results shown are IFNγ levels in 24h whole blood cultures in response to the indicated antigens



SE3033 Housing of naturally infected cattle (field reactors) at VLA for immunological and bacteriological analysis

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/04
End date 30/09/07

Total cost £775,076

Abstract
Whilst *Mycobacterium bovis* infection in cattle can be modelled using experimentally infected cows, the 'natural' temporal development of bovine tuberculosis can only be studied with 'naturally' infected cattle held under observation for prolonged periods of time. Investigation of disease progression in field-reactor cattle not only allows the validation of findings obtained with experimentally infected cattle but also has direct impact on diagnostic and management strategies. To this end, we have housed and sampled so far sampled naturally infected reactor cattle for a period of up to 6 months. Sampling has included twice weekly nasal sampling for detection of *M. bovis* excretion, skin-tests at 60 day intervals, regular blood sampling to measure temporal changes in BOVIGAM IFNγ responses and for use in screening novel candidate antigen panels and finally extensive post-mortem examinations. Results generated to date include the observation that *M. bovis* excretion via nasal secretions is at best an extremely rare event since all nasal samples have been culture negative. Furthermore, measurement of BOVIGAM IFNγ responses before and after a recent skin-test has demonstrated that blood samples may be taken either prior to, or 3 and 10 days post skin-test without adversely influencing the diagnostic outcome of the BOVIGAM test. Study animals have also been used to screen a panel of 530 peptides mapping 42 candidate diagnostic antigens. From these, 8 promising peptides from 2 antigens (Mb2555 and Mb2890) have been identified for further evaluation. Investigations are also currently underway to compare lung and peripheral blood immune responses using endoscopic lavage procedures.

Aims

- To define novel mycobacterial antigens useful for diagnosis or vaccination, and to study the temporal changes in their recognition. In particular, emphasis will be placed on the identification of antigens that complement ESAT-6 and CFP-10.
- To define the effects of repeat short interval tuberculin skin testing on performance of the IFNγ test in *naturally* infected cattle.
- To monitor shedding of bacilli in nasal secretions in order to define how frequently shedding occurs in *naturally* infected animals in the UK, and how many organisms are excreted when shedding occurs.
- To define the temporal development of the *in vitro* IFNγ responses and other immunological parameters in *naturally* infected cattle and to compare immune responses at sites of disease and in the peripheral blood.

Relevance to Defra

Bovine tuberculosis remains an economically important problem in Great Britain with potential zoonotic consequences and Defra has a statutory obligation to control tuberculosis in farm animals in Great Britain. The incidence of bovine tuberculosis in GB has been increasing since 1988 and several policy options and research priorities are being pursued. This project is aimed at several of these objectives.

1. To improve the specificity and sensitivity of diagnostic tests remains a high research priority and this project addresses two aspects of diagnosis, namely, the definition of specific diagnostic proteins by antigen mining complementing other studies, and the refinement of the operational conditions of the BOVIGAM IFNγ assay.
2. Cattle vaccine development is another research priority for Defra, and comparison of local lung responses with those in the peripheral blood will complement planned studies with experimentally infected or vaccinated cattle to define correlates of disease or protection, whose definition will support vaccine development.
3. Determining the nasal shedding frequencies in naturally infected animals will support the risk assessment/modeling of cattle-to-cattle transmission under natural infection conditions.

Methods

- Recruitment and accommodation of up to 10 naturally infected cattle at any one time for a period of up to 6 months (per animal).
- Collection of nasal mucus for detection of *M. bovis* by culture and real-time PCR methodologies.
- Up to 3 short interval skin-tests per animal (60, 120 and 180 days post disclosing skin-test).
- At each skin-test, blood will be taken directly prior to, and 3 and 10 days post, skin-test and tested using the BOVIGAM IFNγ assay.
- Bi-weekly blood sampling of all animals to allow measurement of temporal changes in peripheral immune responses.
- Blood obtained from study animals will be used to screen libraries of overlapping peptides of antigens produced in the context of project SE3028 and SE3222.
- Comparison of immune responses occurring in peripheral blood with responses occurring in the lung by performing bronchoalveolar lavage.
- All animals to undergo extensive post mortem examination to define their pathological status including bacteriological and histopathological examination of tissue samples.

Results

1. <u>Recruitment of study reactors</u>

   To date, 26 tuberculin skin test reactor positive cattle have been recruited and housed at VLA. Whilst the preferred animals selection criteria was to identify animals that provided negative responses to the antigens ESAT-6 and CFP-10 in the BOVIGAM IFNγ test[1,2], such animals have been rarely encountered. Of those animals screened to date, only 4 have been negative to both ESAT-6 and CFP10 at the time of recruitment. Of these, one animal developed responsiveness to ESAT6/CFP10 during the sampling period. Interestingly, the 3 animals that remained negative to ESAT6/CFP10 showed no evidence of disease at post

mortem. *M. bovis* infection has been confirmed in all other study animals that have undergone post mortem examination so far (n=13).

2. Comparative genomic antigen screening
   To date, 530 peptides, broken down into 70 peptide pools (mapping 42 individual antigens), have been tested using the study animals. Using this approach, 8 promising peptides from two antigens (Mb2555 and Mb2890) have been identified for further evaluation. This data has been published in January 2006[3].

3. Influence of a recent skin test on IFNγ responses
   In these naturally infected cattle, we did not observe the reduction in IFNγ signal strength in bloods taken 3 days post skin-test that had previously been observed in experimentally infected cattle.[4] Indeed, BOVIGAM IFNγ responses observed prior to, and 3 and 10 days post, skin-test were comparable (see Figure 1). At each time point we also investigated the influence of storing blood samples overnight prior to testing to simulate a delay in sample testing due to overnight sample transportation. Overnight blood storage resulted in reduced signal strength for all antigens. However, this reduction was not detrimental to the diagnostic outcome when using PPD as test antigens (see Table 1). In fact, overnight storage of blood improved test specificity when applying PPD-A and PPD-B as comparative test antigens since it resolved a PPD-A biased response in a small minority of animals which in turn resulted in higher responder frequencies (see Table 1). In contrast, the drop in signal strength due to overnight blood storage did reduce the sensitivity of ESAT6/CFP10 as test antigens, although this could be overcome by adjusting the detection cut-off from 0.1 to 0.05 OD_{450nm} (see Table 1). A paper has been drafted for publication of these data.

4. Longitudinal study of bacillary shedding in nasal secretions
   We observed a PCR positive result in 3 animals which, in accordance with our local risk assessments, were then immediately killed and examined by post mortem examination. Two of these reactors were NVL/culture negative, whilst infection was confirmed in the third animal. However, retrospective culture of these PCR 'positive' nasal samples failed to culture *M. bovis*. These samples were also sent for culture by the Bactec culture system used at VSD-Northern Ireland but also proved to be culture negative in this assay system. Indeed, to date, we have failed to culture *M. bovis* from any of the nasal samples collected (over 500 samples from 21 animals) indicating that shedding of bacilli, in the nasal mucus at least, is at best an extremely infrequent occurrence with at best low numbers of nasally shed bacilli.

5. Measurement of immune responses at site of disease
   With the aid of a veterinary endoscope, we have developed and refined methodologies to perform serial bronchoalvelar lavages on cattle. Preliminary data demonstrated that the frequencies of PPD-B and ESAT6/CFP10 specific IFNγ ELISPOT's was 4 times greater in lung lymphocytes compared with peripheral blood for animala with active pulmonary disease, but comparable between blood and lung animals in which no evidence of pulmonary disease could be detected. To further characterise and compare the cell populations isolated from lung and peripheral blood, multi-colour fluorescence activated cell

sorter (FACS) analysis methods have been developed to allow identification and quantification of cell subpopulation including those expressing the cell surface markers $CD3^+$, $CD4^+$, $CD8^+$ and $\delta\gamma^+$ TcR (T-cells), ($CD21^+$ (B-cells) and $CD14^+$ ($m\theta$).

Conclusions

- The availability of large volume bleeds from naturally infected cattle held at VLA-Weybridge has rationalised the diagnostic screening of peptide libraries. 8 promising diagnostic peptides from 2 genes (Mb2555 and Mb2890) have been identified.
- Performing the BOVIGAM test 3 days after a skin-test did not compromise the diagnostic outcome of the test in naturally infected cattle.
- Testing blood 24 hours after collection was not detrimental to the diagnostic outcome when using PPD as test antigen in the BOVIGAM assay. However, if applying defined antigens, it may be necessary to apply more stringent test cut-offs or test blood on the day of collection to ensure optimal test sensitivity.
- Shedding of bacilli in nasal mucus is at best an extremely infrequent occurrence in naturally infected cattle.
- Methods have been developed to measure cellular immune responses at the site of disease (in the lung) in cattle and promising results are forthcoming.

Future research

- Screening of diagnostic peptide libraries and further investigation of promising peptide/antigen targets is an ongoing iterative process.
- The bronchoalveolar sampling methods now developed will be applied to investigate immune responses at the site of disease also in other projects (eg the project of developing cattle TB vaccines).
- Assessment of the effects of repeat short interval skin testing on diagnostic tests (skin tests and IFNγ) is ongoing. However, as the number of animals sampled increases, better statistical correlation of the (confirmed) pathological status of animals with the immunological observations will be possible.
- The specificity of mucosal humoral responses (IgG and IgA) in stored nasal samples, and their potential for diagnostic applications, will be investigated.

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1. *Field comparison of the interferon gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis*. Australian Veterinary Journal, 1991 **68**: 286-290. Wood PR, Corner LA, Rothel JS, Baldock C, Jones SL, Cousins DB, McCormick BS, Francis BR, Creeper J and Tweedle NE.
2. *The gamma-interferon assay for diagnosis of bovine tuberculosis in cattle: conditions affecting the production of gamma-interferon in whole blood culture.* Australian Veterinary Journal, 1992 **69**:1-4. Rothel JS, Jones SL, Corner LA and Wood PR.
3. *Antigen mining with iterative genome screens identifies novel diagnostics for the Mycobacterium tuberculosis complex.* Clin Vacc Immunol 2006 **13**: 90-97. Ewer K, Cockle P, Gordon S, Mansoor H, Govaerts M, Walravens K, Marche S, Hewinson G, Vordermeier, M.
4. *Influence of skin testing and overnight sample storage on blood-based diagnosis of bovine tuberculosis.* The Veterinary Record, 2004, **155**:204-206. Whelan AO, Coad M, Peck ZAA, Clifford D, Hewinson RG and HM Vordermeier.

Publications generated from the project

Ewer, K., Cockle, P., Gordon, S., Mansoor, H., Govaerts, M., Walravens, K., Marche, S., Hewinson, G., Vordermeier, M. 2006. Antigen mining with iterative genome screens identifies novel diagnostics for the *Mycobacterium tuberculosis* complex. Clin. Vacc. Immunol. **13**: 90-97.

Paper submitted to the Veterinary Record;

The influence of SICTT and delay in blood testing on BOVIGAM IFNγ test in naturally infected cattle. M. Coad, R.G. Hewinson, D. Clifford, H.M. Vordermeier and A.O. Whelan.

Review comment

A very successful and relevant grant, aided by clear objectives and established technologies. This work uses naturally infected cattle and validates some of the findings from experimentally-infected cattle in previous studies. Access to animals at different stages of natural infection, including those that fail to react in current versions of the IFNγ test, are of major benefit for studies aimed at enhancing the sensitivity of the IFNγ assay. The antigen mining and repeat skin testing are providing some interesting and valuable results, some of which differ from those seen in experimentally-infected animals. There is a need to identify additional specific mycobacterial antigens to complement ESAT-6 and CFP10 and some new candidate antigens look promising. The finding that the IFNγ test works well 3 days after skin testing needs further confirmation, but if true, would provide major cost savings as field testing officers could collect blood samples from animals when they read the skin test. Failure to isolate *M. bovis* from 800 nasal samples from 27 naturally infected cattle is consistent with the findings from the cattle pathogenesis study (SE 3013). Factors which facilitate the spread of TB between cattle are not known, perhaps stressing the animals in some way will stimulate nasal shedding. If the factors which promoted nasal shedding were understood, then some management practices may be possible to reduce it. The strength of reaction by the local lung immune response suggests that consideration possibly be given to developing intra-nasal or intra-tracheal vaccine.

Scores

Conclusions based on sound evidence: 3.2
Quality of science: 3.3
Overall rating: 3.4

Fig 1 Bar and whisker plots of IFNγ responses, measured as PPD-B minus PPD-A and Peptides minus unstimulated control OD_{450nm}, for fresh and overnight stored blood pre- and at days 3 and 10 post-skin test.

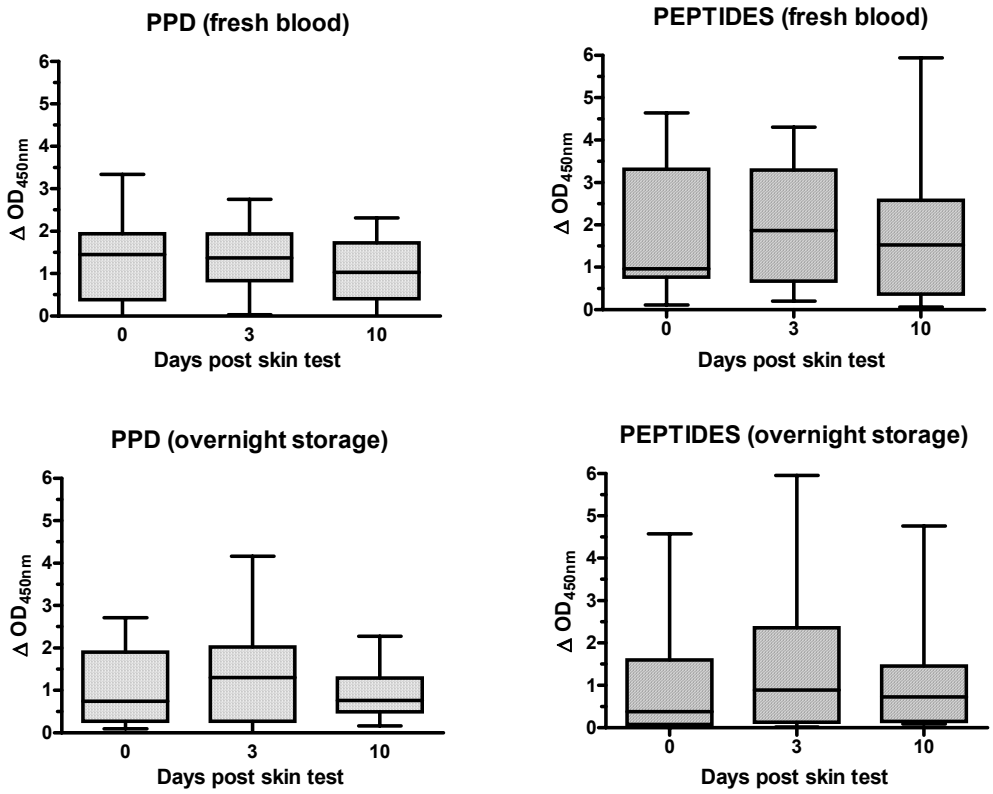

Table 1 Diagnostic outcome of IFNγ tests. Shown are the munber of tests (n=20) where animals were found positive using cut-off of 0.1 (*= 0.05).

Day Post-SICTT	PPD		Peptide Cocktail	
	8hr	24hr	8hr	24hr
0	18/20	19/20	20/20	14/20 (17/20*)
3	19/20	19/20	20/20	15/20 (16/20*)
10	17/20	20/20	19/20	18/20 (20/20*)

SE3013 Pathogenesis and diagnosis of tuberculosis in cattle – complementary field studies

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/10/2000
End date 31/09/2005

Total cost £2,850,729

Abstract
A detailed pathological and immunological investigation was undertaken of 200 reactor and 200 non-reactor, but exposed ("In-contact") cattle. Of the reactor cattle, 105 (52.5%) were shown to be *M. bovis* infected, and 22 (11%) of the in-contacts, the latter all having tested clear at standard interpretation. No evidence was found indicating that trace element deficiencies or inter-current infection increased the severity of disease, but a surprising result was that dairy cattle were more likely to have tested falsely positive to the tuberculin test. The detailed PMEs showed that in the reactor cattle, the lesions were predominately in the chest and largely confined to the lymph nodes. By contrast, few lesions were found in the tonsils and shedding of *M. bovis* in the nasal mucus was either absent or at low levels. The PMEs detected few lesions in the lungs, and most of these would not have been missed by a routine slaughterhouse inspection. Comparison of a host of immunological tests showed the one with best diagnostic performance was the IFNγ assay using the "peptide-mix" of antigens. However, test performance was still sub-optimal, and suspected to be due to the assay being undertaken under field conditions, *viz* 20 to 26 hours following collection.

Aims
- Develop intranet based method for data capture and transfer.
- Undertake 200 detailed post-mortem examinations of reactor cattle.
- Undertake relevant histological, microbiological and clinical pathological studies of samples collected from the autopsied animals.
- Undertake immunological testing on 200 in-contact non-reactor cattle followed by a detailed PME and relevant histological, microbiological and clinical pathological studies.
- Undertake epidemiological and statistical analyses of the total study.

Relevance to Defra
1. Optimal usage of results arising from the experimental and modelling studies, particularly in ensuring that these results truly reflect the field pathology of reactor animals in England and Wales.
2. Development of internet based data capture protocols which will enable essential experience if this technology is to be used for other disease control programs.
3. Generation of novel hypotheses regarding tuberculosis in cattle which will help guide future research.

4. Provide convincing evidence that the infected but skin test negative ("false negative") animals are a significant cause of prolonged breakdowns through cattle to cattle spread, and thus advance the scientific debate concerning the relative importance of this route as against that of wildlife to cattle spread.
5. Improved immunological blood tests to detect false negative (to the SICTT) animals that are currently considered.

Methods

200 tuberculin test positive "reactor" cattle and 200 tuberculin test negative (at standard interpretation) cattle from known infected herds were selected by the SVS between April 2002 and March 2005. The reactor cattle were sent to one of three VLA Regional Laboratories where, following the collection of blood and nasal mucus, they were subjected to a detailed post-mortem examination (PME). The in-contact animals were additionally held for two-months in separate pens where blood and nasal mucus was regularly collected, and were then processed as for the reactor cattle. Following the PME, a standard set of tissues was sent for culture and in addition for those cattle in which a possible TB lesion was detected, for histopathology. A range of diagnostic tests were performed upon the blood samples, to determine, *inter alia*, blood trace-element status and past exposure to antigens of common bovine infections. The immunological assays included lymphocyte transformation assay, the IFNγ test and assays for IL-2 and IL-4, all undertaken using a diversity of antigens. The project had immensely complex logistics, which were successfully overcome through the development of a project-specific web-enabled database, *PathMan* (Durr and Eastland, 2004).

Results

- Of the reactor cattle, 105 (52.5%) were shown to be *M. bovis* infected, and 22 of the in-contacts, the latter all having tested clear at standard interpretation.
- The hypothesis that trace-element deficiencies and/or inter-current infection might result in more severe disease was not supported.
- Dairy cows were shown to be much more likely to be non-visibly lesioned / non-infected than other classes / breeds of cattle, and this was shown not to be due to confounding factors like skin thickness and herd size.
- In the positive reactor cattle, lesions were predominately found in the lymph nodes of the chest, and rarely in the tonsils. In the in-contact animals, lesion distribution was more complex indicating that these might have different infection/pathogenesis dynamic influencing their reduced immunological response.
- No *M. bovis* was isolated from any of the nasal mucus samples, and although this was in part attributed to a sub-optimal decontamination technique, follow-on studies indicate that nasal mucus excretion in naturally infected animals is low or sporadic.
- All infected animals had a single *M. bovis* genotype, indicating that multiple infection events might be uncommon.
- A ROC curve analysis showed that the IFNγ test using the peptide mixture of antigens (Vordermeier *et al* 2001) gave the best diagnostic performance, but was only slightly better than the conventional PPD-M/PPD-A combination. However, both were sub-optimal, particularly with respect to test sensitivity. The reason for this was suspected to be due to the 20-26 hour delay required for overnight

transport from the collecting centers to the processing laboratories (Gormley *et al* 2004).

- In line with current thinking of reciprocal nature of cell and humoral mediated immune responses, animals with advanced pathology were more likely to be serologically positive to bTB antigens.

Conclusions

- Although the existence of exposed animals that test negative to the skin test (at standard interpretation) was confirmed, the practical significance of these will depend upon the herd-testing regime. While these animals are likely to be detected at following short-interval and check tests, in four-yearly testing parishes they may escape detection.
- The IT method of experimental infection is likely to best replicate field-based infection, and some of the previously reported results of the IN route, such as high levels of nasal mucus excretion of *M. bovis*, may have been experimental artifacts.
- Hypotheses suggesting that trace-element deficiencies and intercurrent infection might lead to accelerated disease progression were not supported. The hypothesis that these factors might lead to increased susceptibility to *M. bovis* infection was not tested.
- Despite convincing evidence from experimental studies, detection of *M. bovis* in nasal mucus by culture was indicated as not a robust diagnostic method for detecting animals in early stages of infection. The extent to which nasal mucus shedding occurs in these animals is still to be determined.
- Blood-test diagnostic methods of diagnosing bTB in the field are indicated to be limited when processing, and requires occurring the day following collection. If same day processing is not possible – and this is the norm in GB – then methods to enhance lymphocyte survival/reactivity will need to be explored.
- The current system of animal level diagnosis and confirmation of bTB in Great Britain was broadly validated.

Future research

Four follow-on projects are proposed:

- Further epidemiological studies to explain why dairy cattle are more likely to be false positives;
- A study, based at Boxworth, to examine field responses of cattle to repeated tuberculin injections;
- Histo-pathological studies on the tissue archive collated for the study to better understand cellular and tissue responses to infection; and
- Research on enhancing the survival or effective response time of the blood lymphocytes and monocytes.

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Durr, P.A., Eastland, S., 2004. Use of web-enabled databases for complex animal health investigations. *Revue Scientifique et Technique de l'Office International des Epizooties* **23**, 873-84.

Gormley, E., Doyle, M.B., McGill, K., Costello, E., Good, M., Collins, J.D., 2004. The effect of the tuberculin test and the consequences of a delay in blood culture on the sensitivity of a gamma-interferon assay for the detection of *Mycobacterium bovis* infection in cattle. Vet Immunol Immunopathol **102**, 413-20.

Vordermeier, H.M., Whelan, A., Cockle, P.J., Farrant, L., Palmer, N., Hewinson, R.G., 2001. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. *Clinical and Diagnostic Laboratory Immunology* **8**, 571-578.

Publications generated from the project

Durr, P.A., Eastland, S., 2004. Use of web-enabled databases for complex animal health investigations. *Revue Scientifique et Technique de l'Office International des Epizooties* **23**, 873-84.

Review comment

Generally this was regarded as an excellent applied project. It was a complex, multidisciplinary and collaborative study involving VLA, ADAS, SVS, and IAH-Compton and has delivered a range of valuable scientific results under quite difficult conditions and generated new hypotheses for future testing. However, there is a wealth of data generated that must be analysed and written up – including results on SICCT/IFNγ/VL status and post mortem findings. This makes judgements of its outputs difficult. Despite this the study has shown that that shedding of *M. bovis* in nasal mucus is not a normal feature of natural infection in cattle and its importance may have been overstated in early experimental studies. At best shedding is sporadic and with low bacillary loads. From all those animals with more than one infected tissue, the same spoligotype was detected supporting the hypothesis that multiple infection events in cattle are uncommon. There also appears to be a difference in susceptibility to bTB due to production type though this requires more analysis. The study of “in-contact” reactor animals was very innovative and demonstrated that about 10% of these animals had *M. bovis* infection, despite presenting a negative tuberculin skin test. This emphasises the need for repeated testing of infected herds to clear all infections and may explain why some herds may remain persistently infected for many years. In-contact animals appear to differ slightly in their pathology and this needs more analysis. It is important to determine whether the higher rate of skin test negatives in the in-contact group is significant for control purposes.

Scores

Conclusions based on sound evidence: 2.6
Quality of science: 2.8
Overall rating: 2.6

SE3017 Development and evaluation of strain typing methods for *Mycobacterium bovis*

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/1999
End date 31/03/2005

Total cost £1,359,372

Abstract
In this project the suitability of all current molecular typing techniques for improving our understanding of herd breakdowns in GB was assessed. In addition, more sensitive methods that allow high throughput molecular typing were developed and evaluated. Each technique was performed in a centre of expertise for that technique ie AgResearch, NZ for restriction endonuclease analysis (REA) typing, the Veterinary Sciences Division, Stormont (Queens University Belfast) for RFLP and VNTR analysis and VLA for Spoligotyping, microarray and automated VNTR analysis.

Extended molecular analysis of epidemiologically linked strains did not reveal further polymorphism, over and above that obtained by spoligotyping and VNTR analysis that could be used to identify recent epidemiological links. Extended MIRU/VNTR analysis of a total of 26 loci did little to increase the diversity revealed by combined spoligotyping and 6 locus ETR typing.

As part of this project we typed over 8,000 isolates of *M. bovis* from GB by ETR (VNTR) typing including isolates from the Randomised Badger Culling Trial and as many isolates as possible from 2000 onwards. For strains of spoligotype 9, we found evidence of extreme geographical localisation of VNTR genotype. The geographical localisation of strains has proved invaluable for determining the source of *M. bovis* as a result of farmers purchasing infected animals.

Finally we standardized molecular typing methodology with the PHLS, now HPA, Mycobacterial Reference Laboratory and showed that *M. bovis* infection in humans in GB is largely as a result of immigration or reactivation of infection acquired before pasteurization and TB control.

Aims

- To evaluate the suitability of all current molecular typing techniques (REA, RFLP, spoligotyping and VNTR) for improving our understanding of herd breakdowns in GB.
- To develop more sensitive methods which allow high throughput molecular typing (AFLP, microarray technology, improved RFLP and VNTR and blood based fingerprinting techniques).
- To validate these new methods against existing methods using epidemiologically defined *M. bovis* isolates from GB.

- To standardize fingerprinting techniques between VLA and PHLS to allow co-ordinated surveillance of *M. bovis* infection in animals and humans in Great Britain.
- To conduct an epidemiological study on a well-defined farming cohort in order to identify whether farmers are at increased risk from bTB infection.

Relevance to Defra

The Krebs report, Defra and ISG have highlighted a requirement 'to develop sensitive and specific molecular typing techniques to enable a long-term study of transmission between wildlife, cattle and other species (including man) and to assess the variation of different genomes of *M. bovis*'. The aim of this proposal is to evaluate the suitability of all current molecular typing techniques for improving our understanding of herd breakdowns in GB and to develop more sensitive methods which allow high throughput molecular typing. These new methods will be validated against existing methods using epidemiologically defined *M. bovis* isolates from GB. We also aim to standardize fingerprinting techniques between VLA and PHLS to allow co-ordinated surveillance of *M. bovis* infection in animals and humans in Great Britain thereby supporting government policy to minimize the risks to humans and investigate the potential links with human health. In addition, the project establishes a worldwide collaborative network of workers involved in the molecular epidemiology of *M. bovis* (VLA Weybridge, VSD Stormont, Imperial College of Science, Technology and Medicine, PHLS Mycobacterium Reference Centre, Dulwich, AgResearch, Wallaceville, NZ and RIVM, NL).

Methods

Restriction fragment length polymorphism analysis (RFLP)[1].
Restriction endonuclease analysis (REA)[2].
Spoligotyping[3].
Variable number of tandem repeats (VNTR)[4].
Whole genome microarray analysis [5].
PCR.
Comparative genomic analysis.
Amplified fragment length polymorphism (AFLP).
Peptide synthesis.
Whole blood IFNγ assay for cattle (BOVIGAM).
IFNγ assay to ESAT-6 for humans (QuantiFERON).

Results

The aim of this proposal was to evaluate the suitability of all the leading current molecular typing techniques (REA, RFLP, spoligotyping and VNTR) for the GB situation and to develop more sensitive methods that allow high-throughput molecular typing (AFLP, microarray technology, improved RFLP and VNTR). These new methods were validated against existing methods using epidemiologically defined *M. bovis* isolates from GB. Each technique was performed in a centre of expertise for that technique ie AgResearch, NZ for REA typing, the Veterinary Sciences Division, Stormont (Queens University Belfast) for RFLP and VNTR analysis and VLA for Spoligotyping, microarray and automated VNTR analysis.

Molecular typing

GB isolates were selected to assess the ability of candidate genotyping techniques to address specific epidemiological questions. The panel was compiled by the Epidemiology Department at VLA and the identity of isolates in this panel was blind to the participants in this trial. A second panel *M. bovis* isolates was used to further evaluate the high through-put typing systems developed as part of this project. This second panel comprised 10 strains of the 10 most common spoligotypes chosen to maximize genotypic and geographical diversity.

The application of RFLP and PCR techniques to the set of epidemiologically related strains clearly showed that PCR techniques were superior in ease of use, high through-put, ease of recording results, portability of results between laboratories, as well as standardisation and, most important, identification of genotypic variation. Given the man-hours involved in RFLP techniques they are, without doubt, more expensive than spoligotyping and VNTR typing.

The RFLP techniques that require chromosomal DNA purification (REA, PGRS, IS6110 and DR region RFLP pattern as well as IS6110 copy number) were difficult to perform and, with the exception of REA, revealed equal or less diversity than spoligotyping alone. REA typing identified the major clonal lineages that are also marked by a combination of spoligotyping and six loci ETR typing. REA typing did reveal extra polymorphism within some spoligotypes, in particular, in strains of spoligotype 22. However, REA typing is very difficult to perform, the results are not portable between laboratories and the results are not phylogenetically informative. Therefore, we do not recommend REA typing as a routine surveillance technique for strains of *M. bovis* from GB.

Extended MIRU/VNTR analysis of a total of 26 loci did little to increase the diversity revealed by combined spoligotyping and 6 locus ETR typing (Tables 1 and 2). Although this observation could be explained as a result of analysing identical strains from a series of very closely related outbreaks of bovine TB we frequently found that identical genotypes were recovered from disparate epidemiological groups. This suggests that spoligotyping and 6 loci ETR typing has identified the major clones circulating in the population. For example, 6 loci ETR analysis revealed 6 different genotypes within strains of spoligotype 9. The 6 most polymorphic loci (MV3232, ETR-D, MV2163A, ETR-C, ETR-A, SUPK) identified the same 6 major clones and only added a single extra genotype marked by a single isolate. Although there are good reasons to use the most polymorphic VNTR-MIRU loci in standard surveillance of *M. bovis* in GB these results suggest that the current VNTR typing effort has identified the major clones circulating in the population.

Extended molecular analysis of epidemiologically linked strains did not reveal further polymorphism, over and above that obtained by spoligotyping and VNTR analysis that could be used to identify recent epidemiological links. Strains of spoligotype 17, for example, were recovered from 10 different epidemiologically linked groups and yet only a single genotype was recovered by the analysis of 30 loci using a variety of RFLP and PCR based techniques. For most other spoligotypes limited genotypic diversity or small numbers of isolates confounded any analysis of recent transmission within an epidemiologically linked group. Our results did demonstrate, however, that

cattle and badgers in the same geographic area share the same genotype of *M. bovis*.

To maximise surveillance of *M. bovis* genotypes circulating in GB we therefore recommend a combination of spoligotyping and VNTR typing. Spoligotyping is a simple technique that identifies most of the major clonal lineages of *M. bovis*. Furthermore, it has the advantage of standardisation and international recognition that permits the identification of strains of international origin. Spoligotyping has also been used to identify ecotypes of the *M. tuberculosis* complex with differing host adaptations[6] (Smith *et al* (2005)). Finally, spoligotyping permits phylogenetic inferences that are not readily available with VNTR tying and therefore provides a framework that is essential for the identification of phenotypic correlates and analysis and monitoring of population structure.

Spoligotyping alone, however, does not uncover all of the major genotypes of *M. bovis* in GB. This is especially true when considering the most common spoligotype, type 9. VNTR typing has revealed that a number of genotypes can be identified within this, and other spoligotypes.

As part of this project we typed over 8,000 isolates of *M. bovis* from GB by ETR (VNTR) typing including isolates from the Randomised Badger Culling Trial and as many isolates as possible from 2000 onwards. For strains of spoligotype 9, we found evidence of extreme geographical localisation of VNTR genotype. More than 900 strains from individual outbreaks of bovine TB in cattle caused by spoligotype 9 were analysed and over 20 different VNTR types recorded. The three most common VNTR types of spoligotype 9 in GB, representing over 60% of the isolates, are geographically localised (over 86% for each VNTR type) in one or two, adjacent, counties[7] (Hewinson *et al* 2005). The most plausible explanation for the observed results is that *M. bovis* in Great Britain has undergone a series of 'clonal expansions' (Smith *et al* 2003)[8]. The geographical localisation of strains has proved invaluable for determining the source of *M. bovis* as a result of farmers purchasing infected animals.

Immunotyping

We demonstrated that it is not feasible to generate an immunotyping system in which antibody or T cell responses could provide information about the infecting strain. However, the work showed that many of the PE/PPE proteins are strongly immunogenc during infection. Although the function of these proteins remains unclear, there has been much speculation in the literature that these proteins represent a major source of antigenic variation that enables the organisms to persist within the host by evading the host immune defences. The strong IFNγ responses against these proteins that we observed in blood from *M. bovis* infected cows is consistent with this hypothesis. Therefore this work has been taken forward in a Wellcome Trust funded collaborative project involving Imperial College and VLA to allow us to further investigate this hypothesis by determining whether different PE and PPE epitopes are recognised by the host immune system at different times over the course of an infection.

M. bovis in humans

A further recommendation by the Krebs Report was that the incidence of *M. bovis* TB in humans should be kept under review in light of the increasing incidence in cattle. In order to facilitate this process it is essential that molecular typing techniques for *M. bovis* in Great Britain are standardised between the PHLS and VLA. A secondary aim of this proposal was therefore to transfer and to standardise *M. bovis* typing systems between VLA and the PHLS Mycobacterium Reference Unit at Dulwich, so that the genotypes of *M. bovis* in humans and cattle could be compared. Moreover, an immunological and epidemiological study was undertaken on a cohort of farmers from GB, to determine previous exposure of farmers to bovine tuberculosis and to identify the risk factors responsible for infection.

Spoligotyping of 50 human *M. bovis* isolates obtained between 1997 and 2000 in GB produced 25 different patterns. Fifteen of these spoligotypes have not yet been recorded in cattle and 13 of these types were unique to GB. The unique spoligotypes found in man may reflect the *M. bovis* strains circulating in cattle in GB over 50 years ago. In this context it is interesting to note that only one daughter strain of spoligotype 9 was identified in humans (spoligotype 17) although approximately 58% of all extant strains of *M. bovis* in GB are daughter strains of spoligotype 9 suggesting that the emergence of these clones may have been a recent event. The largest cluster of human *M. bovis* isolates (15 isolates, 30%) had been seen in cattle before and was identified as spoligotype 9 (international type SB140). Spoligotype 9 is the most frequently seen spoligotype of *M. bovis* (over 30% of all isolates have this spoligotype) isolated from cattle and has a wide geographical range in Great Britain (Hewinson *et al* 2005). This predominant spoligotype in humans and cattle was subdivided by VNTR (Gibson *et al* 2004)[9].

The immunological and epidemiological survey in farmers revealed that despite few clinical cases in GB, 103 out of 387 farmers had a positive IFNγ response to ESAT-6, which is a relatively specific antigen of the *M. tuberculosis*-complex that includes *M. bovis*. Farmers that kept dairy as opposed to beef cattle, increased their risk of being ESAT-6 positive. Moreover, the odds ratio for being ESAT-6 positive increased as the size of the dairy herds increased. Drinking unpasteurised cow's milk increased the likelihood of being ESAT-6 positive and those farmers that had a previous diagnosis of TB, had all drunk raw cow's milk. However, a statistically significant association was also found between various types of poultry farming and having a positive ESAT-6 response. Further investigations are required to determine the nature of this association especially with regard to the specificity of ESAT-6 as a diagnostic reagent for the detection of TB infection using blood-based assays.

Conclusions

1. VNTR analysis shows much promise as a high throughput typing assay that can increase the discriminatory power of spoligotyping. The combination of the two techniques might enable long-term studies into the transmission between wildlife, cattle and other species (including man).
2. The geographical localisation of strains has proved invaluable for determining the source of *M. bovis* as a result of farmers purchasing infected animals.
3. Neither of the two data sets analysed here was a true population survey and they have reached conflicting results as to the best VNTR loci to use in a standard surveillance program. We therefore recommend that a population survey of

strains of *M. bovis* be carried out on 1000 isolates, as they arrive at VLA, for the 14 most informative loci. At the end of this exercise we will be able to recommend the most useful VNTR-MIRU loci to be applied in the routine surveillance of British strains.

4. It is not feasible to generate an immunotyping system in which antibody or T cell responses could provide information about the infecting strain. However, the work showed that many of the PE/PPE proteins are strongly immunogenic during infection.
5. *M. bovis* infection in humans in GB is largely as a result of immigration or reactivation of infection acquired before pasteurization and TB control.
6. Farmers that farmed dairy and not beef cattle increased their risk of being positive for ESAT-6. The size of dairy herds kept and the length of time spent farming them both contributed as risk factors. Drinking raw cow's milk also increased the risk of farmers being ESAT-6 positive while no such association was noted from drinking raw goat's milk. It is interesting to note that in The Gambia, 30% of community controls produced IFNγ in response to ESAT-6. Increased proportions of responders and intensities of responses were found in household contacts. Moreover, frequencies of circulating ESAT-6 peptide-specific IFNγ-secreting CD4 T cells have been shown to be higher in latently infected healthy human contacts and subjects with minimal disease and low bacterial burdens than in patients with culture-positive active pulmonary tuberculosis.

Future research

1. The analysis performed as part of this project has shown that the current 6 loci ETR typing used at VLA has been outstandingly successful at identifying the major VNTR genotypes of each spoligotype and that the application of more loci does not substantially increase the genotypic variation identified. These results are to be expected if the geographical localisation of strains in GB is due to 'clonal expansion' (Smith *et al* 2003). However, other MIRU/VNTR loci were shown to have a much higher allelic diversity than many of the 6 ETR loci currently used and therefore should be considered as candidates for a standard MIRU/VNTR assay of British *M. bovis* strains. Neither of the two data sets analysed here was a true population survey and they have reached conflicting results as to the best loci to use in a standard surveillance program. We therefore recommend that a population survey of strains of *M. bovis* be carried out on 1000 isolates, as they arrive at VLA, for the 14 most informative loci. At the end of this exercise we will be able to recommend the most useful VNTR-MIRU loci to be applied in the routine surveillance of British strains.

2. Now that data from the cattle tracing system (CTS) has become available, the combination of CTS data with the molecular typing data set has the potential to throw greater light on the routes and causes of *M. bovis* transmission. This approach should be used to address the following questions:
   a. To define the contribution of cattle-to-cattle transmission in the spread of *M. bovis* within GB;
   b. To clarify the importance of 'amplification' of *M. bovis* within restocked herds and provide clear evidence of the long distance transfer of *M. bovis* by cattle;
   c. To determine whether differences in cattle movements can explain differential rates of clonal expansion;
   d. To identify epidemiologically important phenotypes that may be associated with different strains (eg the ability to evade detection by skin test before

successful transmission, the ability of the strains to persist, disseminate etc); and

e. As highlighted by the ISG, typing of isolates obtained from badger RTA surveys around areas of re-stoked herds would help elucidate the direction of transmission of *M. bovis* and the time it takes to get any infection established in wildlife.

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In addition a Review article has been requested by Nature Reviews in Microbiology. This manuscript is in preparation.

Review comment

This was an ambitious yet highly successful project in terms of scientific endeavour and project management as it involved multiple partners in UK and abroad, each of which were expert in a particular technique. There were a large number of objectives that all appear to have been met and the results are both highly informative and predictive for long-term surveillance of bovine TB in the UK. It is remarkable that this project has been so productive – publications (14); protocol assessments and refinements; standardisation of typing methods for *M. bovis* in the HPA for screening human cases of *M. bovis* infection; assessing and validating the full range of molecular tests available at the time and incorporating new developments in genetic typing as they emerged (e.g. MIRUs, microarrays); establishing extent and geographic distributions of common strain types of *M. bovis* in the UK so as to predict the potential value of genetic tests; modelling data and predicting reasons for epidemiological patterns observed are due to 'clonal expansion', an obvious biological prediction but that was imperative to prove. The project has established a firm basis for typing *M. bovis* that will be of great value for future control programmes

and this must be its greatest achievement. It also addressed key goals from this Bovine TB Research Programme and of the Krebs Report from which this programme draws its aims. It would be potentially useful to ascertain best loci for discrimination in the UK situation and combine the data with CTS to better understand the epidemiology of cattle spread.

Scores
Conclusions based on sound evidence: 3.8
Quality of science: 3.6
Overall rating: 3.6

Table 1 Number of strains for each spoligotype, the number of discrete epidemiological groups with a strain of that spoligotype, and the number of genotypes for each spoligotype using standard 6 loci ETR typing and with the full 30 loci. Data is shown for spoligotypes with more than 3 isolates in the data set. Data generated from panel comprising the epidemiologically linked strains.

	Number of			
Spoligotype	strains	epidemiological groupings	genotypes with 6 loci	genotypes with 30 loci
9	43	8	6	9
11	13	3	1	2
17	33	10	1	1
20	9	3	1	3
21	16	3	2	3
22	20	6	2	4

Table 2 Spoligotypes, number of strains of each spoligotype and the number of genotypes for each spoligotype observed by standard 6 loci ETR typing and with the full set of 16 polymorphic loci. Data was generated using a panel of strains comprising the most common spoligotypes chosen to maximize genotypic and geographical diversity.

	Number of		
spoligotype	strains	genotypes obseved with 6 loci	genotypes observed with 18 loci
9	9	4	6
10	10	1	3
11	9	2	2
12	5	1	1
13	7	2	2
17	10	1	1
20	5	1	2
22	9	1	3
25	10	1	2
35	10	1	2

SE3206 Genome sequence analysis of *Mycobacterium bovis*

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/02/1998
End date 31/03/2005

Total cost £1,181,312

Abstract
The aim of this project was to determine the complete genome sequence of *M. bovi*s AF2122/97, a GB strain isolated in 1997 from a diseased cow in Cornwall and to generate sequence data from the *M. bovis* BCG vaccine strain, the only currently available vaccine against tuberculosis in man or animals. This project is now completed and was undertaken by the VLA, the Sanger Institute and the Institut Pasteur. A database has been set up that allows the *M. bovis* genome sequence to be queried by the International Research Community via the Internet. The BoviList database has proved to be popular, with an average of 3,000-3,5000 hits per month from researchers around the world.

As a result of this project all the genes, proteins, enzymes and antigens present in *M. bovis* have been identified rapidly in a highly cost-effective manner. The genome sequence of *M. bovis* will therefore underpin all future Defra TB research in the development of vaccines and improved diagnostic tests, in the understanding of the molecular basis of pathogenesis and in developing improved tools for molecular epidemiology. Comparative analysis with the genome of BCG has identified antigens not present in the vaccine strain but present in the pathogen, hence providing the basis for differential diagnostics and providing clues to approaches that might improve BCG as a vaccine.

The genome sequence of *M. bovis* AF2122/97 was found to be 4345492 bp in length and was >99.95% identical at the nucleotide level to the genome of *M. tuberculosis*. However, deletion of genetic information led to a reduced genome size suggesting that *M. bovis* has evolved from a progenitor of the *M. tuberculosis* complex as a host-adapted clone. Cell wall components and secreted proteins showed the greatest variation, indicating their potential role in host-bacillus interactions or immune evasion. Furthermore, there were no genes unique to *M. bovis*, implying that differential gene expression may be the key to the host tropisms of human and bovine bacilli.

Aims
- To sequence the genome of *M. bovis.*
- To annotate the fully sequenced genome of *M. bovis.*
- To act as curator of the *M. bovis* genome.
- To sequence the genome of BCG.

Relevance to Defra

Since 1988 the level of bTB in Great Britain has been increasing, with a particular problem in the South-West which has shown an exponential increase over the past 10 years (http://www.defra.gov.uk/animalh/). There is a clear need for improved tools such as diagnostic tests and vaccines to underpin the development of new control strategies if bTB is to be brought under control. Sequencing the genome of *M. bovis* undoubtedly represents the best means of catalysing vaccine research and development as this scientific approach enables all the genes, proteins, enzymes and antigens present in *M. bovis* to be identified rapidly in a highly cost-effective manner. The genome sequence of *M. bovis* will therefore underpin all future Defra research in vaccine and diagnostic development and will form a springboard for post-genomic research on *M. bovis*.

Methods

For the shotgun phase of *M. bovis* AF2122/97 a total of 81,146 reads, or ~7.7 coverage, was generated from pUC18 and M13mp18 small (1-4 kb) insert libraries using dye-terminator chemistry on ABI377 or ABI3700 automated DNA sequencers. Assembly of the shotgun data was performed by PHRAP. The sequence was finished using GAP4 as previously described, with an extra 13,000 reads from the pUC libraries performed on ABI3700 machines for finishing purposes. Annotation was managed through the ARTEMIS (http://www.sanger.ac.uk/Software) tool, with comparisons to public and in-house databases performed using the BLAST suite and FASTA. Comparative genome analysis was achieved using the Aretmis Comparison Tool (ACT; http://www.sanger.ac.uk/Software) with SNP identification performed using the STADEN package (http://www.mrc-lmb.cam.ac.uk/pubseq/staden_home.html). The complete genome sequence and annotation of *M. bovis* 2122/97 was deposited with the EMBL database under the accession number BX248333 and published in 2003 [1].

For *M. bovis* BCG Pasteur, a whole genome shotgun approach was performed as described above that generated 32,982 reads, totalling 17.418 Mb and giving a theoretical coverage of 98.09% of the genome. This data was used by the Institute Pasteur for finishing of the sequence. A further ~35,000 reads were added, with physical gap closure through targeted sequencing of BAC clones. The final assembly generated a single contiguous genome of 4375192 bp.

Results

1. The complete genome sequence and annotation of *M. bovis* 2122/97 was deposited with the EMBL database under the accession number BX248333 and published in 2003 [1].
2. The ability for researchers around the world to access and use the genome sequence of *M. bovis* is essential if we are to analyse research on vaccine development and diagnostics. Hence a key part of the proposal was making the data valuable in an easy-to-use format that would be widely used. The GenoList database model was developed by the Institute Pasteur and has been adopted for a wide range of bacteria, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli, Streptococcus pneumoniae,* but critically for us *Mycobacterium tuberculosis* and *Mycobacterium leprae*. These latter two databases are widely used as the first point of call for researchers working on tuberculosis. Hence, it was logical to adapt the *M. bovis* genome sequence to the

GenoList format. It also meant that hyperlinks could be place in the *M. tuberculosis* and *M. leprae* databases to ensure ease of genome comparison across the species. The first version of BoviList went live in July 2003 and be accessed at http://genolist.pasteur.fr/BoviList/. The database contains the complete annotation of *M. bovis*, as well as links to relevant papers; for example, data on microarray expression analyses is incorporated where information exists for particular genes. Hyperlinks to orthologous genes in *M. tuberculosis* and *M. leprae* is also available, as well as information on SNPs in *M. bovis*. The utility of the database is evidenced by it receiving 3,000-3,500 hits per month from researchers in countries as far a field as New Zealand, Canada, Russia, Spain, Cuba, Norway, India, France and UK.

3. The *M. bovis* genome is 4345492 bp, making it 66039 bp smaller than the genome of *M. tuberculosis* H37Rv (4411531 bp). *In silico* comparisons with the genome of *M. tuberculosis* has revealed that there are 2348 single nucleotide polymorphisms (SNPs) between the two sequenced genomes, consisting of 744 transversions and 1604 transitions. This gives an identity of >99.95% at the base pair between *M. bovis* and *M. tuberculosis*.
4. Many of the changes are predicted to affect the cell surface of *M. bovis*.
5. There is extensive variation between the genes of *M. bovis* and *M. tuberculosis* encoding the repetitive proteins of the PE/PGRS or PE/PPE families as a result of insertion-deletion events (Indels).
6. Of 225 total Indels in *M. bovis*, 124 of these change genes.
7. Deletion events have been a major force in the evolution of the genome. Eleven deletions from the *M. bovis* genome, ranging in size from ~0.8-13 kb have been identified that effect 73 genes. It is clear from the ORF structure at the deletion junction points that these are deletions, rather than insertions in *M. tuberculosis*.
8. There is only one locus, termed TbD1, which is deleted from *M. tuberculosis* H37Rv but present in *M. bovis*. This locus contains genes that may be involved in lipid transport, and hence may play a role in the known difference in phenolic glycolipid content between human and bovine bacilli.
9. An important question to address was whether the genome structure that we had identified in one strain was representative across *M. bovis* isolates. In collaboration with the Prof. Stewart Cole's team at Pasteur, the junction points of the known deletions across the *M. bovis* genome were amplified form 100 *M. tuberculosis* complex strains, including 28 *M. bovis* isolates. This revealed that the presence or absence of these deletions could be used to construct a genealogy of *M. tuberculosis* complex strains (Brosch *et al* 2002). Results from this study showed that *M. bovis* is the most far removed member of the complex from the common ancestor. In the past it was believed that the human tubercle bacillus, *M. tuberculosis*, was derived from *M. bovis* due to the bovine bacillus crossing the species barrier into man at the time of the domestication of cattle 10,000 to 15,000 years ago. However, the genome sequence of *M. bovis* has revealed that this is unlikely to be the case. Since the *M. bovis* genome is smaller than that of *M. tuberculosis*, it is more likely that man gave tuberculosis to cattle or that the two organisms evolved separately from a common ancestor. This work therefore calls for a rethink on our previous understanding of the dynamics between human and animal disease.
10. We also performed *in silico* comparative analysis of *M. bovis*, *M. tuberculosis* and *M. leprae* genomes. Interpretation of the metabolic consequences of the genomic

deletions observed in the genome of *M. leprae* was integrated into the publication of the *M. leprae* genome and has lead to several other publications.

11. The genome of *M. bovis* BCG Pasteur was found to be 4375192 bp. The genome contained 3952 genes, giving a coding density of 90.8%, and with a GC content of 65.64%. Comparison of the genome sequence with that of *M. bovis* AF2122/97 revealed 736 single nucleotide mutations, with the majority being transitions. This relatively small number of point mutations underlines the close relationship between the strains.
12. SNPs and deletions are the major source of variation between BCG and all other members of the complex.
13. The completion of the genome sequences of *M. bovis* and *M. bovis* BCG will allow identification of the molecular basis for attenuation of BCG and insights into how to improve BCG or rationally attenuate *M. bovis* to produce better vaccines than BCG. In addition, comparison of the two genomes readily allows identification of *M. bovis* specific genes that can be used to develop diagnostic tests that differentiate between animals vaccinated with BCG and those infected with *M. bovis*. A number of diagnostic reagents identified in this way have already shown promise for use in cattle when used in conjunction with the BOVIGAM assay (see SE3020 and SE3028). The genome sequence will therefore have a major impact on the generation of vaccine candidates and diagnostic reagents to combat disease.

Conclusions

- The genome of *M. bovis* is smaller than that of *M. tuberculosis.*
- Indels drive plasticity of the genome of the *M. tuberculosis* complex.
- TBD1 is the major region of difference between *M. bovis* and *M. tuberculosis* and hence may play a role in the known difference in phenolic glycolipid content between human and bovine bacilli.
- Many of the genetic differences between *M. bovis* and *M. tuberculosis* are predicted to affect the cell surface of *M. bovis*.
- *M. tuberculosis* did not evolve from *M. bovis*.
- Completion of the *M. bovis* genome sequence generated much positive publicity for Defra in the national and international media and in the scientific Press. It has also helped to bring *M. bovis* research into the mainstream TB and biological research communities.
- The completed genome sequences of *M. bovis* and *M. bovis* BCG will underpin future development of improved vaccines and diagnostics for bovine TB.

Future research

A key future task will be detailed genome comparisons between *M. bovis* BCG and other strains of *M. bovis*. Through other Defra-funded work (SE3020) we have determined the population structure of *M. bovis* in Great Britain, and we know that the sequenced *M. bovis* strains are relatively distant on the phylogeny from the *M. bovis* clade that was used to generate BCG. Hence, we need to be sure that 'loss-of-function' mutations that we have identified in BCG Pasteur (and hence may be linked to attenuation of the strain) are not simply markers of the *M. bovis* clade it was derived from. Similarly, we will need to check SNPs against other *M. bovis* BCG sub strains (ie Russia. Tokyo, Copenhagen) to ensure that mutations are conserved across all BCG strains. This is important if we are to develop diagnostics based on, for example, frame shifts that lead C-terminal truncated proteins in BCG, allowing

peptides against the intact orthologue in *M. bovis* to be used as differential diagnostic reagents. It is also important if we are to identify the molecular basis for the attenuation of BCG in order to produce vaccines with improved efficacy to BCG in a rational way.

The other essential task for the future is maintaining the annotation of *M. bovis* to ensure that it is up-to-date and therefore exploited to its maximum potential by workers in the field. The ever-increasing numbers of bacteria that are being sequenced, coupled with post-genomic analyses, are increasing the functional assignments to genes of previously unknown function. Hence the accuracy of genome annotation decreases with the time since it was last performed.

The completion of the genome sequences of *M. bovis* and *M. bovis* BCG will allow identification of the molecular basis for attenuation of BCG and insights into how to improve BCG or rationally attenuate *M. bovis* to produce better vaccines than BCG. In addition, comparison of the two genomes readily allows identification of *M. bovis* specific genes that can be used to develop diagnostic tests that differentiate between animals vaccinated with BCG and those infected with *M. bovis*.

The exploitation of the genome sequence to understand the molecular basis of pathogenicity of *M. bovis* for cattle is being explored as part of SE3030. Knowledge of the genome sequence has also accelerated the development of molecular typing tools for *M. bovis*. These have been developed successfully as part of SE3017 and SE3020 and will now require validation for use as surveillance tools.

Finally, as a consequence of the Genome Sequencing project (SE3206) VLA has become the European Centre of Excellence for mycobacterial genomics as part of the VENoMYC (Veterinary Network of Laboratories Researching into Improved Diagnosis and Epidemiology of Mycobacterial Diseases) initiative (EU FP6, Scientific Support to Policies; Contract 501903).

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Publications generated from the project

Publications

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The PNAS paper (2 above) outlining the evolutionary scenario for the *M. tuberculosis* complex was 'Editor's choice' – *Science* Vol **295** 29th March 2002.

Websites

Bovilist: http://genolist.pasteur.fr/BoviList/

Sanger Institute: The data is available for download by FTP (ftp://ftp.sanger.ac.uk/pub/pathogens/mb/) and can be searched using the Sanger Institute server (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/m_bovis).

VENoMYC: http://europa.eu.int/comm/research/fp6/ssp/venomyc_en.htm

Media Coverage

Completion of the *M. bovis* genome sequence generated much positive publicity for Defra in the national and international media and in the scientific Press including The Independent, The Guardian, The Daily Express, Nature and The Veterinary Record. Prof. Hewinson also announced the completion of the Genome sequence on Radio 4's 'Today Programme (March 1st 2002).

Review comment

This project was a timely exploitation of technical abilities in 3 Institutions: Sanger Institute, Institut Pasteur and VLA – and delivered high impact genome sequence information for *M. bovis* and its comparisons with *M. bovis* BCG, *M. tuberculosis* and *M. leprae*. It has led to significant shifts in our understanding of the evolution and comparative biology of these important pathogens and has provided an information base upon which to build further studies that underpin Defra's objectives towards vaccine and diagnostic reagent development. This genome sequencing project is of the highest order scientifically with 10 publications including Nature and PNAS(2). The groups involved have international reputations and this project has enhanced the reputation of VLA and Defra and spotlighted *M. bovis* research as a major contributor to world efforts to combat mycobacterial diseases such as human TB. This project scores 'excellent' in all aspects and meets the objectives and brings bovine TB research into the post-genomic era. This project underpins much other bTB research funded by Defra. In the future it will be important to determine if the mutations found in the BCG vaccine are not just markers of the *M. bovis* clade from which it originates and to check for SNPs across the other vaccine strains. This will underpin the critical task of defining genes that can act as diagnostics to differentiate vaccinated from infected cattle.

Scores

Conclusions based on sound evidence: 3.5
Quality of science: 3.9
Overall rating: 3.6

SE3030 Application of post-genomics to reveal the genetic and physiological basis of virulence, pathogenesis and transmissibility of *Mycobacterium bovis*

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/01
End date 31/03/06

Total cost £3,439,982

Abstract
The current sharp increase in bovine tuberculosis in Great Britain has highlighted the need for improved strategies and tools for disease control, including improved diagnostic tests and vaccines for cattle and badgers. The availability of the genome sequence of *M. bovis*, and related tubercle bacilli, provided us with the opportunity to take a global approach to understanding this pathogen, its interaction with the host, and its basic biology. In this project we therefore proposed the development and application of a range of 'post-genomic' approaches to develop rational strategies for defining novel targets for vaccines and diagnostics, and to increase our understanding of the pathogenesis of disease. In pursuing this objective we developed a centre of expertise in post-genomics for Defra at VLA.

Aims
- Obj. 1: To define the comparative virulence of *M. bovis*, *M. tuberculosis* and *M. microti* for bovines using the intratracheal model of infection.
- Obj. 2. To use in silico methods to identify species specific sequences across the *M. tuberculosis* complex, *M. avium* and *M. avium subsp paratuberculosis* as a means to identify putative virulence factors and potential diagnostic antigens.
- Obj. 3 To elucidate the structural relationships, function and basis of antigenicity in the ESAT-6 family.
- Obj. 4. Investigate the role and biogenesis of PE and PPE proteins as a route to understanding their function and putative role in virulence.
- Obj. 5. To establish a DNA microarray facility at VLA.
- Obj. 6. To use DNA microarrays to determine the extent of genomic variation amongst strains of *M. bovis*.
- Obj. 7. To use DNA microarrays to determine the differences in transcriptome between *M. bovis* and *M. tuberculosis* under steady state and pH stress in a chemostat.
- Obj. 8. To develop and apply mathematical tools for the design and analysis of microarray data.
- Obj. 9. To elucidate the metabolic consequences of defined genetic differences between *M. bovis* strains and other tubercle bacilli.
- Obj. 10. To construct defined mutants via allelic exchange, or strains complemented with wild-type alleles, in genes identified through comparative genomics, transcriptomics, or proteomics as a means to evaluate their contribution to virulence in animal models of infection.

- Obj. 11. To hold annual meetings with the steering committee so that progress and research priorities can be assessed.

Relevance to Defra

Defra continues to have a statutory obligation to control tuberculosis in farm animals in Great Britain under the Animal Health Act of 1981, the Tuberculosis Orders, and various EC directives. The current sharp increase in bovine tuberculosis in Great Britain has highlighted the need for improved strategies and tools for disease control including improved diagnostic tests and vaccines for cattle and badgers. Genomic and post-genomics are set to revolutionise molecular biology, our understanding of pathogenesis, and the diagnosis and control of disease. In this project we sought to apply these cutting-edge approaches to *M. bovis* so as to accelerate our understanding of the pathogen and to develop new control tools.

Methods

Virulence studies (Obj 1 and 10):

Infection of cattle with *M. bovis* and *M. tuberculosis* was performed using the IT route as previously described (Buddle *et al* 1995). During the study, animals were housed in a high-security isolation unit under negative pressure and expelled air was filtered. Euthanasia was carried out at 16-20 weeks post infection, and a detailed post-mortem was performed. Samples from individual nodes and lesions were obtained for mycobacterial culture and histological examination. Mouse and guinea pig infections were performed as previously described (Hogarth *et al* 2005; Inwald *et al* 2003).

Comparative genomics (Obj 2):

In silico comparative analyses of mycobacterial genomes was performed with a combination of BLAST, ARTEMIS and ACT software. At the time when the genome analysis was performed, the complete sequence of *M. bovis* was available, with incomplete versions of the *M. avium* subsp *avium* and *M. avium* subsp *paratuberculosis* genomes. For the *M. avium* subsp *avium* sequence we performed a GeneMark and Glimmer analysis to define coding sequences, and from this generated a list of potential proteins encoded in the *M. avium* subsp *avium* genome. We then used StandAlone BLASTP to compare all proteins in *M. bovis* to those in *M. avium* subsp *avium*, identifying *M. bovis* proteins that showed no, or poor, identity against *M. avium* subsp *avium*; this generated a list of potential *M. bovis*-specific proteins. These proteins were then rechecked against the *M. avium* subsp *avium* genome using TBLASTX to ensure that no potential coding sequences had been missed. To refine the list, these proteins were then screened against the *M. avium* subsp *paratuberculosis* genome using the public NCBI TBLASTN server. This led in turn to a list of proteins that was finally checked against *Streptomyces coelicolor* as a means to screen against related soil actinobacteria.

Structural studies (Obj 3):

The coding sequences of ESAT-6 and CFP-10 were cloned into standard expression vectors and expressed and purified from recombinant *E. coli* strains. NMR data were acquired on 600 MHz Varian Inova and Bruker Avance spectrometers at temperatures between 15 and 35 ^{o}C. The 2D NOESY and TOCSY spectra were recorded with mixing times of 100 to 150 ms and 45 ms respectively, with typical

acquisition times of 35 ms in F_1 and 250 ms in F_2. The NMR data were processed using either the Varian VNMR or Bruker XWINNMR packages, as appropriate. For yeast two hybrid experiments, purified PCR products of ESAT-6 family members were cloned into the GAL4 transactivation domain (TAD) vector pGAD-C1 and into the GAL4 DNA binding domain (DBD) vector pGBD-C1 as combinations of bait and prey. Two-hybrid assays were carried out in *Saccharomyces cerevisiae* CG1945 cells.

Microarray work (Obj 5, 6, 7, 8):
The *M. bovis* / *M. tuberculosis* composite microarrays used in these experiments were developed by the Bacterial Microarray Group (St. Georges Hospital, UK) in collaboration with the Veterinary Laboratories Agency. The array consists of 4,410 PCR products printed in duplicate that represent all the genes in the genomes of *M. bovis* strain AF2122 and *M. tuberculosis* strains H37Rv and CDC1551. The microarrays were all scanned with an Affymetrix 428 scanner. Fluorescent spot intensities were quantified using ImaGene 4.1. (Biodiscovery Inc., Marina Del Rey, Calif).

For comparative genomic studies, genomic DNA was used as a template for direct incorporation of fluorescent analogues (Cy3- and Cy5-dCTP) by a randomly primed polymerisation reaction. Data were analysed using GeneSpring (Silicon Genetics) and Mathematica 4.2 (Wolfram Research Inc.). A cut-off for the normalised test/control ratio of < 0.5 was used to create gene deletion lists.

For transcriptome analyses, *M. bovis* and *M. tuberculosis* were grown in a chemostat with pyruvate as the sole carbon source. Complementary DNA synthesised from RNA was fluorescently labelled in a reverse transcription reaction in the presence of Cy5-CTP (Amersham Biosciences). Fluorescent spots on each scanned image were quantified using Imagene and then analysed by GeneSpring and Mathematicia. Genes were selected as being differentially expressed if the difference in normalised expression values in one or more of the post acid shock samples compared to the pre-acid steady state samples was 3 fold or more, and if their p-value was less than 0.05 using the Benjamini Hochberg correction. Fold change expression ratios were calculated using data obtained from three independent experiments.

Metabolic analyses (Obj 4, 9):
For enzyme assays, cells were harvested, washed twice, resuspended in phosphate buffer and disrupted using a Fast Prep system (Hybaid). Glycerol kinase activity was measured by the glycerol 3-phosphate-coupled assay (Pettigrew *et al* 1998). Pyruvate kinase activity was assayed by the method adapted from Wheeler (Wheeler, 1983).

Results

Obj. 1: To define the comparative virulence of *M. bovis*, *M. tuberculosis* and *M. microti* for bovines using the intratracheal model of infection

The virulence of the genome sequenced strains of *M. bovis*, *M. microti*, and *M. tuberculosis* for bovines was assessed using the intratracheal model of infection, followed by detailed post-mortem. As expected, *M. microti* OV254 was attenuated for cattle, while the results for *M. tuberculosis* and *M. bovis* are summarised in Table 1. From this it is clear that the *M. tuberculosis* animals, although infected, did not show

any signs of pathology in this model. This experiment shows that the genetic differences in the genomes of *M. bovis* and *M. tuberculosis* are responsible for their differing degrees of virulence for cattle. It therefore provided the baseline for the comparative aspects of this project.

Obj. 2. To use in silico methods to identify species specific sequences across the *M. tuberculosis* complex, *M. avium* and *M. avium* subsp *paratuberculosis* as a means to identify putative virulence factors and potential diagnostic antigens

78 *M. bovis* genes encoding putative specific-proteins were selected on the basis of iterative genome screens. These proteins encompassed a wide range of putative function, molecular weight, and subcellular locations, so we therefore selected a representative subset of 42 for synthesis as overlapping peptides (Table 2). Screening of these peptide pools revealed that 3 proteins, Mb2890, Mb2555 and Mb3895 contained epitopes that gave responder frequencies of greater than 50% in experimentally infected cattle. These proteins could be further refined to 8 peptides that are now a high priority for screening against naturally infected animals (Ewer *et al* 2006).

Obj. 3 To elucidate the structural relationships, function and basis of antigenicity in the ESAT-6 family

This work sought to determine the structure of ESAT-6 and CFP-10, the two dominant T-cell antigens of the *M. tuberculosis* complex. Previous work has shown that these two proteins interact to form a dimer, and a further extension of this work was therefore to determine whether other members of the ESAT-6 family of proteins also interacted in a pair wise fashion. For this latter work we used a yeast-two hybrid system and concentrated on CFP-10/ESAT-6 and the products of Rv0287/Rv0288 and Rv3019c/Rv3020c (Lightbody *et al* 2004). It was found that tight binding occurs between the products of both Rv0287/Rv0288 and Rv3019c/Rv3020c and that the Rv0287•Rv0288 complex has the properties of a folded protein similar to that previously seen for the CFP-10•ESAT-6 complex. We also found that there is cross talk between some the ESAT-6 family. This work suggests that all genome pairs of CFP-10/ESAT-6 family proteins will bind to each other and indicates that groups of closely related sequences probably have the potential to form complexes with non-genome partners and therefore increase the functional flexibility of this protein family.

The structural analysis of CFP-10•ESAT-6 (Renshaw *et al* 2005) showed that the core of the complex consists of two similar helix-turn-helix hairpin structures formed from the individual proteins, which have an extensive hydrophobic contact surface and lie anti-parallel to each other to form a 4 helix bundle (Figure 1). A striking feature of the complex is the disordered N and particularly C-termini of both proteins which form long flexible arms at both ends of the 4-helix bundle core; these regions may be involved in interactions with a host cell target protein, resulting in stabilisation of the helical conformation.

Obj. 4. Investigate the role and biogenesis of PE and PPE proteins as a route to understanding their function and putative role in virulence

After discussion with the Steering Committee this objective was modified during the course of the project. The new aim was to identify VNTR markers in genes encoding PE and PPE proteins, both to identify new markers for molecular typing and to determine whether there was evidence for variation in these genes between *M. bovis* clades defined in SE3020. PE and PPE genes were screened for internal VNTR sequences, and these were then screened against 100 *M. bovis* strains from the major GB *M. bovis* clades (Smith *et al* 2003). Markers were identified that allowed us to split the major spoligotype groupings of type 09 and 17 with greater resolution that with existing markers. We are currently appraising the best panel of VNTR markers to be used in routine surveillance as part of other projects, therefore underpinning work that was funded as part of SE3017 which is an appraisal of various molecular typing methods.

As part of this objective we also sought to determine whether the PE and PPE genes showed differential expression between *M. tuberculosis* and *M. bovis*, so as to determine whether they are involved in the differential virulence of *M. bovis* and *M. tuberculosis* for the bovine host. We analysed expression data from both chemostat and batch grown *M. bovis* and *M. tuberculosis*, but only two PEs showed consistent differences in expression between the two strains; hence it would appear that differential PE/PPE expression is not a major mechanism of variation across the human and bovine bacilli. We also assessed PE/PPE expression in response to different carbon sources, the logic being to better reflect the *in vivo* environment and hence allow us to select potential antigens for diagnostics. However, no PE or PPE genes showed induced expression in response to a palmitic acid carbon source, while control genes, such as isocitrate lyase, showed clear upregulation.

Obj. 5. To establish a DNA microarray facility at VLA

This objective was key to the delivery of downstream objectives in the project. The successful achievement of this objective depended on a close collaboration with the Bacterial Microarray Group at St George's Hospital, London, who developed the first *M. tuberculosis* spotted-array. We successfully transferred microarray technology from the BUGS group and developed a second generation microarray that contained amplicons which covered the genomes of *M. bovis* AF2122/97 as well as both *M. tuberculosis* strains, H37Rv and CDC1551.

Obj. 6. To use DNA microarrays to determine the extent of genomic variation amongst strains of *M. bovis*

The first target of this work was the *M. bovis* AN5 strain which is used for the production of bovine tuberculin. Despite its widespread use as a diagnostic reagent, *M. bovis* AN5 is poorly defined at the genetic level. Hence we sought to characterise its genome by using DNA microarrays. The hybridisation data for *M. bovis* AN5 disclosed an almost identical set of deletions to those of *M. bovis* 2122/97, showing that *M. bovis* AN5 has not suffered extensive gene deletion events during *in vitro* culture. Our analysis shows that, at least at the gross genomic level, there is no reason to suspect that *M. bovis* AN5 should be suboptimal for the detection of infection by *M. bovis* strains that are currently prevalent (Inwald, Hinds *et al* 2003).

Representative strains from the 10 major spoligotypes found in the UK were screened for deletions using microarrays. The key finding was that there is no evidence for significant gene deletion events across the major spologitype groups. This has implications for the use of defined peptide cocktails as diagnostics markers, since it shows that UK *M. bovis* strains do not have any major divergence in encoded proteins; peptide cocktails should therefore be able to detect infection with extant *M. bovis* strains. However, deletions were identified in spoligotypes 13 and 17 that showed utility as markers of deep phylogeny (Mostowy *et al* 2005). Screening multiple type 17 strains showed that all isolates shared the same deletion of Mb1963-1971; this showed that this deletion occurred once in the common ancestor of all type 17 strains, and acts a marker of this clonal group. The same was true for the deletion Mb3438-3439 in type 13. This work has therefore underpinned our work on the molecular typing of *M. bovis* funded under SE3017 (see final report).

As the current test and slaughter policy removes *M. bovis* strains from the population that illicit strong immune responses, it is conceivable that strains that are less immunogenic, or that modify the immune response in some way, may escape surveillance and expand in the population. This may be achieved through deletion of antigens form the bacillus. To test this, the proteins encoded by the genes deleted from type 17 were checked for immunogenicity. However, there was no evidence that these genes encode strong antigens, suggesting that deletion of major antigens in not a significant force driving *M. bovis* genome evolution.

Obj. 7. To use DNA microarrays to determine the differences in transcriptome between *M. bovis* and *M. tuberculosis* under steady state and pH stress in a chemostat

We chose to use a chemostat for these experiments for a number of reasons. The chemostat provides greater control over the culture conditions, ensuring that gene expression profiles are specific to pH induction. Another advantage of continuous culture is in its reproducibility; one of the greatest sources of variation in microarray is derived from the heterogeneity of samples from replicate cultures. Chemostat-grown cultures are highly reproducible, allowing more accurate determinations of changes in gene expression. Using this system we defined a systematic approach to the discovery of *M. bovis* antigens by (1) identifying genes that were induced *in vitro* in response to acid shock; (2) determining whether these genes were also induced during infection; (3) screening the resulting candidates for their immunogenicity in *M. bovis* infected cattle.

Through this pipeline we identified a total of 71 genes that were expressed at significantly higher levels after acid shock. Real Time qRT-PCR was then used to show that 13 of 27 genes tested showed an increase in expression in infected mouse lung tissues compared with *in vitro* grown *M. bovis*. 11 of the *in vivo* induced genes were then assessed for their immunogenic properties in cattle naturally infected with *M. bovis*. Five of the peptide pools tested showed a positive IFNγ response in at least 40% of cattle tested, showing that these proteins may have potential as diagnostic antigens or as part of subunit vaccines (Golby *et al* submitted).

Parallel experiments on the steady state and acid-shock expression were also performed with *M. tuberculosis*. This data set provides a snap-shot of gene expression differences between *M. bovis* and *M. tuberculosis* and adds a further

layer of information to differences seen at the genome level. Analysis of this data is ongoing.

Obj. 8. To develop and apply mathematical tools for the design and analysis of microarray data

The complexity and volume of microarray data generated through the course of the project has required specialist skills in statistics to ensure that (1) experiments are correctly designed, (2) data is normalised correctly, (3) resulting gene expression differences across test and control conditions are statistically valid, (4) fully annotated datasets are stored on publicly accessible servers. Through our subcontract with Prof Wolkenhaeur we originally envisaged that this statistical help would be through a help-desk type interaction. However, to increase our day-to-day familiarity with microarray data handling we positioned a member of Prof Wolkenhaeur's group, Dr Javier Nunez, here at VLA. This has proved to be a very successful appointment, as the wet bench scientists now get help on a day-to-day basis. While the core microarray analysis tool for the wet scientists remains the 'industry-standard' of GeneSpring (Silicon Genetics), Dr Nunez has also developed and implemented analysis tools in Mathematica (Wolfram Research) and Bioconductor (http://www.bioconductor.org/).

Obj. 9. To elucidate the metabolic consequences of defined genetic differences between *M. bovis* strains and other tubercle bacilli

A range of *in vitro* characteristics can be used to differentiate the members of the *M. tuberculosis* complex; for example, unlike *M. tuberculosis*, *M. bovis* is unable to use glycerol as a sole carbon source. It was our aim to define the genetic basis for this *in vitro* phenotype and determine any relevance to *in vivo* biology. Comparative genome analyses revealed that both the glycerol kinase (GK) and pyruvate kinase (PK) genes in *M. bovis* were disrupted; as these genes are essential for growth on glycerol as a sole carbon source they were targeted for further investigation.

Our genetic and biochemical examination of GK in a range of tubercle bacilli showed that the absence of GK activity was not the sole reason for an inability to catabolise glycerol. Our attention therefore turned to PK which catalyses the final irreversible step in glycolysis, the transphosphorylation of PEP and ADP to pyruvate and ATP. We found that *M. bovis* recombinants containing an active PK were able to grow on glycerol as the sole carbon source, showing that the glycerol-pyruvate link in *M. bovis* is due to a SNP in the pykA gene (Keating *et al* 2005).

Furthermore it is known that *M. tuberculosis* and *M. bovis* can be differentiated on the basis of colony morphology (Schaefer, 1952) with *M. tuberculosis* said to be eugonic, with abundant growth in the presence of glycerol, while *M. bovis* is described as dysgonic with sparse growth on glycerol. We found that *M. bovis* expressing an active PK also showed eugonic morphology (Keating *et al* 2005), indicating a link between carbohydrate metabolism and colony morphology.

Obj. 10. To construct defined mutants via allelic exchange, or strains complemented with wild-type alleles, in genes identified through comparative genomics, transcriptomics, or proteomics as a means to evaluate their contribution to virulence in animal models of infection

Comparative analyses across the *M. tuberculosis* has revealed a range of differences between *M. bovis* and other members of the complex at the gene, transcriptome, and metabolic level. At the gene level, Mb3909c shows variation in the copy number of an internal repeat compared to the 2 *M. tuberculosis* genome sequenced strains. This locus is also deleted from BCG as part of the RD1 deletion, and has been shown to be antigenic in cattle. Hence, we investigated the role of this protein in virulence of *M. bovis* and in parallel with our search for vaccine candidates as part of SE3208 (see final report). Mb3909c was deleted by allelic exchange from *M. bovis* Mb3909c, and the virulence of the mutant assessed in guinea pigs. No difference in the virulence of the mutant and the wild type was evident, suggesting that Mb3909c does not play a role in the virulence of *M. bovis* (Inwald *et al* 2003).

Examination of the transcriptome reveals that the expression of *mpb83* is one of the greatest differences between *M. bovis* and *M. tuberculosis*. To determine the role of mpb83 in the virulence of *M. bovis*, we therefore assessed the virulence of an *M. bovis mpb83* deletion mutant in the mouse model (constructed under SE3208, "Generation of vaccine candidates", see final report). The results from this experiment suggest that *mpb83* may not be a key virulence factor of *M. bovis*.

At the metabolic level we showed that the loss of a functional *pykA* gene in *M. bovis* had profound effects on its carbon metabolism and colony morphology switching *M. bovis* to resemble *M. tuberculosis*. We therefore determined the virulence of wild type *M. bovis* and *M. bovis* complemented with a functional *pykA* gene in the bovine model. This showed that the presence of a functional pyruvate kinase did not attenuate *M. bovis* to the level of *M. tuberculosis* for bovines, suggesting that PK activity is not the core reason for the host tropism of the human and bovine bacilli.

Conclusions

This project has succeeded across a number of areas, including (1) identifying new antigens for use in diagnostic cocktails or as subunit vaccines, underpinning our diagnostic and vaccine work; (2) describing genome variation across UK *M. bovis* isolates and the AN5 diagnostic strain, hence supporting our molecular typing efforts; (3) solving the structure of the key *M. bovis* T-cell antigens ESAT-6 and CFP-10; (4) defining the virulence of *M. tuberculosis* for cattle; (5) explaining the molecular basis for adding pyruvate to cultures of *M. bovis*; (5) setting up microarray technology and associated data analysis skills at VLA, work that has helped support and catalyse other post-genomic projects at VLA..

Future research

Areas for future research include:

1. Determine the transcriptome and proteome of *M. bovis* AN5 as a means to better define the current diagnostic reagent;
2. Exploring genotype-phenotype links across clades of *M. bovis* to establish whether there is any evidence for the evolution of strains of increased virulence/transmissibility; and

3. Studying host-*M. bovis* interactions to identify the key steps in the infectious process and how the pathogen subverts host defences.

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Up to Jan 2006

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Review comment

The most important element of this project has been the establishment within VLA of a bacterial genomics unit providing the capability of studying bacterial gene expression profiles both at the transcript and protein levels. This capability will play an important role in other ongoing studies relating to diagnostics and vaccine development. The studies of selected proteins, although of scientific interest, have less obvious strategic relevance. The aspects that can be studied are almost endless and some of those chosen worked well whilst others less so. Defra might also consider that this work is state-of-the-art, and shows that they are investing in newer areas. It is likely that these projects feed positively into the other work, though not always in a tangible way. They are also likely to help retain top quality researchers in what is an excellent grouping of scientists. Contrary to the suggestion in SE3020, it appears that the genomic work here shows that there is no reason to suspect that *M. bovis* AN5 in PPD is sub-optimal at detecting more recent *M. bovis* strains such as 2122/97. This is a useful practical finding. Further work in this area would be useful to explore the phenotypes of the different strains (spoligotypes) of *M. bovis* to establish whether there is any evidence for the evolution of strains of increased virulence, immunogenicity and infectiousness.

Scores

Conclusions based on sound evidence: 3.3
Quality of science: 3.8
Overall rating: 2.9

Table 1 Virulence of *M. tuberculosis* and *M. bovis* for cattle.

	Animal	**Infectious Dose**	Pathology score[a]				**Culture Score[b]**	**Skin test (mm)**	**Clinical comment**
			Head nodes	Lung nodes	Lung	Total			
M. tuberculosis	2730	2.8×10^6	0	0	0	0	1	30	None
	2731	"	0	0	0	0	5	12	None
	2732	"	0	0	0	0	0	9	None
	2733	"	0	0	0	0	6	28	None
	2734	"	0	0	0	0	0	34	None
						0	**12**		
M. bovis	2723	1.0×10^6	6	9	11	26	27	17	None
	2735	"	1	17	15	33	16	15	Cough
	2736	"	0	3	9	12	15	9	None
	2756	"	0	19	20	39	23	10	Cough
	2757	"	10	20	30	60	55	-	Euthanised at week 5
						170	**136**		

[a]Cumulative pathology score in accordance with Vordermeier et. al., 2002, Infection and Immunity.

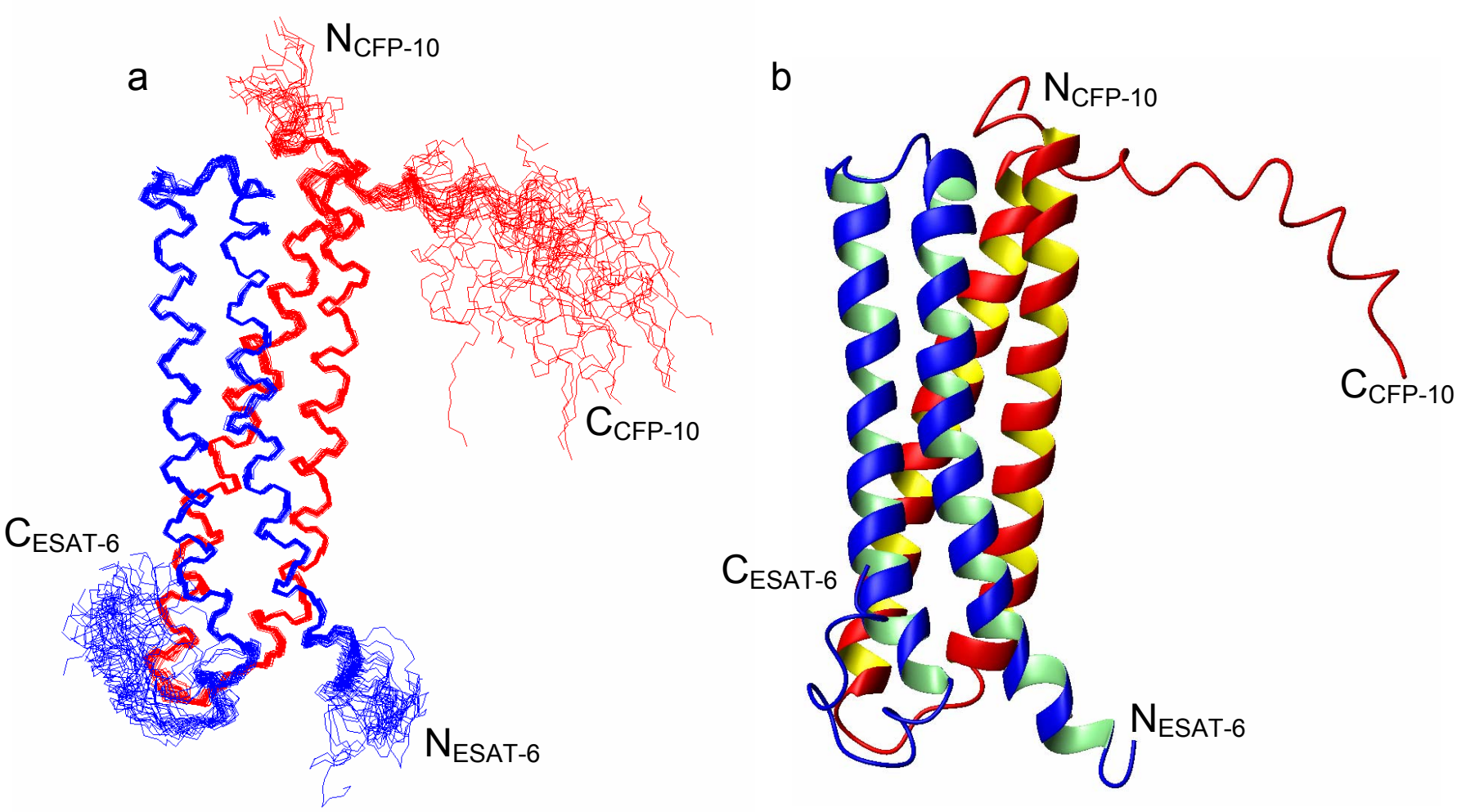
[b]Cumulative culture score based on a ranking of total cfu/plate for each tissue homogenate at highest dilution: 0 = culture negative, 1 = 1-9 cfu, 2 =10-99 cfu,
3 = 100-999 cfu, 4 = 1000+ cfu.

Table 2 Proteins identified from genome screen as potential specific antigens from *M. bovis* with minimal homology to environmental species.

M. bovis protein (Mb)	H37Rv protein (Rv)	Length (a.a.)	e-value vs. *M. avium*	e-value vs. *M. paratuberculosis*	e-value vs. *S. coelicolor*
TRANSPORT/MEMBRANE PROTEINS					
0454c	0446c	256	4.1	1.1	none
0864	0841	80	5.2	5.2	5.2
3024	2999	321	5.7	4.4	none
3881	3851	94	5.2	5.2	6.8
INTERMEDIARY METABOLISM & RESPIRATION PROTEINS					
1580	1555	125	none	none	none
3162	3138	362	5.8	5.8	none
2854c	2830c	71	5.2	3.1	2.3
1991	1956	149	3.5	none	1.2
CONSERVED HYPOTHETICAL PROTEINS					
0101	0098	183	4	4	none
0103	0100	78	4	4	none
0614c	0598C	137	none	4.9	none
0676c	0657c	51	6.9	6.9	none
0679c	0660c	81	none	none	none
0680c	0661c	145	none	none	none
0990c	0965c	139	3.9	1	6.6
1133c	1103c	106	none	none	none
1750c	1721c	75	5.2	5.2	none
1869c	1838c	131	3.4	1.5	none
2579c	2549c	131	4.4	5.7	5.7
2781c	2760c	89	4	4	none

M. bovis protein (Mb)	H37Rv protein (Rv)	Length (a.a.)	e-value vs. *M. avium*	e-value vs. *M. paratuberculosis*	e-value vs. *S. coelicolor*
2840c	2816c	113	4	6.8	none
2854c	2830c	71	5.2	3.1	2.3
2890	2865	93	45	1.4	1.4
2973c	2949c	199	6.1	6.1	none
3210	3188	115	5.2	none	0.043
3711c	3686c	110	3.1	1.4	3.1
3895	3865	103	none	none	none
HYPOTHETICAL PROTEINS					
0081c	0078A	197	none	none	none
0274c	0268c	169	none	none	none
0306	0298	75	none	none	none
1165	1134	78	5.2	5.2	none
1807c	1778c	149	5.9	0.92	5.9
2326c	2304c	69	5.2	6.8	none
2459c	2433c	96	8.9	8.9	5.2
2520	2492	250	6.8	none	none
2555	2526	75	none	none	none
2846c	2822c	124	3.8	3.8	none
3022	2998	153	3.7	2.8	1.3
3219c	3196A	66	8.9	8.9	none
3796c	3770c	191	7.4	9.6	none

Figure 1 The structural analysis of CFP-10•ESAT-6.



SE3220 Molecular and epidemiological characterisation of the PPD diagnostic reagent

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/09/05
End date 31/08/07

Total cost £260,185

Abstract
Since 1988 the level of bTB in Great Britain has been increasing, with a particular problem in the Southwest. The control strategy relies on detecting infected animals through their reaction to a protein preparation from cultures of *M. bovis* and *M. avium*, termed PPD, followed by compulsory slaughter of reactors. As stated in the 2004 Animal Health and Welfare Research Requirements Document, concerns have been expressed that the current *M. bovis* strain used for tuberculin production may not be as effective as it once was in detecting infected animals. We propose a rigorous epidemiological and molecular characterisation of the PPD reagent to determine whether evidence exists to support this concern.

Aims
- An epidemiological analysis of field data on the relation of the molecular type of *M. bovis* to PPD skin test.
- A global gene expression analysis of *M. bovis* AN5 that will show whether the expression of any key antigens is altered compared to recent field isolates.
- A retrospective analysis of PPD-A and PPD-B to determine whether any alterations in potency can me mapped back to production changes.

Relevance to Defra
Within Defra concerns have been expressed that the current *M. bovis* strain used for tuberculin production may not be as effective as it once was in detecting infected animals. The outputs of this proposal will therefore provide evidence for policy decisions on the suitability of the current diagnostic reagent.

Methods
Microarray analysis will be used to define the gene expression profiles of AN5 relative to recent field isolates of *M. bovis*. The methods are the same as those developed during the course of SE3030 (see SE3030 summary report for details).

To determine any links between molecular type and pathology we shall construct a database that will store pathology data extracted from the TB50A form along with the batch number of PPD used. Multivariate logistic regression will be used to determine the relationship between PPD, skin test and molecular type. For the retrospective analysis of tuberculin batches, we will test 20 PPDs from the PPD stocks kept by the Tuberculin Unit in Bovigam IFNγ assays to determine whether batches show any significant variation in potency. Infected animals from this work will be obtained through SE3033 (“Longitudinal study of field reactor cattle”).

Results
The project started in September 2005. The key result to date has been defining the growth curve of *M. bovis* AN5 in Middlebrook 7H9 and BAI media (Obj 2). Growth of AN5 in 7H9 is relatively rapid when compared to field strains of *M. bovis*, and is more similar to that of BCG. This is to be expected considering the many years of *in vitro* cultivation that these strains have undergone. However, growth in BAI is much slower, with AN5 growing as a submerged pellicle and only showing any significant growth after 8-12 weeks. The nature of AN5's growth in this detergent-free medium makes the construction of a growth curve based on OD impractical. Therefore, we shall use 7H9 as the common media for the identification of genes that show differential expression between AN5 and field strains, and then use RT-PCR to determine whether these genes are also differentially regulated in AN5 grown in BAI media. Analysis of TB50A forms and the pathology data contained therein is on-going, although our initial analysis suggests there is a great degree of variation in the quality of data recorded on these forms in terms of pathological description, lesion distributions, etc.

Conclusions
Growth curves for *M. bovis* AN5 have been completed and microarray work is on-going. Extraction of consistent pathology data from TB50A forms is also on-going.

Future research
As this project has only recently started it is too early to determine how our results will impact on future research requirements.

Review comment
There was ambivalence about this project, which is a mix of rather different sub-projects. Overall there was no clear consensus that the hypothesis that AN5 PPD may not be ideal for skin testing cattle in GB was reasonable, although it was agreed that evidence for this was most likely to come from epidemiological investigations to determine the relationship between PPD, skin test and molecular type. The second component, the justification for a global gene expression analysis of AN5 *M. bovis* was also not clear, especially before any evidence was presented that AN5 PPD was ineffective. Additionally variations in the gene expression profiles of AN5 and recent field isolates would not be unexpected, but the biological significance of these findings would be difficult to prove when it is not known what antigens are important in the induction of the skin test response in cattle. However, if the transcriptome studies show this to be the case, and the peptides poorly expressed in AN5 are strongly immunogenic, then the project will have delivered an important advance in developing more appropriate skin test reagents applicable to currently circulating strains. This would be a high priority development. The experimental approach, design and expertise are not in question here. The results will be interesting.

Scores
Conclusions based on sound evidence: 2.8
Quality of science: 2.5
Overall rating: 2.7

Wednesday 5th July 2006

Vaccines/Diagnostics/Immunology

Wednesday 5th July 2006

Venue: The Diskus, Transport House, 128 Theobald's Road, London WC1X 8TN

09:00 – 09:30	Registration and Coffee		
09:30 – 09:40	Introduction		*Chairman*- Richard Drummond
	Code	**Title**	**Speaker(s)**
09:40 – 10:00	SE3008	Detection and enumeration of *Mycobacterium bovis* from clinical and environmental samples	VLA
10:00 – 10:20	SE3118	Review and economic analysis of the use of PCR assays for *M. tuberculosis* complex detection and incorporation into routine bovine TB testing	VLA
10:20 – 10:40	SE3028	The development of improved tests for the diagnosis of *Mycobacterium bovis* infection in cattle	VLA
10:40 – 11:00	SE3222	Development of improved diagnostic tests for the detection of bovine tuberculosis	VLA
11:00 – 11:20	Coffee		
11:20 – 12:00	SE3208	Generation of vaccine candidates against *Mycobacterium bovis*	VLA
	SE3209	Testing of vaccine candidates for bovine tuberculosis using a low dose aerosol challenge guinea pig model	
	SE3212	Testing TB vaccines in cattle	
	SE3224	Continuation of the development for vaccines against bovine TB in cattle	
12:00 – 12:20	**Discussion for SE3208, SE3209, SE3212 & SE3224**		
12:20 – 12:50	SE3227	Evaluation of the protection efficacy of vaccines against bovine TB in a natural transmission setting	VLA
12:50 – 13:35	Lunch		
13:35 – 14:05	SE3217	Kinetics of skin test response in bovine tuberculosis	IAH
14:05 – 14:30	SE3221	Volatile organic compound analysis for the rapid diagnosis of disease	VLA
14:30 – 14:55	SE3215	Development of immunological assays for the detection of *Mycobacterium bovis* infection in badgers	VLA
14:55 – 15:20	SE3223	Development of an oral BCG vaccine bait formulation for badgers	VLA
15:20 – 15:45	SE3216	Development and testing of vaccines against badger tuberculosis	VLA
15:45 – 16:00	SE3228	A safety study of BCG vaccine in wild badgers – preparatory work (to include the follow-on BCG field vaccine development project)	VLA
16:00 – 16:30	Coffee		
16:20 – 18.30	Closed Session – confidential project specific issues. Proposed way forward of the research area		

SE3008 Detection and enumeration of *Mycobacterium bovis* from clinical and environment samples

Organisation Veterinary Laboratories Agency
Weybridge

Start date 04/01/1999
End date 31/12/2004

Total cost £487,325

Abstract
SE3008 was a collaborative project between VLA Weybridge, Imperial College of Science, Technology and Medicine, London and the PHLS Mycobacterium Reference Unit, Dulwich. A number of direct biomolecular methods for detection, quantitation and genotyping of *M. bovis* strains sampled from cattle, badgers and small mammals have been developed. NucliSens extraction has been coupled with a sensitive PCR screening method for the *M. tuberculosis* complex (*IS1081*, 6 copies per genome, detection limit < 1 genome copy). A series of genotyping PCRs for specific deletion events or regions of difference (RDs) within the *M. tuberculosis* complex have also been developed. The sensitivity and specificity of these PCRs were tested on reference strains, spiked samples and on clinical and environmental samples collected in the field. The detection limits for all these single copy methods is 10 genome copies. They can be used as confirmatory assays for classical *M. bovis* and variants (eg *M. bovis caprae*) and for distinguishing other members of the complex (*M. africanum, M. microti*).

Using a test sample of 95 visibly-lesioned cattle lymph nodes, the detection rate of the *IS1081* and RD4 methods was determined to be to 90% and 58% respectively. A standard operating procedure for sample treatment and the PCR assay for detection have been produced and the technology transferred to VLA's routine diagnosis section. The technique is showing much promise for speeding up confirmation of *M. bovis* infection from VL lesions. The success rate of spoligotyping applied directly to the same extracts was around 50% compared with cultures and lower for VNTR analysis.

In a single-blinded study of 50 badger faecal samples spiked with either *M. bovis* (strains AN5 or AF 2122/97), or *M. tuberculosis* strain H37Rv or *M. microti,* 48 / 50 samples were correctly analysed by qualitative PCR. Further evaluation is required on clinical samples taken from badgers.

A mycobactreiophage-based detection system (PhaB) was optimised for use in badger clinical samples by colleagues at the Public Health Laboratory Service Mycobacterium Reference Unit. This assay met with limited success at detecting *M. bovis* from clinical samples from *M. bovis*-infected badgers. Moreover some problems with specificity of the assay were encountered due to the ability of the mycobacteriophage used in this assay (D29) to infect mycobacteria other than members of the *M. tuberculosis* complex.

Aims

SE 3008 was a collaborative project between VLA Weybridge, Imperial College of Science, Technology and Medicine, London and the PHLS Mycobacterium Reference Unit, Dulwich. The aim of the project is to develop methods for the detection and enumeration of *M. bovis* in badger carcasses, excreta and environmental samples. Specific objectives are:

- Design and optimize an *M. bovis*-specific PCR for the detection of *M. bovis* DNA;
- Design and optimize a PCR assay for the detection of badger DNA;
- Develop an economical and reproducible method for recovery of *M. bovis* DNA from visible lesion tissue, pooled lymph nodes, tracheal washings, faecal samples, blood urine and latrine samples;
- Assess whether extracted material is suitable for spoligotyping and VNTR analysis;
- Validate PCR methodology on clinical samples; and
- Develop and validate PhaB assay for enumeration of *M. bovis* in clinical and environmental samples.

Relevance to Defra

The aim of the project was to develop methods for the detection and enumeration of *M. bovis* in badger carcasses, excreta and environmental samples. The development of such assays would allow rapid screening of samples from badger carcasses and monitoring of the environment and badger populations for the presence of *M. bovis*. The technique could also be applied to the detection of *M. bovis* DNA in cattle samples including tissues, nasal mucus, and milk. The technique developed as part of this project is showing much promise for speeding up confirmation of *M. bovis* infection from VL lesions, which is especially useful in confirming *M. bovis* infection in slaughterhouse cases.

Methods

- PCR: The RD7 PCR assay is described in the published paper below[1].
- PCR: IS1081, RD4 and other PCR assays are described in Appendix 4 of the final report for this project.
- Sample processing methodologies including the NucliSens kit are also given in the final report.
- Spoligotyping[2]
- VNTR analysis[3]
- The PhaB (phage amplified biologically) assay[4]**:** 100μl of sample is incubated with 10μl of $CaCl_2$ (1mM) and 10μl of mycobacteriophage D29 (10^9 plaque forming units, [pfu]) for 2 h at 37°C. Extracellular bacteriophage are neutralized with the addition of 12 ml of 100 mM ferrous ammonium sulphate and 100μl of the samples mixed in triple vented 90mm petri dishes with 1 ml *M. smegmatis* (ATCC607) and 1ml oleic acid albumindextrose catalase (OADC) enrichment media and 9 ml of 7H9with 1.5% agar (w/v; at ca. 54oC). Results are read the following day and in some cases at 48 hours, and recorded as pfu/ml.
- *Mycobacterium bovis* culture.

Results

PCR detection from clinical samples: PCR development was performed by Dr G M Taylor, Imperial College, London.

1. Work undertaken as part of this project has resulted in the development of a number of direct biomolecular methods for detection, quantitation and genotyping of *Mycobacterium bovis* strains sampled from cattle, badgers and small mammals. Underlying these series of experiments was the need for a versatile DNA extraction protocol which could handle a variety of tissue samples and biological fluids. DNA extraction with the NucliSens isolation kit (bioMérieux) has been shown to fulfil nearly all of these criteria. A bonus is that mycobacteria are immediately attenuated so minimising the risk to laboratory personnel and the requirement for category three containment. NucliSens extraction has been coupled with a sensitive PCR screening method for the *Mycobacterium tuberculosis* complex (*IS1081*, 6 copies per genome, detection limit < 1 genome copy). A series of genotyping PCRs for specific deletion events or regions of difference (RDs) within the *M. tuberculosis* complex have also been developed. These PCRs identify the occurrence of RDs 4, 7, 8, 9, 10, 12 and 13 (Brosch *et al* 2001). The sensitivity and specificity of these PCRs were tested on reference strains, spiked samples and on clinical and environmental samples collected in the field. Additional methods for confirmation of species include the *oxyR285,* and *pncA157* polymorphic loci. The detection limits for all these single copy methods is 10 genome copies. They can be used as confirmatory assays for classical *M. bovis* and variants (eg *M. bovis caprae*) and for distinguishing other members of the complex (*M. africanum, M. microti*). All the above methods can be readily adapted to quantitative PCR methods with the use of SYBR Green interchelating dye on the RotorGene 3000 platform (Corbett Research).
2. Based on specific sequences in the cytochrome b gene, the *M. bovis* PCR method was successfully multiplexed with one for badger (*Meles meles*) or cattle (*Bos taurus*) DNA, allowing a check on efficiency of extraction procedures in uninfected animals. Restriction enzyme analysis of the 359 bp *cyt*b PCR product with *Taq*I and *Hin*dIII was able to differentiate between *cyt*b products from cattle, badgers and humans.
3. The extracted DNA, which was PCR positive, was also used as a template for spoligotyping and fluorescent VNTR analysis. These experiments showed that DNA recovery was not always suitable for both strain-typing methods.
4. Testing of clinical samples (bovine lymph nodes, n = 109) highlighted two shortfalls of the molecular approach. These are 1. Comparison of *IS1081* PCR with the “gold standard” of culture initially showed a sensitivity of 70%. When RD4 or RD7 PCRs were compared with culture, the detection rate dropped to 50%. Secondly, the success rate of spoligotyping applied directly to the same extracts was around 50% compared with cultures. Recent work indicates that this deficit was due to delays in processing of archival tissues as well as with difficulty with lysis of the tough mycobacterial cell wall (Takade *et al* 2003), rather than to inhibition of PCR or efficiency of DNA recovery itself. Either of these factors may restrict application of other more discriminant typing methods eg VNTR. In the closing months of SE3008 a means of increasing the efficiency of mycobacterial lysis was devised and tested using a further pool of 95 cattle lymph nodes. After modification of the extraction protocol, detection rate of the *IS1081* and RD4 methods increased to 90% and 58% respectively. The *IS1081* PCR is therefore a

realistic screening method for rapid identification of positive cases. Single-tube nested PCR offers a means of increasing sensitivity of the RD methods. Spoligotyping of extracts processed in this further sample are currently under study at VLA Weybridge. Three automated or semi-automated DNA extraction systems were evaluated; the NucliSens Extractor and Minimag (both from bioMerieux) and the Qiagen EZ1 Robot and the latter was found to be the most promising platform for the development of high throughput PCR testing.

5. In a single-blinded study of 50 badger faecal samples spiked with either *M. bovis* (strains AN5 or AF 2122/97), or *M. tuberculosis* strain H37Rv or *M. microti,* 48 / 50 samples were correctly analysed by qualitative PCR.
6. Evaluation of the assay on field samples proved impossible due to the lack of availability of clinical samples either from the culling trial or Woodchester Park. This was caused by the outbreak of the Foot and Mouth epidemic in GB, which prevented badger trapping for much of the year. Consequently, we performed PCR on archival material from Woodchester Park, which had been decontaminated, processed and then stored at –80^{o}C for a number of years. A total of 42 extracts from a number of clinical samples including bite wound swabs, faeces, tracheal aspirates and urine were tested. Although badger *cytb* was detected in 29/42 of the samples, *M. bovis* was only detected in 7 samples indicating that the *M. bovis* DNA had probably degraded with storage. These results highlight the need for further evaluation of the assay on fresh clinical material.
7. A standard operating procedure for sample treatment and the PCR assay for detection have been produced and the technology transferred to VLA's routine diagnosis section. The technique is showing much promise for speeding up confirmation of *M. bovis* infection from VL lesions.

<u>Evaluation of the PhaB assay:</u>

1. Tracheal aspirates and urine from uninfected badgers held at the Natural Environmental Center at VLA Weybridge were spiked with serial dilutions of *M. bovis* in 7H9/10% OADC/1mM $CaCl_2$. 100μl of each sample was processed in the PhaB assay as described above. The spiked urine samples gave a titration curve that would be expected for the assay and confirmed the sensitivity of the assay to be between 10-100 live *M. bovis* per milliliter of sample. The number of plaques did not decrease with increasing dilution of *M. bovis* for the spiked tracheal aspirate perhaps indicating contamination of the sample with environmental mycobacteria that are susceptible to D29 infection.
2. *Evaluation of the assay on clinical samples from badgers experimentally infected with M. bovis:* Tracheal aspirates, bronchial-alveolar lavage (BAL) and urine samples were obtained from badgers experimentally infected via the tracheal route in the Republic of Ireland with 10, 100 or 1000 cfu *M. bovis* as well as from control, uninfected badgers. 100μl of each sample (blinded) was processed in the PhaB assay as described above. All negative control badgers were negative at 6 weeks post infection. One control badger was positive at 9 weeks (but pfu were low). At six weeks the PhaB assay was positive for 4 tracheal aspirates from 1/3 badgers infected with 1000 cfu, 2/3 badgers infected with 100 cfu and 1/3 badgers infected with 10 cfu *M. bovis*. At nine weeks the PhaB assay was positive for 4 urine samples from 2/3 badgers infected with 1000 cfu, 1/3 badgers infected with 100 cfu and 1/3 badgers infected with 10 cfu of *M. bovis*.

The assay met with limited success at detecting *M. bovis* from clinical samples from *M. bovis*-infected badgers. Moreover, some problems with specificity of the assay were encountered due to the ability of the mycobacteriophage used in this assay

(D29) to infect mycobacteria other than members of the *Mycobacterium tuberculosis* complex.

Conclusions

1. Although both methods are showing promise for the detection of *M. bovis* in clinical material and field samples, evaluation of the assays on large numbers of samples is required.
2. One draw back of the PhaB assay is the ability of the mycobacteriophage D29 to infect a variety of *Mycobacterium* species thus limiting its specificity for the detection of *M. bovis*. Further research will be required to improve the specificity of this assay (see below).

Future research

1. *Evaluation of the assays on clinical field samples:* Evaluation of the assays on field samples proved impossible due to the lack of availability of clinical samples either from the badger culling trial or Woodchester Park due to the outbreak of Foot and Mouth disease. Evaluation of the assays on clinical and field samples is therefore required.
2. The ability to detect and fingerprint *M. bovis* from clinical material and environmental samples would provide rapid screening of samples from badger carcasses and allow the monitoring of local levels of *M. bovis* infection and environmental contamination. By using PCR-based molecular fingerprinting techniques it should be possible to determine whether the strain of *M. bovis* detected in badgers is also present in the local cattle population. This rapid provision of information would have substantial bearing on the evaluation of current TB control policies. Further evaluation of this method will be required on completion of method development.
3. Direct molecular typing from clinical samples would allow real-time data generation in order to support on-going investigations and contact-tracing by the SVS.
4. Presently bacterial culture remains the “gold standard” for confirming *M. bovis* infection in cattle and badgers. If a PCR-based test was sensitive and reproducible it could be used for diagnosis in cattle where a rapid determination is desirable eg rapid conformation of *M. bovis* from visibly lesioned material especially from slaughterhouse cases.
5. Combining the PCR assays developed in this project with the field-platform technologies developed by eg Enigma Diagnostics would allow rapid detection of *M. bovis* (under 30 min) in the field. Discussions towards this end are underway.

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Publications generated from the project

Sales MP, Taylor GM, Hughes S, Yates M, Hewinson G, Young DB, Shaw RJ. (2001) Genetic diversity among *Mycobacterium bovis* isolates: a preliminary study of strains from animal and human sources. *J Clin Microbiol*. **39**, 4558-62.

Review comment

This project sets the basis for further PCR work as it evaluated several approaches for the detection of M. bovis in a range of samples. PCR appeared to be the most promising technology and several targets were developed. Overall the project was well received and the difficulties of this area of work were acknowledged. There was a breadth of techniques covered and as a result it was felt that some areas should be taken further forward. In particular, the DNA extraction method for PCR could be researched further and although the PhaB assay did not work in this project, there is some potential to explore this further. PCR seems suitable for lesioned samples but other sample types did not yield promising data with the PCRs tested (faecal samples gave PCR positive results in only two out of four culture positive samples and blood samples were shown to be unsuitable). Overall the PCRs tested were not as sensitive as culture. Disappointingly only 50% of samples were suitable for genotyping and this limits the PCRs potential at present. In terms of outputs, very few publications have arisen however, there are clearly important questions that need to be addressed regarding the sensitivity, specificity and how such a test will be used for the control of bTB. Further work in this area has been undertaken.

Scores

Conclusions based on sound evidence: 3.0
Quality of science: 3.0
Overall rating: 2.6

SE3118 Review and economic analysis of the use of rapid methods for *M. tuberculosis* complex detection and identification in the bovine TB control programme

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/07/05
End date 31/12/05

Total cost £46,506

Abstract
Recently, new methods have been developed for the rapid detection of *Mycobacterium tuberculosis* complex in tissue samples. These methods, mostly based on amplification of bacterial DNA using the polymerase chain reaction (PCR), can produce a result in days rather than the weeks required for culture. Therefore, they have the potential to improve the cost-efficiency of detection and identification of *M. bovis* in cattle tissue samples examined as part of the TB control programme in Great Britain.

This project has two main objectives. The first is to critically review rapid detection methods, including PCR assays, currently available for the detection and identification of *M. bovis*. The second is to compare the costs and benefits of options for incorporating a PCR assay into routine laboratory testing of cattle tissue samples for *M. bovis*. The cost-effectiveness of different options is compared using a decision analysis framework that includes test validity in the analysis.

The main conclusions from the critical review regarding the use of rapid detection methods in a routine diagnostic laboratory are:

1. Rapid screening for *M. bovis* DNA in cattle tissues is best accomplished using a PCR that targets multi-copy genes or repetitive DNA in the *M. bovis* genome.
2. The extraction procedure is at least as important as the amplification efficiency in providing the sensitivity required.
3. Whichever PCR method is adopted, the assay stages, planning and costs are likely to be similar.

It is too early to draw conclusions from the economic analysis, which will not be completed until late February 2006.

Aims

- To produce a critical review of all current rapid detection methods, including PCR assays, available for the detection and identification of *M. bovis*.
- To specify options for incorporating rapid detection methods into routine laboratory testing of cattle tissue samples for *M. bovis* in Great Britain.
- To compare the costs and benefits of the different options for incorporating rapid detection methods into routine bovine TB testing in Great Britain.

Relevance to Defra

Recently, new methods have been developed for the rapid detection of *Mycobacterium tuberculosis* complex (which includes *M. bovis*) in tissue samples. These methods, most of which are based on amplification of bacterial DNA using PCR, can produce a result in days rather than the weeks required for culture. Therefore, they have the potential to improve the cost-efficiency of detection of *M. bovis* in cattle tissue samples submitted to the Veterinary Laboratories Agency (a Defra agency) as part of the TB control programme. In some situations, more rapid diagnosis of tuberculous herds may also benefit government and industry by speeding up the imposition of movement restriction and reducing the risk of TB spreading to other herds. Information from the project will help Defra policy-makers and VLA managers to make an informed decision, and to respond to questions, about the use of the new rapid detection techniques in the TB control programme.

Methods

Critical review

A literature search of all published PCR methods relating to *M. tuberculosis* (MTB) complex organisms in general and *M. bovis* species in particular was undertaken using the entrez pub med facility at http://www.ncbi.nlm.nih.gov/. The key words used were *Mycobacterium bovis* and PCR, producing 19 pages with 365 entries, *Mycobacterium tuberculosis* complex + PCR (26 pages, 510 entries). These searches were further refined with other terms such as cattle, rapid diagnosis and real-time PCR. The publications were reviewed with regard to their suitability for providing a rapid test for *M. bovis* in a diagnostic laboratory setting. This included consideration of sensitivity, specificity and the potential for high throughput analysis if required. All possible DNA extraction methods were also critiqued as part of the review as well as technologies other than PCR for the rapid detection of *M. bovis*.

Information was also sought from university departments and agencies using PCR for diagnosis of MTB complex organisms in order to benefit from their experience using unpublished in-house methods. These included Central Science Laboratory (York), Health Protection Agency, DSTL (Porton Down, Wiltshire), AgResearch at Wallaceville Animal Research Centre (Upper Hutt, New Zealand) and Universities of Surrey, Bristol and Oxford in the UK.

Specification of options

Regular visits have been made to VLA Weybridge to discuss possible options for PCR testing in the new laboratory facilities. These have included discussion of likely screening and typing assays, preliminary costing for consumables and staff time needed per batch. The front-end processing of tissue samples for culture and DNA extraction is problematical and labour intensive, so recent discussions have focussed on options for tissue homogenisation and robotic DNA preparation.

The current laboratory testing regime and options for incorporating PCR testing into routine laboratory testing for the detection and identification of *M. bovis* in bovine tissue samples have been described using a decision tree analysis framework (Rushton *et al* 1999). The framework also includes the possible outcomes for the TB control programme from changing the laboratory diagnostic mix.

Economic analysis

The costs and benefits of incorporating a PCR assay into routine laboratory testing of cattle tissue samples for *M. bovis* have been described and where possible, will be estimated in monetary terms. Costs refer to the inputs or resources needed to undertake a specified option. Benefits refer to the outputs or outcomes achieved under each option. The decision analysis framework is used to take the sensitivity and specificity of the different diagnostic tests into account in the analysis. The monetary value of options incorporating PCR testing is compared with that of the current laboratory diagnostic testing regime.

Results

Critical review.

Key word searches revealed over 500 entries for PCR methods related to detection of MTB complex mycobacteria. A review of the relevant papers showed that methods may be divided into those amplifying repetitive DNA, single copy genes or those which have multiplexed several loci into one assay. Invariably, these are less sensitive than the first two categories. PCRs may be further sub-divided, depending on whether the primers were designed before or after information became available from the various genome sequencing projects. Some earlier methods should now be discounted on the basis of lack of specificity, that is, the presence of homologs of target genes in mycobacteria other than tuberculosis (MOTT) (Hughes *et al* 2005, Woolford *et al* 1997). Similarly, some early methods that claimed to be able to distinguish *M. bovis* from *M. tuberculosis* have proven fallible (Weil *et al* 1996).

PCR methods based on regions of difference (RD) in the *M. bovis* genome offer a uniquely specific way of identifying this pathogen. In practice the sensitivity is unsatisfactory for use on paucibacillary samples, detecting around 60% of known culture positives (SE3008 final report). This lack of sensitivity in paucibacillary samples is intrinsic to all methods amplifying single-copy genes. However, the use of primers flanking deletion regions remains the most logical approach for devising specific assays for *M. bovis* (Parsons *et al* 2002) and deserves further development to increase their sensitivity (see 3 below).

The most promising PCR contender for routine testing is the multi-copy element *IS1081* (Wards *et al* 1995). This is present in 6 copies across the MTB complex and has a sensitivity of 91% (compared to culture) when applied to cattle tissue samples (SE3008 final report). Other leading assays include single tube nested versions of the *IS6110* PCR (Taylor *et al* 2003) and the *hupB* gene (Rv2986c, Mishra *et al* 2005). Many researchers have used the *IS6110* assay, including the USA laboratory testing for *M. bovis* in white-tailed deer in Michigan (O'Brien *et al* 2004). There are several real-time versions of this available using either Taqman or FRET probe chemistries. Quantitative PCR for mycobacterial DNA and RNA has been described by many workers using either probe or non-probe methodologies.

A critical review of the literature stresses the importance of the extraction process and PCR methods should be assessed as a combination of both DNA recovery and amplification of genomic loci. The extraction procedure must deliver: 1. Efficient lysis of mycobacteria; 2. Recovery of the DNA from a complex mixture of tissue debris; and 3. Removal of PCR inhibitors. There are several possible methods but some are more suitable than others for automation and transfer to routine testing.

Other potential rapid detection systems.

1. Mycolic acids. Characteristic long-chain mycolic acids can be used as prime biomarkers for tuberculosis. Expertise is needed and the extraction is expensive. This has greater potential for identification of cultures as nanograms of mycolic acids are required.

2. E-nose (Fend *et al* 2005). An electronic "nose". Chemical sensors of volatile compounds linked to changes in physical properties of the sensor surface and production of electrical stimuli. These are analysed by a pattern recognition system. The e-nose has been used on serum and is being tested with cultures. The test is easy to perform, relatively inexpensive but still under development.

3. B-cell assay. This involves the genetic modification of B cell lines (M12g3R) engineered to express cytosolic aequorin, a calcium sensitive bioluminescent protein from the *Aequoria victoria* jellyfish and membrane bound antibodies to pathogen of interest. Cross-linking of antibody and pathogen results in an increase in intracellular calcium and fluorescence. This must be read in luminometer within 5 minutes. Sensitivities of developed tests to date are Y*. pestis* 50 copies, *B. anthracis* 1000 cfu (Rider *et al* 2003).

4. Nanotechnology. Magnetic nanoparticles with specific DNA or protein or antibody are used to bind to target pathogen. A second particle with multiple DNA reporting molecules attached is used to pull out the pathogen (Georganopoulou *et al* 2005). Of all the alternative technologies, this approach offers with greatest potential for development of a tuberculosis screening test in the short term as it has the ability to detect specific DNA or proteins from complex substrates and link these to amplification steps, which allow detection of targets in the femtomolar or attomolar range. Realistically, the development of such a test for TB is probably a few years in the future.

In summary, whilst there have been intriguing advances in methods for pathogen detection, there are no obvious candidates which will replace DNA amplification methods in the immediate future or which provide the basis for further epidemiological analysis.

<u>Specification of options</u>

VLA Weybridge has expressed a preference for 96-well plate PCR assays. Therefore, this format has been given priority in planning. A guanidinium / silica based method is envisaged for initial processing. This attenuates mycobacteria early in the extraction process, is versatile in its ability to deal with a variety of sample types and has the potential for high throughput using DNA robots.

Currently, a trial of 3 robots has been organised (Qiagen, bioMérieux and Corbett Research) to assess automation. Initially, samples will be extracted manually. For project SE3008, homogenates were prepared by hand using pestles and mortars but a tissue disaggregator, the Medimachine, is now available from DAKO. This will homogenize and filter crude extracts using disposable "Medicons". To provide sufficient samples for 96 well plates, 2 members of staff will be required to generate extracts from tissues over a 1-2 day period. Days 3-5 will be spent on DNA extraction from the homogenates, PCR set-up and analysis of the products by gel electrophoresis. This period may be shortened by eventual use of a DNA robotic system and also by design of a specific probe to report formation of the PCR product on a real-time platform, dispensing with the need for agarose gels (See Future Research 1, below).

An estimate of the DNA extraction costs and one screening PCR is approximately £17 for reagents and disposable plastic ware. This does not include staff time or purchase of capital equipment such as the robotic system (£25,000-£30,000) tissue homogeniser (£2,000) and a high-throughput gel tank and power source needed for 96-well plates (£500).

Economic analysis

The economic analysis of options for the use of PCR testing to detect and identify *M. bovis* in bovine tissue samples is not yet completed. Currently, the incorporation of a PCR assay into the diagnostic testing routine in the laboratory appears to be of most benefit to speed up the confirmation of TB in clear herds from which an animal presents at the slaughterhouse with a visible lesion. PCR testing may also be of benefit in herds that have non-visible lesioned reactors at the disclosure test. Tangible benefits from its use in herds with visible-lesioned reactors at the disclosure test are more difficult to identify but there may be indirect benefits from convincing farmers that their herds are truly infected with *M. bovis*. Using a PCR assay in addition to culture and/or histology increases the sensitivity of laboratory confirmation of the skin test and post-mortem examination results.

On the laboratory cost side of the equation, PCR testing may be done in addition to histopathology and culture of bovine tissue samples or it may replace histopathological examination of visible lesions. Both options are being subjected to economic analysis. Currently, PCR testing could not be used to replace culture as the latter is required to obtain sufficient DNA for genetic typing (eg, spoligotyping) of *M. bovis* isolates.

Conclusions

1. Rapid screening for *M. bovis* DNA in cattle tissues is best accomplished using a PCR that targets multi-copy genes or repetitive DNA in the *M. bovis* genome.
2. The extraction procedure is at least as important as the amplification efficiency in providing the sensitivity required.
3. Whichever PCR method is adopted, the assay stages, planning and costs are likely to be similar.

It is too early to draw conclusions from the economic analysis, which will not be completed until late February 2006.

Future research

1. Enigmadiagnostics at DSTL Porton Down has an ultra rapid PCR platform and is about to embark on development of methods for human and bovine tuberculosis, amongst others. They use novel probe chemistries for confirming production of amplicons, which may be useful for routine PCR diagnosis of *M. bovis*. It would seem practical to maintain close links with this group, if possible, to co-ordinate the test(s) they develop with the test that might eventually be used by the VLA and to identify areas in which the Enigma platform might be of use to Defra.

2. A critical review of the literature, experience from project SE3008 and informal discussion with scientists in agencies mentioned above all indicate that single copy PCR methods are unlikely to provide sufficient sensitivity for the detection of *M. bovis* DNA in paucibacillary tissue extracts. Multiplex PCRs also suffer from a lack of sensitivity and this problem worsens as the number of targets increases. Multiplex methods would be useful as secondary tests confirming species and for genotyping of *M. bovis* strains. The problem of poor sensitivity have been tackled by some workers by "chessboard" optimisation of all the assay components (Gunson *et al* 2003) with reports of over 40 regions successfully amplified in one reaction (Goguet de la Salmoniere *et al* 2004). It is proposed that a series of PCRs for deletion regions would be useful for confirmation of classic *M. bovis* and other ecotypes (RD4, RD9, RD12, RDmicroti, etc) and that these be optimised and combined for use as a secondary test.

   Similarly, spoligotyping applied directly to mycobacterial DNA extracts was only successful in a maximum of 50% of cases. Spoligotyping, although amplifying multiple copies of the direct repeats (DR), is effectively a multiplex PCR in that around 40 regions must be successfully amplified to yield a full fingerprint of the *M. bovis* isolate. Unsuccessful spoligotyping experiments probably reflect a form of allele "dropout" due to poor quality or quantity of DNA extracted from paucibacillary samples. It should be possible to increase the sensitivity of spoligotyping by using a modified PCR. Linear after the exponential phase PCR (LATE PCR) is a technique where one primer is limiting and exponential amplification ceases after a determined number of cycles (Pierce *et al* 2005). Thereafter the excess primer preferentially amplifies one strand above the other. This could be applied to spoligotyping by preferentially amplifying the biotinylated strand which hybridises to the membrane. This should improve sensitivity by decreasing the likelihood of reannealing of the labelled strand with its partner.

   Although not completed, the economic analysis has shown the need for more data on the sensitivity and specificity of all the laboratory tests used to detect *M. bovis* in bovine tissue samples examined as part of the TB control programme. Information on test validity in cattle populations with different skin test and post-mortem examination results are particularly lacking. Such data are important in this study because the benefits from introducing PCR testing are related to the TB testing history of the herds.

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Review comment

This contract is in an area of great potential and although a number of Defra-funded projects have practically examined the use of PCR to detect Mycobacteria, this critical review of rapid detection methods takes into account all published methods. It has confirmed that methods in use at VLA are appropriate for Defra's requirements. A full economic assessment of the potential consequences of speeding up detection rates was impossible within the project time-frame but the limited analysis was reasonable in the time available and reports on the costs and benefits of implementing rapid detection methods into routine cattle testing in the UK. Further work would be needed on analysis of the impacts on national resource use, both for farms and in implementing veterinary controls, taking into account the array of benefits accruing nationally from reduced spread of infection consequent on gains from more rapid detection. This is a very different issue from consideration of the cost effectiveness of new detection methods. Further experimental work is required to standardise both the PCR technique and assess the area where it is best to apply it.

Scores

Conclusions based on sound evidence: 1.8
Quality of science: 2.1
Overall rating: 1.9

SE3028 The development of improved tests for the diagnosis of *Mycobacterium bovis* infection in cattle

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/02
End date 31/03/05

Total cost £417,783

Abstract
The incidence of bovine tuberculosis in GB has been increasing since 1988 despite the use of a control strategy based on tuberculin skin testing and slaughter of animals that react positively to the test. To improve the specificity and sensitivity of diagnostic tests is therefore a high research priority for Defra. In this project, we applied a combination of *in silico* prediction of specificity to shortlist antigens for testing, peptide chemistry, and *in vitro* screening of peptide pools using the BOVIGAM assay with blood from infected, vaccinated, or control as a rapid high-throughput system to prioritize potential diagnostic antigens for further evaluation. Firstly, 29 antigens encoded in the RD1, RD2, and RD14 regions of the *M. bovis* genome that have been deleted from the BCG Pasteur genome, were assessed. The antigens [redacted], [redacted] and [redacted] were recognized strongly and frequently by infected, but not by BCG vaccinated cattle. Other diagnostic antigens assessed were the ESAT-6-like proteins [redacted] and [redacted]. Immunodominant peptides of these proteins were selected and, together with peptides from ESAT-6 and CFP-10 formulated into a cocktail, which was more sensitive in identifying skin test negative cattle with confirmed TB than tuberculin and ESAT-6/CFP-10 alone without loss of specificity. This peptide cocktail was also not recognised by BCG vaccinated calves. Secondly, 42 possible antigens, selected because they had no or only low homology with proteins encoded within the *Mycobacterium avium avium* (MAA) and *Mycobacterium avium paratuberculosis* (MAP) genomes, were tested with the aim to identify antigens that improved the specificity of the assay compared to tuberculin-based reagents. Several antigens that were frequently recognised by *M. bovis* infected cattle were identified and await further characterisation.

Aims
To identify and characterize novel diagnostic antigens to improve test specificity and to serve as reagents to differentiate BCG vaccinated and infected cattle (differential diagnosis) by:

- Objective 01. Apply comparative genome analysis to identify specific antigens detecting bovine TB in cattle;
- Objective 02: Validate diagnostic potential of antigens indentified in objective 01; and
- Objective 03: Exchange information between VLA and VSD, DARDNI, Stormont.

Relevance to Defra

Bovine tuberculosis is a growing and economically important problem in Great Britain with zoonotic consequences. As such, Defra continues to have a statutory obligation to control tuberculosis in farm animals in Great Britain under the Animal Health Act of 1981, the Tuberculosis Orders, and various EC directives. The incidence of bovine tuberculosis in GB has been increasing since 1988 despite the use of a control strategy based on tuberculin skin testing and slaughter of animals that react positively to the test. To improve the specificity and sensitivity of diagnostic tests is therefore a high research priority. The benefits of this approach are three-fold. First, improved sensitivity, particularly when novel diagnostic assays are used in parallel with the tuberculin skin test, would have a major benefit in reducing the economic burden of disease control. It has been estimated that an increase from 70 % to 90 % in test sensitivity would be equivalent to reducing the testing interval by a third with appreciable reduction in prevalence. Secondly, increased test specificity would have a further economic benefit by reducing the numbers of false-positive animals that may be slaughtered needlessly. Thirdly, in order to allow cattle vaccination to become a viable control policy option, diagnostic tests are required that can differentiate between infected and vaccinated cattle (*differential diagnosis*).

Methods

- Antigen mining by comparative *in silico* genome analysis (*M. bovis*, BCG Pasteur, MAA, MAP).
- Experimental *M. bovis* infection, experimental BCG vaccination.
- Testing naturally infected cattle (field reactors).
- ROC analysis.
- BOVIGAM IFNγ assay.
- Peptide synthesis for high-throughput antigen screening.
- Epitope mapping.
- BoLA class II restriction analysis (DR/DQ).
- T cell subset analysis using magnetic sorting techniques.
- IFNγ ELISPOT analysis.
- Production of recombinant proteins.

Results

1. RD regions antigens

Potential antigens from the RD1, RD2 and RD14 regions that were deleted from the genome of BCG Pasteur (Behr,1999; Mahairas,1996) were prepared as sets of overlapping synthetic peptides and screened together with two ESAT-6 like proteins, Rv0288 and Rv3019c, for reactivity in the blood of *M. bovis* infected, BCG vaccinated, or environmentally sensitized cattle (avian PPD responders). In total, 29 potential RD1, RD2, and RD14 regions antigens were assessed for immunogenicity and the antigens [redacted], [redacted], and the RD1 region antigens [redacted], [redacted], and [redacted] (Table 1) improved the sensitivity of the IFNγ assay significantly when used in combination with ESAT-6 and CFP10 as diagnostic antigens. The comparative testing of the selected antigens in *M. bovis* infected, BCG vaccinated and un-infected, yet environmentally sensitized (avian PPD responders), cattle allowed the further differentiation of immunogenic proteins into candidates for differential diagnosis, 'specific' diagnosis by improving on tuberculin, and into subunit vaccine candidates (Table 1).

With the application of peptide technology it was possible to select the most immunogenic regions of the most frequently recognized proteins as well as 'specific' epitopes from otherwise cross-reactive, 'non-specific' antigens. These peptides, together with similarly selected peptides from ESAT-6 and CFP-10 (Vordermeier,1999; 2001) were formulated into a diagnostic cocktail (Table 2) and tested in field samples using cattle with confirmed tuberculosis and cattle from herds free of bovine tuberculosis. After diagnostic cut-offs were defined by ROC analysis, we were able to apply these cut-offs to demonstrate that the defined cocktail was particularly effective in identifying cattle that were skin-test negative but had culture and pathology confirmed tuberculosis (Table 3). This was likely due to the fact that the inclusion of the novel antigens resulted in the detection of animals at early stages of disease and infection when skin test responses were not yet fully developed. This peptide cocktail was also not recognized by BCG vaccinated calves and could thus be used not only as a reagent for improving the specificity of PPD *per se*, but also for the differentiation of vaccinated from infected cattle.

2. *M. bovis*-specific antigens

With a view to applying comparative genome analysis to the identification of further antigens with potential for the specific diagnosis of bovine TB in the face of cattle exposed to environmental mycobacteria (avian PPD responders), potential antigens were prioritised for biological analysis after comparing the genome of *M. bovis* to that of *(*MAA*),* (MAP) and *Mycobacterium smegmatis*. Forty-two *M. bovis* open-reading frames that displayed no or low homology with genes found in the genomes of the other mycobacterial species were identified by performing blast analysis *in silico*, the respective proteins were then prepared as sets of overlapping peptides as described above. A number of highly immunogenic antigens were thus identified (Figure 1), and three of these ([redacted], [redacted], [redacted]) were selected for additional studies on the basis of their high response frequencies in *M. bovis* infected cattle. This subsequent analysis showed that they were not or only at low frequencies and response strengths recognised by BCG vaccinated and non-vaccinated/non-infected (avian PPD responders) control animals, but were cross-reactive with antigens from MAP. Their epitopic peptides were determined and these 20mer sequences compared to the MAA and MAP genomes, which revealed small areas of homologies between other, unrelated, MAP, but not the MAA proteins despite the fact that no significant homology to other genes were found when the complete proteins sequences were assessed (Figure 2). This again demonstrated that T cell cross-reactivity cannot be predicted from the primary protein sequences as it is inherent in small (<20 amino acid residues) sequence areas (as described previously in Cockle,2002).

3. Validation of antigens identified in SE3028

Please note that some aspects of the validation of selected antigens has been described already in the context of objective 01 (above section 1), including the testing of conventionally synthesised peptides in field samples, BCG vaccinated animals and Map infected or vaccinated cattle as well as the formulation of defined peptide cocktails composed of the immunodominant peptides from RD regions antigens, ESAT-6 family members and [redacted], [redacted], [redacted]. The results described in this section will therefore concentrate on the comparison of responses of defined peptide pools with responses achieved after stimulation with recombinant proteins. Recombinant proteins and sets of conventionally synthesised peptides were

prepared from 6 selected antigens and IFNγ responses compared. Our results confirmed that synthetic peptides are useful for the rapid immunological screening for antigenicity, and give equivalent responses compared to recombinant antigens when used as complete overlapping sets representing the complete protein sequences of smaller proteins, or immunodominant regions of longer proteins with only one immunodominant domain (Table 4). Longer proteins whose epitopes are distributed more evenly, however, need to be prepared as sets of a large number of overlapping peptides. However, this would mean the preparation of larger peptide sets and we conclude that it would be more advantageous to identify their individual epitopes and to formulate only these selected peptides into diagnostic cocktails (as described above). I would also like to point out that as part of project SE3212, we have validated a programme predicting human HLA-DR binding peptides to predict bovine BoLA-DR binding peptides (Vordermeier, 2003).

Conclusions

Bioinformatics is a powerful tool to identify potential antigens for differential and specific diagnosis for subsequent immunological analysis. The peptide-based immunogenicity screen applied proved to be highly successful and is a true high-throughput approach. The results have shown that further antigens can be found that complement the prototype antigens ESAT-6 and CFP10 by increasing the sensitivity that is needed to detect animals earlier after infection compared to tuberculin and ESAT-6/CFP-10, to detect animals that do not respond to ESAT-6 or CFP-10, and to detect animals better that going through response 'troughs', by increasing signal strengths.

However, *in silico* analysis cannot, at least with the present state of knowledge, predict the immunogenicity of a protein, mycobacterial or otherwise. Thus, subsequent to the *in silico* step, immunological experiments are an absolute requirement to assign immunogenicity/antigenicity values to the individual proteins. In addition, the absence of a gene from non-*M. bovis* mycobacterial species or BCG does not necessarily guarantee specificity as the minimum unit of immunological cross-reactivity is between 10 and 15 amino acid residues long, and like active sites of enzymes, such areas of homology can be found frequently in unrelated proteins. As T cell cross-reactivity cannot be predicted yet from the primary amino acid sequence of a protein, 'wet' immunological analysis remains an absolute requirement to define an antigen's specificity and immunogenicity.

Future research

Building on the results described here, we propose that these studies should be extended in the following way:

1. Perform field evaluation of the antigens discovered in objective 02;
2. Extend current antigen mining studies using comparative genomics. This should also take into account the recently completed genome sequence elucidation of BCG Pasteur. This should also include SNP data which allows the identification of proteins, whose genes are not completely deleted, but have been modified, or truncated by the presence of such single nucleotide changes leading either to amino acid changes or to gene product truncations;
3. Extend current antigen mining studies using comparative transcriptomics by determining expression profiles *in vitro* after infection of macrophages and dendritic cells with *M. bovis*, or BCG, as well as *in vivo* expression profiles; and

4. Consider any antigens identified also for its potential as subunit vaccine candidate.

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Review comment

This was a very well received project. It was based on direct and scientifically sound Programme needs, was well argued as a scientific case with appropriate methods employed, and has achieved outstanding success with the results obtained. The project was based on the fact that the Krebs review directed Defra towards vaccination as a control strategy for bovine TB and that therefore new diagnostic reagents that distinguish vaccinated from infected cattle are needed. Any new reagents need to include ancillary testing formats to the skin tests which will increase sensitivity and specificity. This project proceeded in a logical manner and explored the use of blood IFNγ assays and showed such tests will have great value as adjuncts to skin testing. This project used comparative genomics to identify antigens, then synthesised overlapping peptides for testing in blood assays. They showed that: blood assays could enhance detection with possibly greater sensitivities and specificities; differentiate vaccinated from infected animals, and from environmental exposure to non-pathogen mycobacteria; and importantly show that blood assays can also detect disease earlier than skin reagents. This is a very important finding as early detection is the key to disease control. However, there remain issues to be resolved. More extensive field testing is required to validate the test and it is not known whether the test will distinguish clearly between infection with diverse *M. bovis*

isolates and diverse environmental mycobacteria (or other cross-reacting organisms). It is also not yet clear whether it will work as well in cattle with all MHC alleles, although the selected epitopes are promiscuous. More thought also needs to be given on when the test is deemed “good enough”.

Scores
Conclusions based on sound evidence: 3.8
Quality of science: 3.7
Overall rating: 3.7

Table 1 List of most frequently recognised RD1, RD2, RD14 regions antigens[a].

Designation	1 Responder Frequency %			Potential Application
	[b] *M. bovis* Reactors	[c] BCG Vaccinated	[d] *Avian PPD* responders	
[redacted]	**75**	0	0	**Differential Diagnostics:** Recognized in *M. bovis* infected, but not BCG vaccinated or avian PPD responders
[redacted]	**50**	0	0	
[redacted]	**31**	0	0	
Combined	**81**	0	0	
ESAT-6/ CFP-10[e]	**94**	0	**10**	
[redacted]	**69**	**17**	0	**Specific diagnostics:** Recognised by *M.bovis* infected and BCG vaccinated but not avian PPD responders
[redacted]	**50**	**33**	0	
[redacted]	**44**	**17**	0	
Combined	**75**	**33**	0	
[redacted]	**88**	**67**	**30**	**Vaccines:** Recognised by all three animal categories
[redacted]	**67**	**33**	**40**	

[a] Only antigens recognised by >30% of *M. bovis* infected animals are listed.
[b] Results from 16 cattle experimentally infected with *M. bovis.*
[c] Results from 6 BCG vaccinated cattle.
[d] Results from 10 environmentally sensitised cattle.
[e]Results obtained with ESAT-6/CFP-10 derived peptide cocktail.

Table 2 withheld

Table 3. Diagnostic performance of reagents using animals from the field

		Field Animals Confirmed *M. bovis*	VL SICTT +ve	VL SICTT -ve	VL SICTT IR
[a]Area Under ROC Curve (0 – 1.000)	Cocktail 1	0.960	1.000	**0.769**	1.000
	Cocktail 2	0.980	0.998	**0.905***	0.990
	B - A	0.893	0.944	**0.640**	0.951
[b]Number of Cattle		[+]58	38	10	7

[a]Area under the ROC curve, generated for the 6 different groups, ROC analysis and statistical calculations was performed using Analyse-it™ software (Leeds, UK).

[b]Number of cattle tested in each of the 7 different groups.

*Area under ROC curve generated using Cocktail 2, is significantly different than that using B – A (PPD-B – PPD-A) (p=0.0372).

[+]Includes three animals that were NVL, but *M. bovis* culture positive.
Cocktail 1: ESAT6/CFP-10 only, Cocktail 2 as described in table 2.

Table 4 withheld

Figure 1 withheld

Figure 2 withheld

SE3222 Development of improved diagnostic tests for the detection of bovine tuberculosis

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/09/05
End date 30/06/08

Total cost £1,907,392

Abstract
The incidence of bTB in GB has been increasing since 1988 despite the use of a control strategy based on tuberculin skin testing and slaughter of animals that react positively to the test. To improve the specificity and sensitivity of diagnostic tests is therefore a high research priority for Defra, particularly to differentiate between infected and animals vaccinated with novel TB vaccines developed in an accompanying proposal. The aim of this proposal is to develop specific diagnostic reagents using comparative genome and transcriptome analysis to identify potential antigens that are then produced as peptide cocktails and evaluated using the IFNγ assay. In this proposal we aim to complete our antigen screen by applying a combination of comparative genome and comparative transcriptome analysis to identify species-specific proteins. Proteins identified in this way will be tested in cattle using peptide-based rapid screening techniques in combination with the IFNγ assay, a test permitted under EU law as an adjunct to the tuberculin skin test in cattle. It is a rapid and practical test potentially detecting animals at an earlier stage of infection, but has slightly lower specificity than the tuberculin skin test used in the UK. The outcome of this project will be diagnostic reagents that allow the differentiation of vaccinated and infected cattle, that reach test sensitivities approaching that of tuberculin and which improve the specificity of the IFNγ assay. In addition, antigens identified during this antigen mining operation will also be assessed for their suitability as potential subunit vaccine candidates.

Aims
To identify and characterize novel diagnostic antigens to improve test specificity and to serve as reagents to differentiate vaccinated and infected cattle (differential diagnosis) by:

- Completing antigen mining using comparative genome analysis;
- Performing antigen mining based on comparative transcriptomics to measure early gene expression in *M. bovis* and BCG following infection of macrophages;
- Determining the kinetics of antigen recognition following experimental infection with *M. bovis*;
- Evaluating the of use of antigens prioritized in objectives 1-3 to improve sensitivity and specificity of the IFNγ assay and to allow differential diagnosis in vaccinated animals; and
- Continuing the collaboration with VSD, Stormont, Northern Ireland and AgResearch, Upper Hutt, New Zealand in order to to optimise, standardize and evaluate antigen cocktails for use in diagnosis in GB, NI and NZ.

Relevance to Defra
Bovine tuberculosis remains an economically important problem in Great Britain with potential zoonotic consequences. As such, Defra continues to have a statutory obligation to control tuberculosis in farm animals in Great Britain under the Animal Health Act of 1981, the Tuberculosis Orders, and various EC directives. The incidence of bovine tuberculosis in GB has been increasing since 1988 despite the use of a control strategy based on tuberculin skin testing and slaughter of animals that react positively to the test. To improve the specificity and sensitivity of diagnostic tests is therefore a high research priority. The benefits of this approach are three-fold. First, improved sensitivity, particularly when novel diagnostic assays are used in parallel with the tuberculin skin test, would have a major benefit in reducing the economic burden of disease control. It has been estimated that an increase from 70 % to 90 % in test sensitivity would be equivalent to reducing the testing interval by a third with appreciable reduction in prevalence. Secondly, increased test specificity would have a further economic benefit by reducing the numbers of false-positive animals that may be slaughtered needlessly. Thirdly, in order to allow cattle vaccination to become a viable control policy option, diagnostic tests are required that can differentiate between infected and vaccinated cattle (differential diagnosis).

Methods
- Antigen mining by comparative genome analysis (*M. bovis*, BCG, *M. a. avium, M. a. paratuberculosis*): Improved specificity, differential diagnosis.
- Antigen mining by using comparative transcriptom analysis (BCG, *M. bovis* infected alveolar macrophages, *in vivo* expression): Differential diagnosis, subunit vaccine candidates.
- Experimental *M. bovis* infection, experimental BCG vaccination.
- Testing naturally with *M. bovis* infected cattle (field reactors).
- BOVIGAM IFNγ assay.
- Peptide synthesis for high-thoughput antigen screening.
- Production of recombinant proteins.

Results
The project has only recently been initiated and therefore only a limited amount of data is available at the time of preparing this report (December 2005/January 2006). However, the project is fully on schedule as contracted and significant progress has been made in the short time this project is active.

I would also like to emphasize that intellectual property considerations prevent me from listing details of potential proteins selected for analysis in objective 01 (comparative genomics).

<u>Objective 01: Extended antigen mining using comparative genomics</u>
The elucidation of the genome sequences of *M. bovis*, *M. tuberculosis*, *M. bovis* BCG Pasteur, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* have been completed, while the sequences of *M. microti*, *M. ulcerans*, *M. marinum*, and *M. smegmatis* are currently being deciphered. Annotation of the BCG Pasteur sequence has also been recently completed and the data is available for our analysis. A file of all proteins encoded by the 3592 genes of *M. bovis* has been generated and used to search the available mycobacterial genome sequence data using the BLAST suite of

programmes. Briefly, stand-alone BLASTP and TBLASTN was used to search the *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* sequences to identify *M. bovis* proteins with low score and E values, ie potentially *M. bovis* unique proteins.

In addition, the file of 3592 *M. bovis* gene was also searched for proteins whose genes were absent from the BCG Pasteur genome, or BCG genes with frame-shift mutations resulting in either truncated gene products (frame-shift to introduce a stop codon). The potential specificity of these selected *M. bovis* genes was then confirmed by demonstrating the absence of sequence homology with other *M. bovis* genes.

In total 40 genes have been selected by applying these criteria: 10 genes without homology to *M. a. avium* and *M. a. paratuberculosis* genes, thus potentially applicable to improving tuberculin specificity; 15 genes with frame-shifting point mutations in BCG Pasteur that lead to truncated gene products; potentially applicable to differential diagnosis; and 15 genes deleted entirely from the genome of BCG Pasteur. These gene products constitute therefore also potential antigens for differential diagnosis.

These gene products will be screened as described by us previously (Cockle,2002; Mustafa,2002). Briefly, sets of overlapping synthetic peptides will be produced and screened in the form of pools of about 10 peptides using the *in vitro* IFNγ test as it is used in the field. Blood will be obtained from cattle infected experimentally with *M. bovis* (strain 2122/97, low dose intratracheal route), BCG vaccinated cattle (BCG Copenhagen or Pasteur, 10^6 cfu, s.c,) as well as uninfected/unvaccinated control cattle or cattle responding to avian tuberculin (20 animal of each group) to select suitable antigens. We intend also to use field reactor animals in addition to the experimentally infected cattle. These peptides are being synthesized at present.

Objective 2: *Antigen mining based on differential gene expression between M. bovis and BCG inside bovine macrophages (comparative transcriptomics) to identify antigens for differential diagnosis.*

The transcriptome is the complete gene expression profile of an organism under defined conditions. We propose to describe the transcriptome of BCG Pasteur and *M. bovis* 2122/97 during growth of these bacilli in bovine macrophages as a route to antigen identification. We shall follow a modification of the methodology of Schnappinger and colleagues (Schnappinger,2003) that was used to define the transcriptome of macrophage-grown *M. tuberculosis*. Microarrays containing all genes from *M. bovis* and *M. tuberculosis* have been developed at VLA in collaboration with the BUGS Group at St George's Hospital, London (see bugs.sghms.ac.uk/index.php). RNA from bacilli extracted from the macrophages will be applied to these arrays, using three technical replicates per sample. Although this project has only very recently (September 2005), significant progress has been towards achieving this objective by developing the experimental *in vitro* infection systems and by preparing RNA for later micro-array analysis.

An additional systematic transcriptome-based approach to prioritize potential diagnostic or vaccine antigens for immunological assessment relevant for this project has been applied recently by us, namely the combination of global *in vitro* expression profiling and *in vivo* transcript analysis. The expression profiling experiments were

performed in project SE3030, but the results of the immunological screen performed have relevance for project SE3222, and I will therefore refer to them below. Acid shock was used as an *in vitro* surrogate of an *in vivo* stress and followed the acid shock response of *M. bovis* over 24 hours. Using this approach 71 genes were listed that were up-regulated *in vitro* in response to an acid shock of pH 5.5. To determine whether these genes were also up-regulated *in vivo,* quantitative RT-PCR was used to measure the expression of acid shock responsive genes in infected mouse lung tissues relative to *in vitro* grown bacilli. Genes shown to be up-regulated in mouse tissues were finally assessed for their immunogenic properties using cattle naturally infected with *M. bovis*. Through this approach we identified novel *M. bovis* antigens that have potential as diagnostic antigens or for use in subunit vaccines (see Table 1 and Figure 1). The most immunogenic antigens (eg [redacted], [redacted]) defined in Figure 1 will be characterized in future studies in respect to specificity and potential as subunit vaccine.

Conclusions

So far only limited data is available to draw far-reaching conclusions. However, our initial data confirm that bioinformatical approaches like comparative genome analysis is a powerful tool to select and prioritise potential protein antigens for immunological analysis.

Future research

Project has just started.

Diagnostic antigens that have been identified during this project should be considered for field trials.

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Publications generated from the project

Project has only started in September 2005, and therefore no publications have been generated at the time of writing this report (December 2005).

Review comment
This has the potential to generate extremely valuable data of practical use, and that is unlikely to be systematically generated elsewhere. Good collaborative links were set up initially and now are maintained. Progress has been made from the early antigen mining experiments in 2002 (SE3028), using genome sequence information from SE3206 and progressing to experimental testing of selected reagents that appear close to the required specifications for diagnostic antigens for bovine TB control. This has been an excellent use of expertise, resources and new scientific developments exploiting genomic data. This project has as its objectives further antigen mining strategies based on comparative genomics to complete the antigen screen and define further peptides to increase both specificity and sensitivity. Although further mining was necessary there has to be a finite antigen mix and from the evidence presented it has almost already been achieved. We should expect a new list of peptides that improve on those already defined in SE3028. A second major approach is to identify genes that are differentially expressed in alveolar macrophages infected with BCG or *M. bovis*. The underlying, and reasonable, hypothesis is that these genes will also be well recognised by T cells. The more important tasks of testing these peptides in skin and blood interferon assays both in field and experimental situations will be carried out in this project, and this is a key series of experiments, especially those looking at early detection. The antigens will also be assessed as vaccine candidates.

Scores
Conclusions based on sound evidence: 3.3
Quality of science: 3.6
Overall rating: 3.8

Table 1 Peptide pools used for antigen screening.

Gene Designation [2]	Gene [3]	Predicted size (amino acids) [4]	Peptide pool
[redacted]		274	1-3
[redacted]		160	4-5
[redacted]		274	6-8
[redacted]	[redacted]	606	9-15
[redacted]	[redacted]	428	16-20
[redacted]	[redacted]	218	21-23
[redacted]	[redacted]	578	24-30
[redacted]	[redacted]	468	31-36
[redacted]	[redacted]	195	37-38
[redacted]		393	39-43
[redacted]		268	44-46

The antigenicity of these gene products was assessed by preparing overlapping sets of synthetic peptides which were formulated in pools of up to 10 peptides. These peptides were then tested in the BOVIGAM IFNγ assay using blood from *M. bovis* infected cattle (see Figure 1 for results).

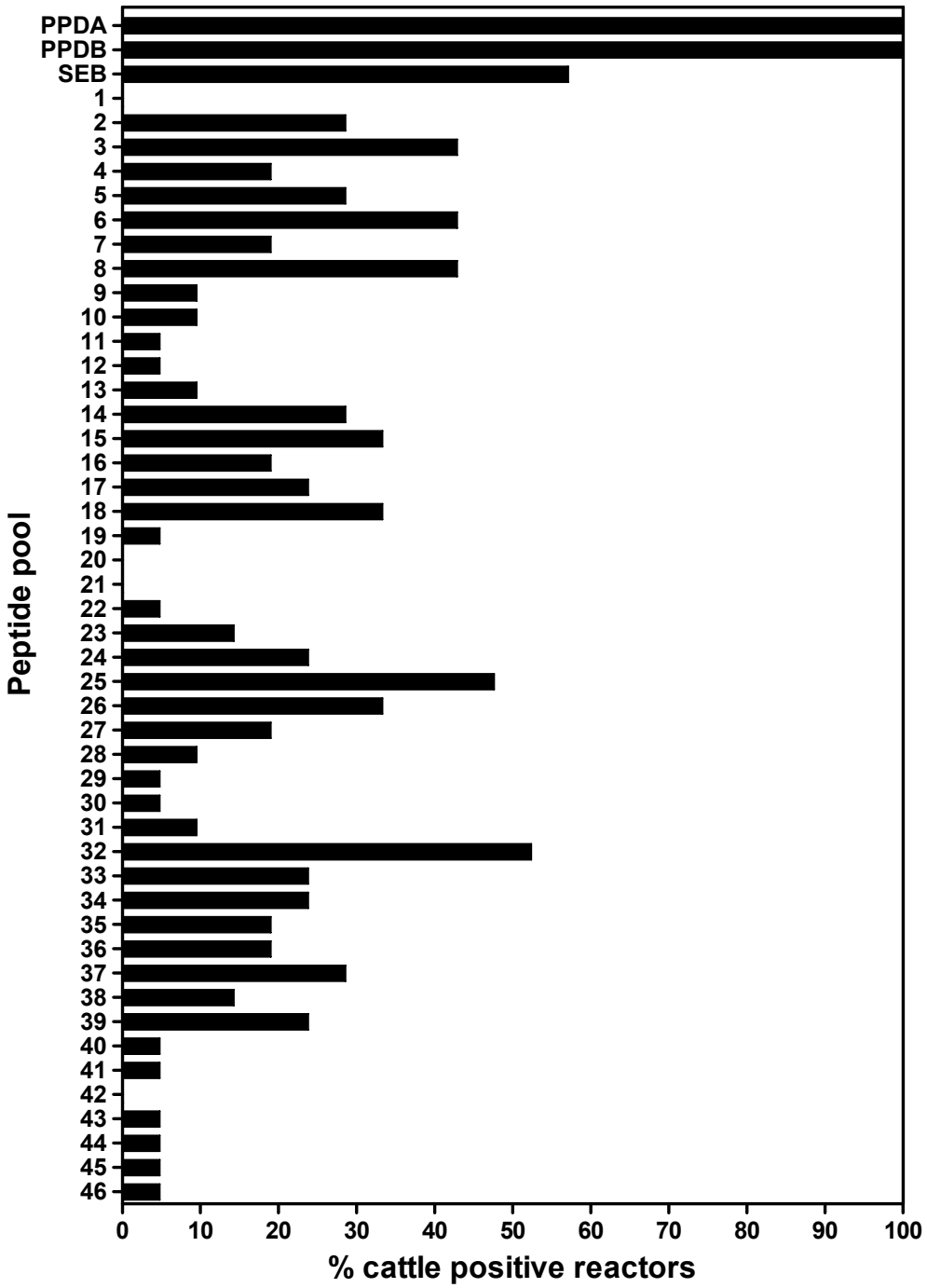

Fig 1 Percentage number of cattle that responded positively to antigens tested. The dashed vertical line indicates the positive cutoff value of 40%. PPDA is purified protein derviative from *M. avium*, PPDB is purified protein derivative from *M. tuberculosis*, and SEB is Staphylococcal enterotoxin B.

SE3208 Generation of vaccine candidates against *Mycobacterium bovis*

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/1999
End date 31/03/2005

Total cost £1,678,597

Abstract
The aim of this project was to generate vaccine candidates against bovine tuberculosis. Promising candidates were first tested in small animal models (mice [this project, SE3210 and SE3216] and guinea pigs [SE3209]) and then cattle (SE3212). Since it cannot be predicted with certainty which approach to vaccine development will yield the best candidate vaccine, we took a three-pronged approach to vaccine development. The first approach, based on the BCG paradigm, was the attenuation of *M. bovis*. This approach generated a number of promising vaccine candidates that gave at least as good protection as BCG against *M. bovis* challenge in guinea pigs. The second approach was based on induction of immune responses to mycobacterial components delivered in the form of subunit vaccines. These vaccines were based in immunodominant antigens of *M. bovis* identified by projects SE3005 and SE3028. Although these vaccines showed promise in the murine model of tuberculosis they conferred poor protection in guinea pigs and cattle. A final approach was to combine live and sub-unit vaccination in a prime-boost regime. This approach resulted in the generation of two vaccination regimes that gave superior protection to BCG-mediated immunity against *M. bovis* challenge in mice and cattle. Finally we established mechanisms with which to co-ordinate human and bovine TB vaccine development so that promising vaccine candidates developed for use in humans can also be tested for efficacy against *M. bovis* in cattle.

Aims
The aim of this project was to generate vaccine candidates against bovine tuberculosis. Diagnostic assays that can differentiate between vaccinated animals and those infected with *M. bovis*, including those that have become infected even after vaccination, are being developed in tandem as part of a co-ordinated vaccine development programme at VLA. Since it cannot be predicted with certainty which approach to vaccine development will yield the best candidate vaccine, we took a three-pronged approach to vaccine development.

1. The first approach, based on the BCG paradigm, was the attenuation of *M. bovis*.
2. The second approach was based on induction of immune responses to mycobacterial components delivered in the form of subunit vaccines. These vaccines were based in immunodominant antigens of *M. bovis* identified by projects SE3005 and SE3028.
3. A final approach was to combine live and sub-unit vaccination in a prime-boost regime.

Finally, we established mechanisms with which to co-ordinate human and bovine TB vaccine development so that promising vaccine candidates developed for use in humans can also be tested for efficacy against *M. bovis* in cattle.

Relevance to Defra
This project addresses the Government policy objective of developing vaccines that protect cattle and badgers against *M. bovis* by generating vaccine candidates. Promising candidates were first tested in small animal models (mice [SE3210, SE3216] and guinea pigs [SE3209]) and then cattle (SE3212).

Methods
- Transposon mutagenesis using the conditionally replicating phage delivery vehicles phAE77 and phAE94 to deliver the transposon Tn5367 to BCG and *M. bovis*[1].
- DNA extraction and Southern analysis.
- The technique of ligation mediated PCR (LMPCR) [2] was used to amplify the genomic DNA flanking the Tn*5367* insertions. Sequencing of the resulting amplicons allowed the mapping of transposon insertions on the genome of *M. bovis* BCG and *M. bovis* AF2122/97.
- Auxanographic analysis of mutant libraries.
- Homologous recombination. To maximize the chances of success a range of options for mutant construction have been pursued, including the use of suicide vectors, counter-selectable markers, and mycobacteriophage delivery systems.
- Construction of DNA vaccines.
- Expression and purification of recombinant proteins.
- 2D gel electrophoresis.
- Western blot analysis.
- Matrix-Assisted Laser Desorption-Ionization (MALDI) mass spectrometry.
- Nanoelectrospray (NanoES) mass spectrometry.
- Aerosol challenge of guinea pigs with *M. bovis*[3].
- Isolation of *M. bovis* from guinea pig lesions by percoll gradient centrifugation.
- Culture of *M. bovis.*
- Preparative anion exchange chromatography.
- Intravenous challenge of mice with *M. bovis*.

Detailed methodologies are available in the final report and in the numerous publications generated by this project are listed below.

Results
The development of effective vaccine candidates is still by and large an empirical process especially against ‘stealth pathogens’ such as TB, HIV and malaria and there is no guarantee of success. However, there are a number of approaches to vaccine development which have proved successful against other organisms and the aim of this project was to exploit recent advances in vaccinology, manipulation of *M. bovis* DNA, the understanding of the immune response against *M. bovis* infection and the recently completed genome sequences of *M. tuberculosis*, *M. bovis* (SE3206) and BCG (SE3206) to generate and test vaccine candidates against *M. bovis* infection.

The first approach that we took, based on the BCG paradigm, was the attenuation of *M. bovis* to produce a live TB vaccine. In this approach key genes essential for the growth and survival of *M. bovis* within the host are inactivated, thereby disabling the micro-organism. For a successful vaccine, the disabled micro-organism will be sufficiently robust to induce a protective immune response but be contained or eliminated such that the disease process is self-limited. A number of methods were used to generate live, attenuated vaccines including transposon mutagenesis, illegitimate recombination and targeted mutagenesis to knock out key genes identified by either genomics, proteomics or detailed study of important metabolic pathways. These approaches generated a number of promising vaccine candidates that gave at least as good protection as BCG against *M. bovis* challenge in guinea pigs. These included an MPB83 knockout mutant, an *M. bovis* RD1 knockout mutant, two as yet uncharacterised mutants WAg556 and WAg563, and a transposon mutant, WAg533, that failed to produce PDIMs (phthiocerol and phthiodiolone dimycocerosate esters) or glycosylphenol–PDIM as a result of disruption of gene Mb0100 by the transposon. Future work will be required on these new vaccines: 1.) To confirm the vaccine efficacy results 2.) To better characterise their stability and genetic functionality, 3.) To investigate their vaccine potential in hosts other than guinea pigs, 4.) To characterise the host response after vaccination and challenge in appropriate hosts; and 5.) To remove antibiotic resistance genes and incorporate another attenuating mutation.

The second approach to generating candidate vaccines for *M. bovis* was based on induction of immune responses to mycobacterial components delivered in the form of a subunit vaccine. This approach is preferable in relation to quality control and safety considerations and has produced a number of effective vaccines against diseases other than TB. In this project we constructed DNA or protein subunit vaccines using antigens that had been identified as immunodominant in cattle as part of projects SE3212 and SE3210. Although a number of these vaccines, especially a DNA vaccine encoding MPB83, showed beneficial effects against *M. bovis* challenge in mice and guinea pigs, none of these gave protection against *M. bovis* challenge in cattle (see SE3212). Moreover, cattle vaccinated with MPB70 first as a DNA vaccine and then used as a protein vaccine in incomplete Freund's adjuvant to boost the immune response before challenge with *M. bovis* had a significantly higher proportion of animals with severe lung lesions (>100 lesions) after challenge than the MPB70 DNA alone or the control group (see SE3212).

In our final approach we combined both approaches described above in a vaccine strategy based on priming the immune response with one type of vaccine and then boosting with the other (a heterologous prime-boost vaccination strategy). In this case the live vaccine was BCG. By using this strategy, we sought to improve the efficacy of BCG by boosting BCG-induced immunity with subunit vaccines. Two advances in our vaccine development programme at VLA had allowed us to pursue this strategy. First, we had developed specific diagnostic tests that differentiated between cattle that had been vaccinated with BCG and animals that were infected with *M. bovis* using antigens that are present in *M. bovis* but absent in BCG (see SE3210 & SE3212). Second, we had demonstrated that a cocktail of DNA vaccines could enhance the protection afforded by BCG in cattle (SE3212). In this current project, the vaccines that we used to boost BCG immunity were either (I) the same cocktail of DNA vaccines that had proved efficacious in cattle ie DNA vaccines

encoding [redacted], [redacted] and [redacted] or (ii) a protein vaccine consisting of protein [redacted] emulsified in an adjuvant consisting of MPL®-TDM and dimethyldioctadecyl ammonium bromide (DDA). We found that boosting the immunity conferred by BCG with either DNA vaccines or protein sub-unit vaccines in adjuvant provided superior protection to BCG alone in mice. Interestingly, in both prime-boost experiments we found that use of either the control DNA vector or adjuvant alone also improved the protective immunity provided by BCG. These data therefeore demonstrate the potential for improving the efficacy of BCG via adjuvant induced mechanisms possibly involving the action of innate immune factors.

The results of these studies also demonstrated that the mouse model could be used to predict the efficacy of prime-boost vaccination strategies in cattle and validated this model for future vaccine screening.

A final outcome of this project was the development of strong links with those involved in the development of Human TB vaccines. Effective links were established through a number of mechanisms including membership of the WHO Working Group on Preclinical Research Activities for the Development of New TB Vaccines, inclusion in the EU Vth Framework TB Vaccine Cluster and organising international conferences (Third International Conference *on Mycobacterium bovis* (*M. bovis* 2000) and the first "TB Vaccines for the World", conference in Montreal 2003). Professor Hewinson also edited special editions of the journal *Tuberculosis (*81:1-2 and 85 1-2) dedicated to these conferences. The extensive collaborative framework established during this project has ensured access to vaccines that are entering Phase I human clinical trials for testing in cattle and these will be tested in SE3224.

Conclusions

- In general, live vaccines have been more effective than sub-unit vaccines at protecting against aerosol challenge with *M. bovis* in the guinea pig model.
- For live vaccines, persistence of the organism or high doses of an organism that is subsequently cleared, are required to stimulate a strong enough T cell response to sensitize animals to the tuberculin skin test and provide good protective immunity.
- DNA vaccines that have showed promise in murine challenge models have not been as effective in the low dose *M. bovis* aerosol challenge model in guinea pigs and even less effective in cattle.
- Used alone sub-unit vaccines have shown little promise compared with live attenuated vaccines.
- A heterologous prime – boost strategy based on DNA vaccine priming followed by BCG boosting of cattle resulted in better protection against *M. bovis* challenge than by vaccination with BCG alone. This was true for experiments in mice and cattle but not for experiments in guinea pigs.
- The mouse model, but not the guinea pig model (SE3209) could be used to predict the efficacy of prime-boost vaccination strategies in cattle.
- Effective mechanisms with which to co-ordinate human and bovine TB vaccine development have been established and we have gained access to vaccines entering human clinical trials for testing in cattle.

Future research

Based on these results, the focus of our TB vaccine development has shifted to vaccine strategies that supplement immunity conferred by BCG rather than finding a replacement for BCG. This is in line with current vaccine development strategies for human TB vaccine development. Interestingly, in both prime-boost experiments we found that use of either the control DNA vector or adjuvant alone also improved the protective immunity provided by BCG. These data demonstrate the potential for improving the efficacy of BCG via adjuvant induced mechanisms possibly involving the action of innate immune factors. This suggests that in the effort to improve on BCG, it may prove fruitful to not only concentrate on the identification of novel antigens for inclusion in a prime-boost strategy but that detailed investigations of the role of components of adjuvants themselves may prove useful. The results of these studies also demonstrated that the mouse model could be used to predict the efficacy of prime-boost vaccination strategies in cattle and validated this model for future vaccine screening.

Since a number of live vaccines generated as part of this project also showed promise, future work will be required on these new vaccines:

1. To confirm the vaccine efficacy results;
2. To better characterise their stability and genetic functionality;
3. To investigate their vaccine potential in hosts other than guinea pigs;
4. To characterise the host response after vaccination and challenge in appropriate hosts; and
5. To remove antibiotic resistance genes and incorporate another attenuating mutation.

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* The efficacy of the vaccine candidates was assessed in small animal models and cattle as part of a co-ordinated programme of vaccine development at VLA (proposals SE3208, SE3209, SE3210 and SE3210). Therefore, publications may appear in more than one report where candidates generated in SE3208 were testing in another project.

Review comment

This was a well received project that has tried a number of approaches to generating cattle vaccine candidates. Overall, the project has made considerable progress, though not achieving fully all its objectives. The programme has encountered a number of technical difficulties in the planned approaches and has been hampered by the delay in availability of mycobacterial genome information. During the project it has also become clear that there is a difficult balance between attenuation and vaccine immunogenicity. An important output is the finding that efficacy studies in guinea pigs are not predictive of efficacy in cattle. The project appears to have been well managed, with a rational approach to assessing the data output and re-defining

objectives where necessary. The quality of the VLA science is excellent but it is also of particular note that the international collaborations in place are having a direct impact on the delivery of the project. Several candidates have been generated and tested in small animal models. As yet no candidates have generated overwhelming evidence of an improvement over BCG, when used alone. However, prime boost strategies show some promise. The conclusion that the way forward is to focus on enhancing the efficacy of BCG rather than seeking a replacement is not only logical scientifically, but is also likely to be the most successful strategy for delivering a cattle vaccine in the shortest possible time period. Further work is obviously required in this area and both SE3224 and SE3227 are progressing from this project.

Scores
Conclusions based on sound evidence: 3.4
Quality of science: 3.6
Overall rating: 3.5

SE3209 Testing of vaccine candidates for bovine tuberculosis using a low dose aerosol challenge guinea pig model

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/07/1999
End date 30/06/2004

Total cost £1,245,205

Abstract
The overall aim of this proposal is to utilise the low-dose aerosol guinea pig challenge model to screen vaccine candidates for their ability to protect the host against *M. bovis* infection acquired via the aerosol route. In all experiments, saline was included as a negative control and BCG Pasteur as a positive control. Various classes of vaccine were tested in this model including: two strains of BCG, live attenuated vaccines, boosted live attenuated vaccines, killed vaccines in adjuvant, culture filtrate in adjuvant, protein sub-unit vaccines in adjuvant, DNA vaccines, DNA-MVA prime-boost vaccine regimes and a BCG-DNA prime-boost regime that had proved more efficacious than BCG alone in cattle. A number of promising live attenuated vaccines were identified that gave at least as good protection as BCG against *M. bovis* challenge in guinea pigs. In contrast, both DNA vaccines and protein subunit vaccines proved disappointing and not one candidate gave protection equivalent to BCG or better. Better success was achieved using formalin-inactivated BCG mixed with non-phospholipid liposome adjuvants (Novasomes). Finally, we demonstrated that the use of heterologous prime-boost immunisation regimes improved upon the protection conferred by single subunit vaccines and highlighted a clear strategy for future TB vaccine development. However, we also demonstrated that the guinea pig model was unable to predict the results that we obtained in cattle (and mice) where priming with a DNA vaccine improved the protective efficacy of BCG using a heterologous prime-boost vaccination strategy.

Aims
This project addresses the policy objective of developing vaccines that protect cattle and badgers against *Mycobacterium bovis*. The overall aim of this proposal is to utilise the low-dose aerosol guinea pig challenge model to screen vaccine candidates for:

- Their ability to protect the host against *M. bovis* infection acquired via the aerosol route (equally applicable to cattle and badgers); and
- Their suitability for field study (in terms of safety and development and evaluation of a differential diagnostic test).

This data will be used to identify at least one vaccine candidate with improved efficacy/suitability over the ‘gold standard’ BCG Pasteur vaccine to go forward to field trials in cattle and badgers.

Relevance to Defra
This project addresses the policy objective of developing vaccines that protect cattle and badgers against *Mycobacterium bovis*.

Methods
Vaccine candidates were tested for efficacy in guinea pigs using a low dose aerosol challenge model developed as part of SE3202 and SE0130[1]. In all experiments, saline was included as a negative control and BCG Pasteur as a positive control. Various classes of vaccine have been tested in this model including: two strains of BCG, live attenuated vaccines, boosted live attenuated vaccines, killed vaccines in adjuvant, culture filtrate in adjuvant, protein sub-unit vaccines in adjuvant, DNA vaccines and DNA-MVA prime-boost vaccine regimes. Tuberculin skin testing, bacterial enumeration and postmortem examination are described in published papers given below.

Results
The aim of this 5 year project was to test vaccine candidates in the low dose aerosol challenge guinea pig model so that suitable candidates could be taken forward for testing in cattle. The model was developed in collaboration between VLA and Health Protection Agency, Porton Down, Salisbury (formerly CAMR, Porton Down). All the aerosol challenge work was performed at Health Protection Agency, Porton Down.

The development of effective vaccine candidates is still by and large an empirical process especially against 'stealth pathogens' such as TB, HIV and malaria and there is no guarantee of success. However, there are a number of approaches to vaccine development which have proved successful against other organisms and the aim of this project was to test vaccines developed as part of SE3028 in the guinea pig model. Vaccines candidates were developed in SE3208 by exploiting a number of recent advances in (i) vaccinology, (ii) manipulation of *M. bovis* DNA, (iii) the understanding of the immune response against *M. bovis* infection and (iv) the recently completed genome sequences of *M. tuberculosis*, *M. bovis* (SE3206) and BCG (SE3206).

The first approach that we took, based on the BCG paradigm, was the attenuation of *M. bovis* to produce a live TB vaccine. In this approach key genes essential for the growth and survival of *M. bovis* within the host are inactivated, thereby disabling the micro-organism. For a successful vaccine, the disabled micro-organism will be sufficiently robust to induce a protective immune response but be contained or eliminated such that the disease process is self-limited. A number of methods were used to generate live, attenuated vaccines including transposon mutagenesis, illegitimate recombination and targeted mutagenesis to knock out key genes identified by either genomics, proteomics or detailed study of important metabolic pathways. Early studies with auxotrophic mutants ie those attenuated due to disruption of key metabolic genes suggested that protective immunity to tuberculosis may, at least in part, be achieved without sensitisation to the tuberculin skin test[2]. We therefore investigated this phenomenon further using either mutants that persisted for different times within the host or by using different doses of the attenuated vaccine. Our results suggest that either persistence of the organism, replication of the organism or high doses of an organism that is subsequently cleared are required to stimulate a strong enough T cell response to sensitise animals to the tuberculin skin

test and provide good protective immunity. Armed with this knowledge we then targeted genes involved in virulence of *M. bov*is that might give improved protection over auxotrophic strains of BCG. These approaches generated a number of promising vaccine candidates that gave at least as good protection as BCG against *M. bovis* challenge in guinea pigs. These included a knockout mutant of the *M. bovis* glycosylated lipoprotein, MPB83 and an *M. bovis* mutant lacking the original attenuating lesion of BCG, RD1. Future work will be required on these new vaccines: 1.) To confirm the vaccine efficacy results; 2.) To better characterise their stability and genetic functionality; 3.) To investigate their vaccine potential in hosts other than guinea pigs; 4.) To characterise the host response after vaccination and challenge in appropriate hosts; and 5.) To remove antibiotic resistance genes and incorporate another attenuating mutation. However, we recommend that both vaccines should be tested in cattle. Of particular priority would be the *M. bovis* RD1 knockout mutant because vaccination of cattle with this recombinant strain would also allow differential diagnosis using RD1 antigens that include ESAT6 and CFP10 which have shown promise as diagnostic antigens for use in the Bovigam IFNγ assay. In this way vaccinated cattle could be differentiated from animals infected with *M. bovis*.

One of the recommendations of the WHO/FAO/OIE consultation on animal tuberculosis vaccines in 1994 (WHO/CDS/VPH/94.138) was that the efficacy of all candidate vaccines for bovine tuberculosis should be compared against that of BCG Pasteur. Therefore, the efficacy of BCG vaccination against disease induced by *M. bovis* was always assessed in the guinea pig alongside any vaccine candidate. We also compared the efficacy of BCG Pasteur with other strains of BCG and demonstrated no statistical difference between the protection conferred by these strains.

The second approach to generating candidate vaccines for *M. bovis* was based on induction of immune responses to mycobacterial components delivered in the form of a subunit vaccine. This approach is preferable in relation to quality control and safety considerations and has produced a number of effective vaccines against diseases other than TB. In this project we tested killed vaccines in adjuvant and DNA and protein subunit vaccines based on antigens that had been identified as immunodominant in cattle as part of projects SE3212, SE3028 and SE3222. Before testing protein vaccine candidates in guinea pigs we first identified adjuvants suitable for use in guinea pigs[3]. Both DNA vaccines and protein subunit vaccines proved disappointing and not one candidate gave protection equivalent to BCG or better.

Better success was achieved using killed organisms[4]. In these experiments formalin-inactivated BCG was mixed with non-phospholipid liposome adjuvants (Novasomes) and administered to guinea pigs as a single subcutaneous inoculation. All formulations were well tolerated and one conferred a significant survival advantage against lethal aerogenic challenge with *M. bovis*. We have therefore provided preliminary evidence that it is possible to achieve protection against TB using a killed whole mycobacterial cell vaccine administered once in an appropriate adjuvant. These vaccine formulations are cheap and safe to produce (being already based on BCG) and had no reactogenicity in the naive guinea pig. Future studies should address titration of the vaccine dose, identification of the optimum Novasome™ composition and the effect of boosting. Given that the profile of expressed mycobacterial proteins alters during their culture in liquid media, there is scope to optimize the conditions in which the BCG are grown prior to harvest and formalin

inactivation and in this way maximize the number and concentration of protective antigens in the final preparation. We also recommend that the lead candidate from this study should be taken forward for assessment and optimisation in cattle.

In our final approach we combined both approaches described above in a vaccine strategy based on priming the immune response with one type of vaccine and then boosting with the other (a heterologous prime-boost vaccination strategy). First we tested a combination of DNA vaccination followed by boosting with a recombinant vaccinia virus, MVA, expressing the same antigens as the DNA vaccine [5]. This demonstrated that the use of heterologous prime-boost immunisation regimes did improve upon the protection conferred by single subunit vaccines and highlighted a clear strategy for future TB vaccine development. We then sought to improve the efficacy of BCG by boosting BCG-induced immunity with subunit vaccines. Two advances in our vaccine development programme at VLA had allowed us to pursue this strategy. First, we had developed specific diagnostic tests that differentiated between cattle that had been vaccinated with BCG and animals that were infected with *M. bovis* using antigens that are present in *M. bovis* but absent in BCG (see SE3212, SE3028 & SE3222). Second, we had demonstrated that a cocktail of DNA vaccines could enhance the protection afforded by BCG in cattle (SE3212)[6]. In this current project, the vaccines that we used to boost BCG immunity in guinea pigs were the same cocktail of DNA vaccines that had proved efficacious in cattle ie DNA vaccines encoding HSP65, HSP70 and Apa. The results showed that the cocktail of DNA vaccines used alone had no protective effect against *M. bovis* challenge. Furthermore the guinea pig model was unable to predict the results obtained in cattle (and mice) ie that priming with DNA vaccine could improve the protective efficacy of BCG in an heterologous prime-boost vaccination strategy. ***For this reason we decided that in future the mouse model and not the guinea pig model should be used to screen vaccines for use in prime-boost strategies to improve the protective efficacy of BCG.*** This is now our strategy for testing vaccine candidates and has been taken forward in SE3224.

Conclusions

- In general live vaccines have been more effective than sub-unit vaccines at protecting against aerosol challenge with *M. bovis* in the guinea pig model.
- BCG Pasteur, BCG Tokyo and an RD1 knockout of *M. bovis* and an MPB83 knockout mutant of *M. bovis* have all proved equally protective against *M. bovis* challenge.
- Boosting with homologous live vaccines does not improve protection in the guinea pig model.
- *M. bovis* strains lacking MPB83 or *uma*A1 are significantly attenuated for guinea pigs and are therefore promising in-house vaccine candidates.
- For live vaccines, persistence of the organism or high doses of an organism that is subsequently cleared, are required to stimulate a strong enough T cell response to sensitise animals to the tuberculin skin test and provide good protective immunity.
- Killed vaccines in suitable adjuvants can be as effective as BCG in protecting against *M. bovis* challenge.
- There is an urgent need to identify adjuvants that enhance protective immunity conferred by sub-unit vaccines.

- DNA vaccines that have showed promise in murine challenge models have not been as effective in the low dose *M. bovis* aerosol challenge model in guinea pigs. Guinea pig challenge models are therefore a more stringent test of DNA vaccine efficacy than murine models.
- Heterologous prime-boost immunisation strategies show promise as a method for improving the efficacy of sub-unit vaccines.
- The guinea pig model was unable to predict the results obtained in cattle (and mice) ie that priming with DNA vaccine could improve the protective efficacy of BCG using a heterologous prime-boost vaccination strategy.

Future research

1. The identification of adjuvants that enhance protective immunity conferred by sub-unit vaccines is an area of high priority for vaccine development.
2. Continue screening promising vaccine candidates. Promising vaccines identified by this study should be tested in cattle using the low dose intratrachae*l M. bovis* challenge model. Of particular priority would be: (i). the *M. bovis* RD1 knockout mutant because vaccination of cattle with this recombinant strain would also allow differential diagnosis using RD1 antigens that include ESAT6 and CFP10 which have shown promise as diagnostic antigens for use in the Bovigam IFNγ assay. In this way vaccinated cattle could be differentiated from animals infected with *M. bovis*. (ii). The lead candidate from the formalin-fixed BCG in Novasomes study should be taken forward for assessment and optimisation in cattle.
3. A number of promising live vaccine candidates have been generated. Future work will be required on these new vaccines: 1.) To confirm the vaccine efficacy results; 2.) To better characterise their stability and genetic functionality; 3.) To investigate their vaccine potential in hosts other than guinea pigs; 4.) To characterise the host response after vaccination and challenge in appropriate hosts; and 5.) To remove antibiotic resistance genes and incorporate another attenuating mutation.
4. The mouse model and not the guinea pig model should be used to screen vaccines for use in prime-boost strategies to improve the protective efficacy of BCG.

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* The efficacy of the vaccine candidates was assessed in small animal models and cattle as part of a co-ordinated programme of vaccine development at VLA (proposals SE3208, SE3209, SE3028 and SE3212). Therefore, publications may appear in more than one report.

Review comment

This project has been fundamental in screening vaccine candidates in guinea pigs to assess the degree of immunity conferred and, the level of protection evidenced, when vaccinated animals are administered low-level aerosol challenges. The project aimed to identify at least one candidate with at least as good as efficacy as BCG to go forward into field trials and has done so. Not all of the project objectives were achieved but it has served to identify several difficulties inherent in the screening (dose/attenuation, species specificity of effects). Overall it is agreed that guinea pigs should not be used to evaluate prime/boost vaccination regimes as they were shown not to predict efficacy in cattle. Of concern was the comparative assessment of vaccine efficacy in a small animal model in which the ranking of candidates may be

different to that in cattle. It was not clear what criteria were applied in deciding antigen load levels, nor was there any information giving assurance of the quality and the consistency of the BCG Pasteur vaccine used as a positive control. The main technical failure was in implementation of the luminescent assay for bacterial burden which would have provided valuable information. However, the main theoretical concern is that this project report describes a 'promising vaccine candidate' '*at least as good as' but not an improved efficacy/suitability over the gold standard BCG*. This leads to the idea that there is a need to consider the benefit of the research in terms of the value of an incremental improvement on use of BCG. What real difference will a marginal increase in efficacy really make in a campaign?

Scores
Conclusions based on sound evidence: 3.0
Quality of science: 3.2
Overall rating: 3.2

SE3212 Testing TB vaccines in cattle

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/99
End date 31/03/05

Total cost £1,609,963

Abstract
Bovine tuberculosis remains an economically important problem in Great Britain with potential zoonotic consequences. In 1996, an independent scientific commission chaired by Professor John Krebs concluded that the development of a cattle vaccine and associated diagnostic test had the best prospect of controlling the disease in cattle. Major advances have been made in this Defra-funded project by VLA and our collaborators (especially AgResearch, NZ) and consequently Defra's TB vaccine programme is broadly on track with the timeline outlined by the Krebs' Report for the development of cattle TB vaccines. The advances can be summarized as follows:

1. Standardized experimental challenge model has been adopted (intratracheal route);
2. BCG vaccination of cattle resulted in significantly fewer visible lesions, and fewer bacteria recovered from lymph nodes. BCG vaccination was more effective when delivered to neonatal calves than to older animals;
3. A vaccine strategy based on priming the immune response with BCG followed by boosting with either DNA vaccines or protein sub-unit vaccines in adjuvant have been found to be superior to BCG alone (BCG heterologous prime-boost);
4. Heterologous prime-boosting using recombinant viruses (MVA) or recombinant adenoviruses and BCG also resulted in significantly improved immunity compared to BCG alone;
5. Prototype diagnostic tests that differentiate between vaccinated and infected cattle (eg ESAT-6, CFP-10 in conjunction with the BOVIGAM test) have been developed;
6. Immunological correlates of disease severity that can be used to predict vaccine efficacy after *M. bovis* challenge (ie ESAT-6/CFP-10 specific IFNγ responses post-challenge) have been defined; and
7. Close collaboration with human TB vaccine effort has been highly profitable.

Aims
The Krebs report concluded that the development of a cattle vaccine would have the best prospects of controlling bovine tuberculosis. Vaccination will also require an associated diagnostic test to distinguish infected from vaccinated animals (*differential diagnosis*). In addition, studies on the immune responses of cattle to *M. bovis* infection are required to identify novel antigens and to define possible correlates of protection. SE3212 addresses these points specifically by pursuing the following specific objectives:

- To adopt the intratracheal *M. bovis* cattle challenge model;
- To perform a BCG vaccination experiment to establish *vaccine gold standard;*

- To define antigen or peptide cocktails (eg MPB64, 70, 83, ESAT-6), which can discriminate between *M. bovis* infected and (BCG) vaccinated cattle (*differential diagnosis*);
- To determine if *M. bovis* infection of BCG-vaccinated cattle will compromise such protein and peptide-based reagents for differential diagnosis;
- To identify novel antigens of *M. bovis,* which stimulate strong IFNγ responses by screening antigens prioritised by comparative genomics;
- To determine if novel *M. bovis* antigens are suitable for differential diagnosis;
- To study cytokine responses as possible correlates of protection in *M. bovis*-infected, BCG-vaccinated animals, or BCG vaccinated and challenged cattle;
- To determine protective efficacy of vaccine candidates (DNA or protein subunits) in collaboration with Dr Buddle, AgResearch, NZ (to be subcontracted to Dr B. Buddle, AgResearch, NZ);
- Immunological assessment of vaccine candidates and adjuvant/delivery systems (DNA. Protein, and viral subunit vaccines); and
- To study the specificity of $CD8^+$ T cell responses in vaccinated and *M. bovis*-infected cattle.

Relevance to Defra

Bovine tuberculosis remains an economically important problem in Great Britain with potential zoonotic consequences. As such, Defra continues to have a statutory obligation to control tuberculosis in farm animals in Great Britain under the Animal Health Act of 1981, the Tuberculosis Orders, and various EC directives. In 1997, an independent scientific commission chaired by Professor John Krebs to review the situation of bovine TB in GB concluded that the development of a cattle vaccine and associated diagnostic test had the best prospect of controlling the disease in the National Herd. This conclusion has been re-affirmed in the House of Commons Environment, Food and Rural Affairs Committee's report on Bovine TB (2004) and by the findings of the Independent Scientific Group Vaccine Scoping Sub-committee, which highlighted that work on development and testing of vaccines should be maintained in order to produce a vaccine that is more effective than BCG in cattle. Vaccination will also require an associated diagnostic test to distinguish infected from vaccinated animals (*differential diagnosis*) and the definition of correlates of protection. The recommendations of the 'Krebs report' were accepted by Defra and formulated as research needs in its research requirements document released in 1998. SE3212 directly addressed these research requirements.

Methods

- Experimental infection with *M. bovis* (intra-tracheal route).
- Experimental BCG vaccination of cattle.
- DNA vaccination of cattle.
- Protein vaccination of cattle.
- Heterologous prime-boost vaccination of cattle.
- Production of DNA vaccines, recombinant vaccinia viruses, recombinant antigens and synthetic peptides.
- Collection of blood samples from naturally *M. bovis* infected cattle (field samples).

- Preparation of PBMC and blood-derived monocytes and macrophages.
- Collection of lymph nodes and preparation of lymph node cells.
- T cell proliferation assay.
- IL-2 and IL-4 bioassays.
- Cytokine RT-PCR (qualitative and real-time, quantitative PCR).
- Cr^{51}-release assays.
- Short and long-term T cell lines and T cell clones.
- TGF-β ELISA and bioassay.
- Positive selection of T cell subsets using magnetic beads.
- Collection of nasal mucus.
- IFNγ ELISA (BOVIGAM) and ELISPOT assays.
- Post-mortem examinations (PME).
- Culture of *M. bovis* from tissue samples collected at PME and histological analysis of tissue sections.
- Cultured ELISPOT analysis to probe central memory responses.
- Tuberculin skin test.

Results

Please note that only major findings are listed due to space constraints. For more details refer to final project report submitted to Defra in May 2006, and published literature.

1. The experimental *M. bovis* challenge of cattle via the intra- tracheal route was established (technology transfer from AgResearch, New Zealand).

2. At early stages of experimental *M. bovis* infection of cattle, the cellular immune responses in the peripheral blood reflect those occurring at the sites of active disease (eg retropharyngeal lymph nodes (3)).

3. Experimental infection of cattle with *M. bovis* results in a TH0 phenotype (mixed IL-4 and IFNγ responses) early after infection ($\leq$ 2.5 months post-infection), when TH1 (IFNγ) responses begin to dominate (>2.5 months post-infection) (4).

4. Antigens, selected on the basis that they are expressed prominently in *M. bovis*, but not, or only at low levels in the vaccine strain *M. bovis* BCG Pasteur (MPB64, MPB70, MPB83, ESAT-6, and CFP-10) were able to differentiate between *M. bovis*-infected and BCG-vaccinated cattle in the blood based IFNγ assay: Diagnostic protein and peptide cocktails detected only *M. bovis* infected, but not BCG vaccinated or non- infected cattle (7-9).

5. Several novel antigens potentially capable of supplementing ESAT-6 and CFP-10 in the differential diagnosis of vaccinated and infected cattle have been identified (1).

6. BCG of cattle in our hands results in about 75 % protection against subsequent *M. bovis* challenge (7).

7. Proliferative T cell and IFNγ responses towards the *M. bovis* specific antigen ESAT-6, whose gene is absent from BCG, were generally low in vaccinated animals, but high in all non- vaccinated calves (7).

8. Correlates of protection/disease severity:
   At present there are no reliable immunological surrogates or molecular markers of protection (ie *that predict the success of vaccination after completion of the vaccination protocol but before exposure to the pathogen*) for tuberculosis. The recent BCG vaccination experiments in cattle performed by Dr Buddle suggested that an immunological profile characterised by strong cell mediated immunity (IFNγ, IL-2) peaking 2-4 weeks post-vaccination, indicated that BCG vaccination had provided some level of protection against challenge, whereas lower responses or slower response kinetics may indicate a poor outcome to a subsequent challenge experiment. In addition, our results suggest that whilst high levels of IFNγ produced after vaccination does not necessarily predict that a vaccine will be protective, vaccines that did not induce strong IFNγ responses invariably failed to protect against challenge, and that IFNγ is therefore a valuable marker to prioritise vaccines for further study. In addition, preliminary results using cultured IFNγ ELISPOT analysis of central memory T cell frequencies lead us to the hypothesis that these central memory responses could correlate with the outcome of vaccination. Specifically:

   8.1. The amount of ESAT-6 and CFP-10 specific IFNγ measured by ELISA after *M. bovis* challenge, but not the frequency of responding cells, correlated positively with the degree of pathology found after infection (Figure 1). This marker is prognostic of vaccine efficacy (Figure 2) (5, 7);
   8.2. The amount of anamnestic MPB70 and MPB83-specific IgG responses (2); and
   8.3. Central memory responses have in a small pilot study shown promise as surrogate markers for protection (ie predicting vaccine efficacy after vaccination BUT before challenge (10)), and this should be investigated further.

9. Diagnostic reagents based on antigens not present in BCG (see above), like ESAT-6 and CFP-10, were still able to distinguish BCG vaccinated/diseased animals from BCG vaccinated animals without signs of disease (Figure 3, (7)). Comparative genome analysis is useful to identify additional candidates (7).

10. Heterologous prime-boosting vaccination approaches improved efficacy of BCG:

    10.1. Priming with DNA vaccines (HSP65, HSP70, Apa) followed by BCG boosting, as well as priming with BCG followed by DNA vaccine boosting improved the protective effects of BCG (5, 6) (Table 1);
    10.2. Priming with BCG followed by boosting with protein subunit vaccines delivered in adjuvant (CpG ODN in Emulsigen™) also improved efficacy of BCG vaccination alone (12, 13) (Table 2); and

10.3. Heterologous prime-boost strategies of BCG in combination with recombinant viral vectors expressing the mycobacterial antigen Ag85A used in human TB vaccine research (recombinant MVA or adenovirus) improved cellular immune responses significantly compared to BCG alone (10, 11).

Conclusions

General remarks:

A number of new vaccine candidates have been tested in this project; and a vaccine strategy based on priming the immune response with BCG followed by boosting with either DNA vaccines or protein sub-unit vaccines in adjuvant have been found to be superior to BCG alone (BCG heterologous prime-boost). Based on these results, the focus of our TB vaccine development has shifted to vaccine strategies that supplement immunity conferred by BCG rather than finding a replacement for BCG. This is in line with current vaccine development strategies for human TB vaccine development and is the approach recommended by Defra's Vaccine Programme Advisory Group. This project has also highlighted the need for better adjuvants for subunit vaccines in cattle and identified a number of promising adjuvant candidates that require optimization. In addition, we have recently shown that heterologous prime-boosting using recombinant viruses (MVA, adenovirus) and BCG also resulted in significantly improved immunogenicity compared to BCG alone, and the protective efficacy of these viral vaccines should be determined as a matter of urgency. It has also been highly profitable to closely coordinate with the global human TB vaccine development effort and to test promising lead vaccine candidates that are entering human clinical trials in cattle.

Specific conclusions:

1. Heterologous prime-boost scenarios based on a combination of protein, DNA or viral subunits and BCG resulted in improved protection and/or immune responses compared to BCG vaccination alone.
2. Immunological correlates of disease severity, and therefore inverse correlates of protection have been identified (RD1 antigen specific post-challenge IFNγ responses) that are useful as prognostic markers of protection.
3. Prototype diagnostic reagents based on peptides derived from RD1 regions antigens have been designed that can distinguish between vaccinated and infected cattle.
4. Close collaboration between national and international groups developing cattle TB vaccines has been highly advantageous and allowed us to provide synergy (eg VLA, IAH, AgReserach).
5. Close collaboration between human and bovine TB vaccine programmes and efforts has also been highly productive and synergistic.

Future research

- To continue to develop heterologous prime-boost vaccination regimens based on boosting BCG with either protein subunit vaccines in adjuvant or recombinant viral vectors. Particular emphasis should be placed on the optimization of adjuvant formulations and viral delivery systems. MVA and adenoviral subunit vaccines need to be assessed urgently for their protective efficacy in cattle.
- To use a combination of genome-based bioinformatics with high-throughput peptide screening in the BOVIGAM assay to define potential subunit candidates.

- To develop improved recombinant BCG by introducing genes not or under-expressed in BCG compared to *M.bovis*.
- To continue close coordination with the global human TB vaccine development effort and to test promising lead vaccine candidates that are entering human clinical trials in cattle.
- To continue with the close synergistic collaboration with AgResearch (New Zealand), IAH (Compton) and VSD (Belfast).
- Neonatal vaccination studies.
- To perform vaccination experiments under conditions of natural transmission using naturally infected animals as donor animals to challenge vaccinated and unvaccinated sentinel cattle. This will help to validate results obtained with experimental *M. bovis* challenge systems.

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Review comment

The work was well received and encompassed the core activities of the cattle vaccine development programme. The first major goal was to establish a reproducible experimental challenge model to evaluate the effect of vaccination. This was achieved and the model is now used routinely in vaccine testing. The most difficult aspect was to establish quantitative endpoints for comparison of different candidates. In small animal models this is generally done by measuring bacterial load in selected organs, though most studies now combine this with some measure of pathology (often time to death). The bovine model currently relies on a semi-quantitative score based mainly on the number of lesions in different tissues. This is a reasonable solution that allows the project to move forward: ultimately, it is likely that more robust quantitative endpoints will be required. The second goal was to use the model to identify improved vaccines. Some success has been achieved in this.

Neonatal BCG was shown to have advantages in comparison to the same vaccine delivered to adult animals, and heterologous prime-boost protocols achieved some detectable improvement over BCG alone. A wide variety of other vaccine strategies, including live vectors, recombinant proteins and different adjuvants were tested for immunogenicity in cattle using either *in vitro* IFNγ based assays or *in vivo* challenge. It was disappointing that more dramatic improvements were not found, but these results are comparable to those of the much larger efforts that have been invested in attempts to improve BCG as a human vaccine. The third goal was to develop discriminatory diagnostic tests. Again, considerable success was achieved in this, and it is realistic to propose that the approaches developed in the project could indeed be used to customise a diagnostic test suitable for use in combination with a BCG-based vaccine strategy. It was not possible to identify "correlates of protection" although evidence was generated to support the use of immunological assays for early detection of disease in future field trials. Overall this represents a huge amount of work, and significant progress in a project that presents formidable scientific and technical challenges. This work has enabled progression to the next stage of use in naturally infected cattle, which will be a more realistic test of efficacy.

Scores
Conclusions based on sound evidence: 3.0
Quality of science: 3.5
Overall rating: 3.6

Table 1 Pathological and microbiological parameters of protection against experimental infection with *M. bovis*.

Group	No. of animals with lung lesions/ total no.	No. of animals with lymph node lesions/ total no.	Mean lung score[a]	Mean lymph node score[b]	Mean no. of lymph nodes with lesions per animal	No. of *M. bovis* culture-positive animals/ total no.	No. of retropharyn-geal and thoracic lymph nodes *M. bovis* positive/ total no.	Mean $\log_{10}$ cfu of *M. bovis* for retropharyngeal and thoracic lymph nodes
Nonvaccinated	9/12	12/12	2.67 ± 0.56	3.75 ± 0.33	2.42 ± 0.42	12/12	32/72	1.89 ± 0.43
DNA	8/12	12/12	2.92 ± 0.67	4.00 ± 0.33	2.58 ± 0.29	12/12	35/72	2.03 ± 0.44
DNA/BCG	3/12[c]	5/12[d]	0.42 ± 0.26[d]	1.08 ± 0.43[e]	1.08 ± 0.49[c]	9/12	22/72	1.36 ± 0.34[d]
BCG	5/12	7/12[c]	1.33 ± 0.54	1.92 ± 0.56[d]	1.33 ± 0.41	10/12	21/72	1.51 ± 0.42

[c] Significantly different from the value for the nonvaccinated group ($P < 0.05$).
[d] Significantly different from the value for the nonvaccinated group ($P < 0.01$).
[e] Significantly different from the value for the nonvaccinated group ($P < 0.001$).
Significantly different parameters are highlighted in red.
From reference (5).

Table 2 Pathological findings following challenge of calves with *M. bovis*[c].

Vaccine group	No. of animals with macroscopic lesions/total no. of animals		Mean lung score[a] ± SEM	Mean total LN score[b] ± SEM	Mean no. of LNs (± SEM) with macroscopic TB lesions per animal
	Lung lesions	LN lesions			
No vaccine	9/10	10/10	3.40 ± 0.48	7.10 ± 1.04	2.50 ± 0.22
CFP/Emulsigen/CpG ODN	10/10	10/10	4.30 ± 0.21	8.60 ± 1.30	3.00 ± 0.37
CFP/Emulsigen/CpG ODN + BCG	4/9*	4/9*	1.56* ± 0.76	2.22* ± 1.06	1.11* ± 0.46
BCG	5/10	8/10	2.00 ± 0.76	3.50* ± 1.17	1.70 ± 0.40

[a] Lung lesion scores: 0, no lesions; 1, 1 to 9 lesions; 2, 10 to 29 lesions; 3, 30 to 99 lesions; 4, 100 to 199 lesions; 5, ≥200 lesions.
[b] Total lymph node (LN) lesion scores for individual animals based on scores for individual nodes: 0, no lesions; 1, 1 to 19 small lesions (1- to 4-mm diameter); 2, ≥20 small lesions; 3, medium-sized lesion (5- to 9-mm diameter); 4, large lesion (≥10-mm diameter).
[c] *, significantly different from nonvaccinated group ($P < 0.05$).
From reference (13).

Fig 1 Positive correlation between ESAT-6-specific IFNγ and disease severity. IFNγ concentration was determined by EIA in supernatants from ESAT-6-stimulated (5 µg/ml) whole blood cultures performed 11 weeks after *M. bovis* infection. Responses of individual cattle are shown in relation to the severity of disease observed at the post-mortem (pathology score). Closed symbols, BCG-vaccinated animals; open symbols, unvaccinated control animals. From reference (7).

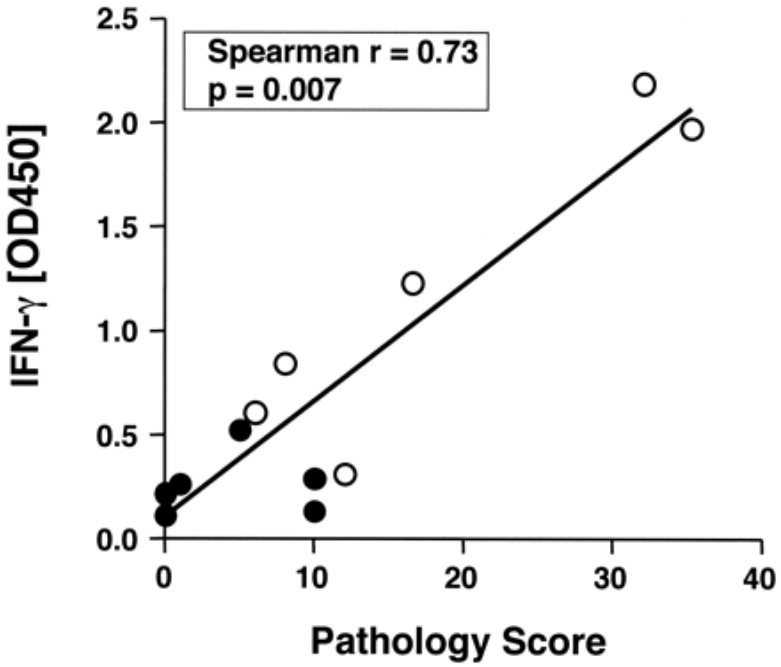


Fig 2 IFNγ responses to an ESAT-6-CFP-10 peptide cocktail and bovine PPD as indicators of protection. IFNγ responses to an ESAT-6- and CFP-10-derived peptide cocktail (A) or bovine PPD (B) were determined 10 weeks after challenge. The values are means, and the error bars indicate standard errors of the means. An asterisk indicates that the value is significantly different from the values for the nonvaccinated and DNA-vaccinated groups ($P < 0.05$).From reference (5).

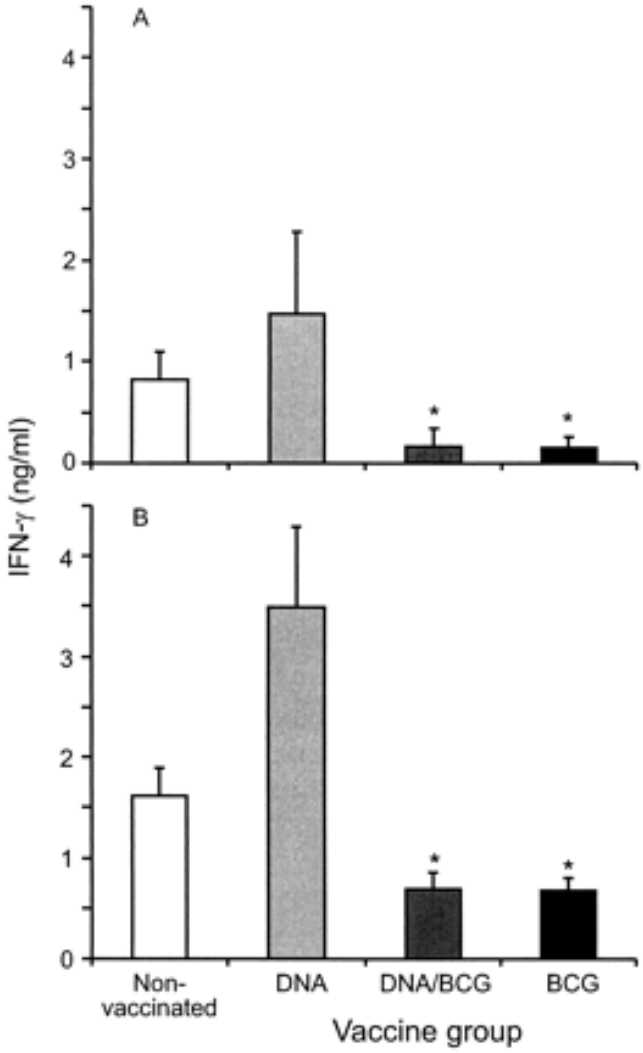
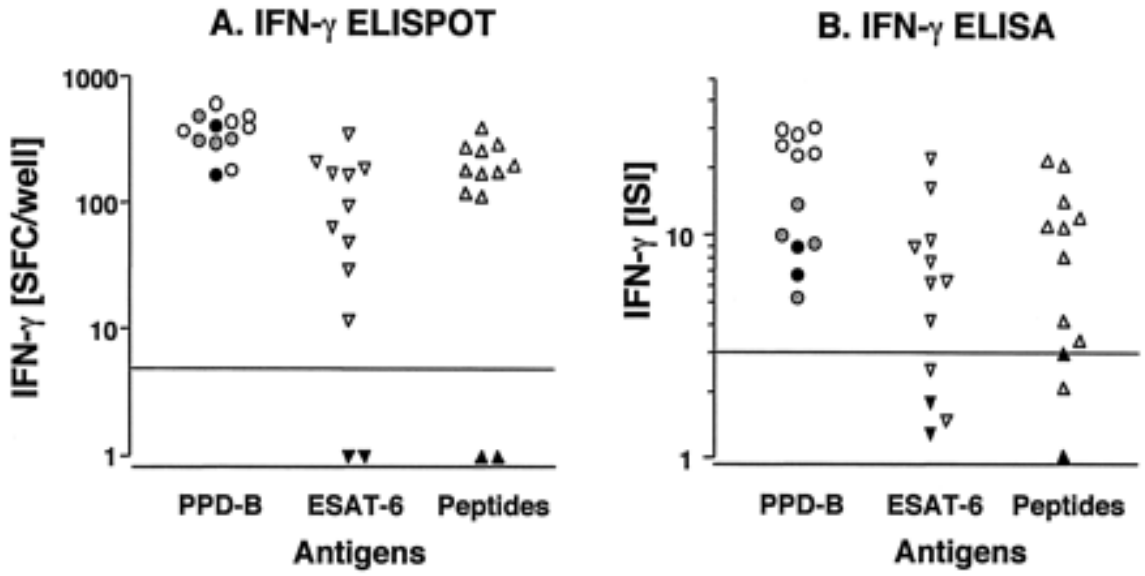


Fig 3 Differential diagnosis to differentiate vaccinated, diseased cattle from vaccinated, protected cattle. IFNγ responses were determined 16 weeks postchallenge using either ELISPOT (A) or EIA (B) as readout systems. Antigens were as follows: PPD-B (10 μg/ml), recombinant ESAT-6 (5 μg/ml), or a synthetic peptide cocktail (5 μg of each peptide/ml). Filled symbols, BCG-vaccinated animals without signs of disease; shaded symbols, BCG-vaccinated animals with confirmed bTB; open symbols, unvaccinated controls; horizontal lines, cutoffs for positivity as determined by testing of uninfected animals. ISI, IFNγ stimulation index; ELISA, enzyme-linked immunosorbent assay. SFC, spot-forming cell. From reference (7).



SE3224 Continuation of the development of vaccines against bovine TB in cattle

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/05
End date 30/09/08

Total cost £5, 622,823

Abstract
The results obtained in Defra's vaccine programme to date have demonstrated the principle that prime-boost strategies using subunit vaccines to boost BCG can confer superior protection to BCG. There is now a need to optimise both the antigens and adjuvants used to formulate such subunit vaccines. This is the aim of the current project. Moreover, there is growing evidence that BCG may be most effective when given to neonates. Therefore as part of this project we will establish the duration of immunity to neonatal vaccination with BCG to provide the model for a prime boost analysis. In addition, we aim to exploit the success of the *M. bovis* and BCG genome sequencing projects to improve the vaccine efficacy of BCG itself.

We also intend to take advantage of recent developments in the development of vaccines against human tuberculosis by testing three candidates that are now moving into clinical trials. Comparison of the immunogenicity and protective efficacy of these candidates in the cattle model with results in humans and non-human primates will provide insights into the extent to which vaccine studies can be extrapolated between species. In addition, these studies will generate information that will inform subsequent rounds of candidate production, and may identify one or more candidates suitable to take forward as a cattle vaccine.

Aims
A. VLA OBJECTIVES (1-8)

- *Objective 1:* Evaluation of antigens identified in antigen mining project as subunit vaccine candidates.
- *Objective 2:* Selection of most potent adjuvant for protein delivery.
- *Objective 3:* Determination of protective efficacy of subunit vaccine candidates in mice.
- *Objective 4:* Establish and validate immune correlates of protection and pathology.
- *Objective 5:* Compare vaccine efficacy of protein/adjuvant subunit vaccines developed in 02 and 03 with proteins delivered by viral vectors in cattle.
- *Objective 6:* Assess the immunogenicity and protective efficacy in cattle of vaccines developed for the human TB vaccine effort now entering human clinical trials.

- *Objective 7:* Improving BCG through understanding of genome dfferences between BCG and *M. bovis*.
- *Objective 8:* Develop private/public partnership (PPP) with Pfizer Animal Health.

B. IAH OBJECTIVES (9-13)

- *Objective 9:* Compare the immunity induced in neonatal calves to BCG Danish and BCG Pasteur.
- *Objective 10:* Establish the duration of immunity to neonatal vaccination with BCG to provide the model for a prime boost analysis.
- *Objective 11:* Determine whether animals vaccinated with BCG as neonates can be effectively boosted at a later time point with either BCG or an alternative antigen in a prime boost strategy.
- *Objective 12:* Compare alternative antigens and immunological assays to distinguish between vaccinated animals that are totally immune from vaccinated animals that are infected or diseased.

Relevance to Defra

In 1996, Government tasked an independent scientific committee, chaired by Professor John Krebs, to review the problem of bovine TB in GB. One of the recommendations put forward by the committee was that vaccination of cattle offered the best long-term solution for controlling the disease in the National Herd and that priority should be given to the development of a cattle vaccine against bovine TB and an associated sensitive and specific diagnostic test. This conclusion has been re-affirmed in the House of Commons Environment, Food and Rural Affairs Committee's report on Bovine TB (2004) and the findings of the Independent Scientific Group Vaccine Scoping Sub-committee, which highlighted that work on development and testing of vaccines should be maintained in order to produce a vaccine that is more effective than BCG in cattle.

Methods

- Experimental infection with *M. bovis* (intra-tracheal route).
- Experimental BCG vaccination of cattle.
- DNA vaccination of cattle.
- Protein vaccination of cattle.
- Heterologous prime-boost vaccination of cattle.
- Production of DNA vaccines, recombinant vaccinia viruses, recombinant adenovirus vaccines, recombinant antigens and synthetic peptides.
- Collection of blood samples from naturally *M. bovis* infected cattle (field samples).
- Preparation of PBMC and blood-derived monocytes and macrophages.
- Collection of lymph nodes and preparation of lymph node cells.
- T cell proliferation assay.
- IL-2 and IL-4 bioassays.
- Cytokine RT-PCR (qualitative and real-time, quantitative PCR).
- Cr^{51}-release assays.
- Short and long-term T cell lines and T cell clones.
- TGF-β ELISA and bioassay.

- Positive selection of T cell subsets using magnetic beads.
- Collection of nasal mucus.
- IFNγ ELISA (BOVIGAM) and ELISPOT assays.
- Post-mortem examinations (PME).
- Culture of *M. bovis* from tissue samples collected at PME and histological analysis of tissue sections.
- Tuberculin skin test.

The testing of live genetically modified vaccines will be performed at VLA and IAH. The evaluation of the protective efficacy of sub-unit vaccines used alone or in combination with BCG will be evaluated through collaboration with AgResearch in New Zealand.

Detailed experimental protocols are given in the original proposal.

Results
At the time of writing contracts have just been signed.

Future research
Promising vaccine candidates ie those that are shown to give superior protection to BCG against intratrachael challenge of cattle with *M. bovis* will need to be assessed in a more realistic challenge model before taken to field trials. A natural transmission model is being established in project SE3227 as part of Defra's TB vaccine programme.

Review comment
This expensive project (£8.2M) is a continuation of vaccine discovery programmes spanning 7 years of Defra funding and the width and depth of the proposal are impressive. The project involves a number of agencies and partnerships (VLA, IAH, AgResearch in NZ), including 'Big Pharma' and together create a scene set for successful exploitation of the fruits of this project – namely improved protective vaccines. A strength of this proposal is the synergy between the human TB vaccine community and the Defra programme, making direct use in cattle of new vaccines being tested in human phase I trials. This was a recommendation of the Krebs' report and has been implemented expeditiously. As an exploratory rather than confirmatory programme, it will require a further 5 year horizon if research findings from this programme are to be refined and translated into something which could be a promising vaccine for use in the field. The project will also look at neonatal vaccinations and assess adjuvant activity in cattle. The experimental strategies for the proposals are well planned and argued, being based on recent progress in antigen mining using genomic screening. The interdependence and sequential progress of multiple Defra projects has been very well managed and has provided a wealth of scientific information that will directly support this current vaccine discovery and vaccine trial project. Although the prioritisation of antigens will be based around interferon response assays, not all antigens will be able to be studied in cattle for resource reasons. Mice will be used for vaccination and challenge experiments and the scientific rationale for this is well argued, but not ideal. There will therefore be some candidate vaccines that work well in mice that fail in cattle. Starting with 30-40 candidate antigens ensures some will be found. A concern is that some potentially good candidates for cattle may be lost through the mouse screening process. There

are some areas that were criticised; adjuvants remain a problem for sub-unit vaccines as those identified in small animal studies do not work sufficiently well in cattle. Also, it is proposed to use 3 adjuvant doses in mice for the screen but assessing the immune response after one or two doses may be more discriminating. Hence although praised, there are significant risks for delivering against the milestones identified with some scientific aspects of the work, which are not insignificant scientific challenges in their own right. As a result, there may have been an underestimate of the time requirements to execute procedures and achieve objectives identified.

Scores
Conclusions based on sound evidence: 3.4
Quality of science: 3.7
Overall rating: 3.6

SE3227 Evaluation of the protection efficacy of vaccines against bovine TB in a natural transmission setting

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/10/05
End date 31/05/11

Total cost £6,862,888

Abstract
Significant scientific advances have been made by VLA and our collaborators towards developing a TB vaccine for cattle. These advances have meant that Defra's TB vaccine programme is broadly on track with the timeline outlined by the Krebs' Report for the development of cattle TB vaccines. However, as highlighted by Defra's Vaccine Programme Advisory Group (VPAG), a major barrier to progress in cattle vaccine research is the absence of experimental systems to measure vaccine efficacy in a natural transmission setting. Without this information, it is difficult to assess whether, "laboratory", advances will have any significant impact in the field. Therefore, the aim of this proposal is to assemble a herd of reactor cattle in a contained setting. This facility will then be used to determine the efficacy of promising vaccine candidates under conditions of natural transmission. This will be done by introducing sentinel vaccinated and control animals into the reactor herd and leaving them in-contact with reactor animals for 10-12 months. The protective efficacy of vaccine candidates will be determined by comparing disease rates between vaccinated and unvaccinated cattle. The first vaccine to be tested will be BCG given to neonates. Further vaccines to be tested in this way will be prioritised on the basis that they have been shown to induce better protection against experimental challenge in cattle than BCG. This design was presented to and approved by VPAG at its meeting on 12 May 2005.

Aims
To determine the protective efficacy of novel TB vaccines for cattle in a natural transmission setting by:

- Developing a logistical framework for project;
- Performing a proof of concept experiment to establish transmission rates;
- Determining the protective efficacies of cattle TB vaccines under conditions of natural transmission; and
- Evaluating reagents for differential diagnosis.

Relevance to Defra
Bovine tuberculosis remains an economically important problem in Great Britain with potential zoonotic consequences. As such, Defra continues to have a statutory obligation to control tuberculosis in farm animals in Great Britain under the Animal Health Act of 1981, the Tuberculosis Orders, and various EC directives. In 1996, an independent scientific commission chaired by Professor John Krebs to review the situation of bovine TB in GB concluded that the development of a cattle vaccine and associated diagnostic test had the best prospect of controlling the disease in the

National Herd. This conclusion has been re-affirmed in the House of Commons Environment, Food and Rural Affairs Committee's report on Bovine TB (2004) and by the findings of the Independent Scientific Group Vaccine Scoping Sub-committee, which highlighted that work on development and testing of vaccines should be maintained in order to produce a vaccine that is more effective than BCG in cattle. Defra's VPAG highlighted that a major barrier to progress in cattle vaccine research is the absence of experimental systems to measure vaccine efficacy in a natural transmission setting. Such an experimental system would be part of the vaccine development 'pipeline' by closing the gap between vaccine development using experimental infection models and field trials. The need to assess the ability of promising TB vaccine candidates to protect cattle against natural transmission of *M. bovis* was announced by the Animal Health Minister Ben Bradshaw to the House of Commons on 9 June 2005.

Methods

- A herd of reactor animals (infected donors) will be established from field reactors selected on the basis of tuberculin skin test positivity and IFNγ responses to ESAT-6 and CFP-10.
- Experimental vaccination of neonates (BCG, and BCG based heterologous prime-boost approaches): sentinel animals.
- In-contact infection of vaccinated and unvaccinated controls.
- Immunological follow-up throughout vaccination and in-contact phase (IFNγ assays, serology, other assays of cell-mediated immune responses).
- Post-mortem examination of vaccinated animals and culture of *M. bovis* to determine protective efficacy of vaccines tested.
- Histological and microbiological assessment of infection status.

Results

The experimental phase of the experiment has not begun yet. However, we have been able to modify the animal housing at VLA as specified in the grant proposal and the proposal is, despite delays in approving funds, on track as planned originally.

I will in the following paragraphs briefly outline the principle of the experiments. Our approach will be to establish a facility for generating natural transmission of *M. bovis* between cattle by assembling reactor cattle in a contained setting and then introducing sentinel vaccinated and control animals. These sentinels will be monitored for immune responses to *M. bovis* infection and the extent of disease will be determined at post mortem. The protective efficacy of vaccine candidates will be determined by comparing disease rates between vaccinated and unvaccinated cattle under conditions of natural transmission. The vaccines to be tested will be prioritised on the basis that they have previously been shown to induce improved protection against experimental challenge in cattle compared to BCG. This design was presented to and approved by VPAG on its meeting on 12 May 2005 and is very loosely based on a similar study conducted in the 1950s (Hubrig,1958).

Donor animals (naturally infected). Skin test reactor animals will be identified in collaboration with the SVS, bought on and transported to VLA. The animals will be selected from farms with previously confirmed bovine TB. Reactors will be replaced when necessary according to animal welfare considerations. We envisage and have planned for a 25% turnover in reactors per annum.

Vaccination experiment. In the initial vaccination experiment, BCG (Danish) vaccination using the freeze-dried commercial vaccine preparation supplied by SSI (Copenhagen, Denmark) will be compared to a heterologous prime-boost protocol based on BCG prime followed by protein subunit boost. We and our collaborators have recently shown that this prime-boost protocol induced protection superior to that of BCG alone (Wedlock,2005). However, this vaccination regime could be replaced if a more promising vaccine candidate becomes available in the period before this experiment is due to start. Sentinel animals will be recruited from farms that are known to be TB free and located in non-endemic parts of GB. They will be bought from these herds within 4 weeks of birth, and BCG vaccinated at around 6-8 weeks of age (Buddle,2003), or kept as un-vaccinated controls. The heterologous prime-boost group of animals will be boosted with the protein subunit vaccine when around 6 months old. Sentinel animals will be kept in isolation from adult animals at a site distant from the farm area housing the reactor animals. Regular (monthly to 6-weekly) blood-based immunological analysis (Bovigam IFNγ test, serology) will be performed. A tuberculin skin test (SICCT) will be performed 1 month before the sentinels are introduced into the donor (reactor) herd. The sentinel animals will be introduced into the reactor herd when they are approximately 9 months old, so that they are old enough to mix well with the older animals. Sentinels will be introduced at a donor-to-sentinel ratio of 1:1. This means that each year we will introduce 20 BCG vaccinated sentinels, 20 BCG/protein subunit vaccinated and 20 unvaccinated control sentinel animals into the reactor herd. Following introduction into the donor herd, blood samples will be obtained regularly from the sentinels for immunological analysis (Bovigam using bovine and avian PPD and antigens that discriminate between BCG vaccination and *M. bovis* infection, such as ESAT-6 and CFP-10 (Vordermeier,2001) and others (Cockle,2002) to assess differential diagnosis, serology); SICCT will be performed every 6 months. After an in-contact period of 10-12 months, sentinels will be slaughtered and post-mortem examinations, as well as histo-pathological and microbiological analysis will be performed as described by Vordermeier *et al* (Vordermeier,2002) to establish the disease status of these animals. One interpretation to be applied will be whether animals would have passed a routine slaughterhouse meat inspection. This, together with the results of the immunological analysis, will allow a dynamic assessment of vaccine efficacy and will lead to an appreciation of the protective efficacy of the vaccines tested (see also *Annexe*). New sentinels will be fed into the donor herd yearly until statistically significant results have been achieved. The numbers of animals introduced into the herd will depend upon the transmission rate of *M. bovis* from infected cattle to sentinel animals as described below.

NB. The initial in-contact experiment will be performed in the first Coombeland-2 building to become available and the aim of the experiment will be to determine the transmission rate of *M. bovis* from reactor animals to uninfected sentinels. Twenty skin test reactors will be held in contact with 9 months old unvaccinated sentinel animals for up to 12 months. This data will be used to refine the subsequent experimental protocols.

Conclusions
Not applicable, experimental phase of project has not started yet.

Future research
Vaccines that have been proven to be effective in this model should be considered for field trials, which should be designed in principle well before the end of this project.

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Publications generated from the project
Project has only started in November 2005, and therefore no publications have been generated at the time of writing this report (January 2006).

Review comment
This project represents a logical progression from the vaccine development work undertaken in SE3212. Previous studies have characterised the performance of vaccines in an experimental challenge model based on a protocol that ensures 100% of the animals develop progressive disease. While this represents a useful initial screen, it is possible that significant differences will be observed under conditions which reproduce natural routes and doses of infection. The critical unknown variable

in the proposed study is the in-contact transmission rate. The provisional study design is based on estimates from old literature and the review of the study design and project objectives once results are available from the initial proof of principle experiment, due March 2007, are important. Thereafter it may be necessary to alter animal numbers or the length of experiments in order to ensure statistical robustness. An important aspect of the study design is that, in addition to vaccine testing, it provides an excellent opportunity for further testing and refinement of diagnostic assays.

Scores
Conclusions based on sound evidence: 3.0
Quality of science: 3.5
Overall rating: 3.4

Annex: Approaches to assessment degree of protection

1. Failure to pass routine abattoir meat inspection criteria (contingency tables = chi-square, Fisher's exact test).
2. Gross pathology and culture to establish number of diseased animals/group (contingency tables = chi-square, Fisher's exact test).
3. Lesion scores (gross pathology) and granuloma scores (histo-pathology) to determine degree of pathology and disease progression (non-parametrical statistical methods, ie Mann-Whitney).
4. Immunological assessment: Conversion rate to positivity in skin test, IFNγ and serological assays ('survival curve' plotting non-recurrent events, ie proportion of animals remaining BOVIGAM or serology tests negative). These immunological readouts will allow a temporal, dynamic assessment of protection.

SE3217 Kinetics of skin test response in bovine tuberculosis

Organisation Institute for Animal Health
Compton Laboratory

Start date 01/04/04
End date 31/03/05

Total cost £252,100

Abstract
The aims of this project were; to determine the kinetics of the tuberculin skin test (TT), the development of anergy to a second TT, and the effect of the TT on the immune responses of infected cattle.

Cattle infected intranasally with *M. bovis* 2122/97 developed positive TT at three weeks post infection; no responses were detected at weeks 1 or 2. Only 1 animal, out of 35, became non-responsive in a second test at 28 days post application of the first TT, indicating that anergy may occur at an individual level, not at the herd level.
The earliest PPD-B-specific IFNγ responses were detected at week 3, which coincides with the development of the skin test.

The TT affected the immune responses to mycobacteria; one week after TT, IFNγ responses to PPD-A were boosted to a greater extent than those to PPD-B, in some cases compromising the diagnostic efficacy of the IFNγ test. This has implications for the timing of blood sampling for diagnosis in the field after skin testing.

The TT boosted antibody responses at weeks 3, 4, 6 and 10, but not at weeks 1 and 2. Thus, the antibody response occurs only after priming by the infection.

The development of IFNγ responses to specific mycobacterial antigens, ESAT-6 and CFP-10, was detected from 3 onwards; no responses against these antigens were observed at weeks 1 and 2, which is similar to the kinetics for PPD.

Experimentally, the skin test can detect infected animals three weeks after inoculation.

Aims
- To determine the kinetics of the development of reactivity in the comparative intradermal skin test (TT) and other laboratory based assays in animals infected with *M. bovis.*
- To determine the period of anergy after an initial tuberculin test.
- To determine the effect of the TT on the immune response and its correlation to time of testing.

Relevance to Defra

This investigation aimed to provide more accurate knowledge of the kinetics of the immune responses detected by diagnostic tests for bTB, which in turn would aid strategies aimed at improving the detection of infected animals. The proposal addressed questions posed by Defra (MAFF at that time) and the Independent Scientific Group (ISG) in the Animal Health Research Requirements 2000-2001 document on Bovine Tuberculosis, namely:

- What are the kinetics of development of reactivity in the tuberculin test and other laboratory based assays in animals infected with *M. bovis*;
- How long following an initial tuberculin test do animals remain non-reactive in a second test; and
- The investigation was also to address how the different immunological responses produced as a result of infection with *M. bovis* are differentially affected by the skin test and how this relates to the time after exposure that the skin test is used?

Methods

The following experimental design was arrived at to allow the objectives above to be answered. It resulted from a number of proposals and discussions between IAH, Defra and the ISG.

	Weeks for applying the TT							
Groups	0	1	2	3	4	6	10	19
1	Inoc	TT					TT	
2	Inoc		TT				TT	
3	Inoc			TT			TT	
4	Inoc				TT		TT	
5	Inoc					TT	TT	
6	Inoc						TT	TT
7	Inoc						TT	TT
8	Mock							TT

Each group consisted of five cattle aged about 6 months. Animals were inoculated intra-nasally (I/N) at time zero with 10^4 colony forming units (cfu) *M. bovis* strain AF2122/97 actively growing log phase organisms via a short canula of about 10 cm into the back of the naso-pharyngeal area.

Groups were skin tested by the comparative intradermal skin test (indicated as TT in the tables and subsequently) at the indicated times. This involved the intradermal inoculation of 100 μl of bovine and avian PPD (called PPD-B and PPD-A respectively) into the neck according to EC Regulation Number 1226/2002. Skin test thickness was measured before inoculation and 3 days later. Increase in skin test thickness in mm is given in the results.

Animals were bled weekly to assess the immune responses by in-vitro assays (listed below).

At the end of the experiment the animals were necropsied. The standard post mortem examination included the removal and macroscopic and microscopic

examination of the following tissues. The left and right submandibular, parotid and retropharyngeal lymph nodes and tonsils. The nasal turbinates and trachea. The lung was examined by making approximate 1 cm slices throughout to look for granuloma. Samples from left and right apical, medial and diaphragmatic lobes and the intermediate lobe were taken into formalin for microscopic (histological) examination. The mediastinal and four bronchial lymph nodes were removed and similarly examined. The pre-scapular lymph nodes were examined in the same way. A macroscopic examination was made of other body tissues including kidney, spleen, liver and heart. Pathology scores were as described (Hope *et al* 2005) slightly modified from Vordermeier *et al* (2002).

All animal experiments were approved by the local ethics committee and were in accordance with UK Home Office regulations.

Immunological assays

Blood was taken into heparin or for serum. Aliquots were incubated with (1) PPD-B (2) PPD-A, (3) control medium for 24h. Supernatants were removed after centrifugation and stored at -20^{0}C for subsequent assays of IFNγ responses. These supernatants were also assayed for production of IL-10.

IFNγ responses to recombinant antigens or peptide pools supplied by VLA, Weybridge were also assessed in blood from animal group 7 incubated with, (a) recombinant ESAT-6, (b) recombinant CFP-10, (c) ESAT-6/CFP-10 peptide pool, (d) "supermix" (Vordermeier *et al* 2002; Pollock *et al* 2000, Cockle *et al* 2002).

Proliferative responses of lymphocytes to PPD-B and PPD-A, or medium, were assessed in blood diluted 1/10 in RPMI medium and incubated for 5 days at 37^{0}C (Thom *et al* 2004). mRNA was isolated from blood diluted with RPMI and incubated with PPD-B for 3 days at 37 C (Thom *et al* unpublished).

The assessment of IFNγ was routinely performed using a quantitative ELISA and results expressed as pg per ml of supernatant (Kwong *et al* 2002, Thom *et al* 2004). Some samples were also assayed using the commercial IFNγ kit (CSL Animal Health, Australia) which relies on differences in OD between plasma from blood cultured with PPD-A and PPD-B to make a diagnosis of infection. IL-10 Elisa was as described (Kwong *et al* 2002).

Results

1. Lesions at necropsy

   Table 2 shows the extent of lesions seen at necropsy. All of the animals had lesions typical of TB in at least some of the tissues with no differences being evident between the different groups.

2. Time taken to develop a response in the comparative intradermal skin test post infection and effect of previous skin tests

   Table 3 summarises the results of the TT in the groups of calves. Details of individual animals are given in Tables 4(a), 4(b), 4(c).

   By 3 weeks post inoculation there was a clear response evident in the TT in 5/5 animals tested (Group 3, Tables 3 and 4a). The response to PPD-B was greater

than that seen with PPD-A. No response in the TT was convincingly evident in animals in groups 1 and 2 that were TT at weeks 1 and 2 post inoculation respectively. For animals TT 4 weeks and 6 weeks post inoculation 10/10 animals responded in the test and the increase in skin thickness is similar and greater than that seen at week 3.

The results of skin testing groups of calves 10 weeks after infection are summarised in Table 3 and detailed for individual animals in Table 4b. Group 6 had not been previously skin tested and is the positive control group for assessing whether a previous TT given in the early stages of disease development affected subsequent skin tests. For the other groups, with the exception of animal 887, the skin test responses were the same for animals subjected to the TT 4 weeks (group 5), 6 weeks (group 4), 7 weeks (group 3), 8 weeks (group 2), and 9 weeks (group 1) previously.

Animals in groups 6 and 7 were subjected to the TT 19 weeks after inoculation of *M. bovis* (Table 3 and Table 4c). Animals in group 6 had previously been subjected to the TT 9 weeks earlier which was at week 10 post-inoculation. Animals in group 7 had not been previously skin tested. No difference was evident between the groups. Animals in group 8 were non-infected controls and did not show any response in the TT.

Animal 887 that did not respond in its second TT on week 10 was re-tested on week 19. At this time it did respond but weakly compared to other animals.

3. Time taken to develop an IFNγ response post infection and effect of the comparative intradermal skin test on the response

The IFNγ responses given as pg of IFNγ in supernatants of whole blood incubated with medium, PPD-A, or PPD-B are shown in Figure 1.

In all animals IFNγ production in response to the addition of antigen to cultures was evident by 3 weeks post challenge. At this time responses to PPD-A were in some cases as great as those seen with PPD-B (discussed and extended below). By 4 weeks post infection, and subsequently, responses to PPD-B were greater than those seen with PPD-A in the absence of a TT.

Skin testing had no obvious effect in animals if they were skin tested on weeks 1, 2 or 3 after challenge. But, in animals TT 4, 6 or 10 weeks after challenge a boost in the IFNγ response was evident which was particularly noticeable for cultures incubated with PPD-A.

4. Comparison of commercial IFNγ test

The results above were assessed with the quantitative ELISA, subsequently called Q-ELISA (Kwong *et al* 2002) and results expressed as pg per ml. The commercial IFNγ ELISA (subsequently called the C-ELISA) as used to detect infection with *M. bovis* simply compares OD values for blood samples incubated with PPD-A and PPD-B. The results of comparing the C-ELISA and Q-ELISA are shown in Table 5.

The interpretation of whether animals were, “positive,” or “negative,” has been done in 3 ways. For the C-ELISA it has been based on an OD value with PPD-B

being 0.05 or 0.1 OD units greater than seen with PPD-A. This is as recommended by the manufacturer (CSL) or Buddle and colleagues (Ryan *et al* 2000) respectively. For the Q-ELISA it is based on a comparison of the pg of IFNγ obtained with blood from non-infected control calves or with PPD and samples from infected animals. Based on this for the Q-ELISA a difference of 250 pg IFNγ allowed the distinction of infected from non-infected animals.

For this analysis "positive" is taken as indicating infection by *M. bovis* because the response to PPD-B was greater than that seen with PPD-A. "Negative," could indicate no response or a response in which the difference between PPD-A and PPD-B was not interpreted as being sufficient to be meaningful for diagnosis.

The C-ELISA detected an immune response at 3 weeks post challenge, the same as the Q-ELISA. All 19/19 pre-infection samples were negative by all 3 methods as were 23/23 samples taken 2 weeks after infection (Table 5).

Three weeks post infection 13/24 samples were positive based on an OD of 0.1, 14/24 samples were positive based on an OD of 0.05 and 13/24 samples were positive based on 250 pg. The samples that were not positive at week 3 did show an immune response had developed, compared with time zero, but the response to PPD-A was greater than or equal to that seen with PPD-B. At 4 weeks post inoculation 14/14 samples were positive by all 3 tests.

When the IFNγ assay is used post TT and the animals in groups 6 and 7 are compared 3/10 animals that were positive before TT were no longer positive based on an OD of 0.1 or 0.05 and 1/10 was no longer positive based on 250 pg.

5. Proliferation assay
   Results of proliferation assays are shown in Figure 2. Proliferative responses were evident by week 3, and peaked at week 4 with no obvious effect from TT.

6. Recombinant antigens
   The opportunity was taken to test several recombinant antigens supplied by VLA Weybridge to determine whether the kinetics of the response was the same using these antigens (Figure 3). The pool of ESAT-6 and CFP-10 peptides gave the most robust response. As with PPD the response to infection was initially detected 3 weeks after exposure to *M. bovis* indicating a similar potential for detection of infected animals.

7. Antibody response
   Figure 4 shows the antibody responses of the calves to PPD-B used as antigen to coat plates. As with previous studies over the period studied post infection there was no or very little antibody response to infection in the absence of the TT. An antibody response was produced by the calves given the TT 3 weeks after infection but not in calves give the TT one or two weeks after infection. Thus, the antibody response to the TT does not occur until animals are already mounting an immune response to the infection ie it is post priming by live bacteria. The intensity of the antibody response was greater when animals were TT at week 4 and even greater for animals TT at week 6. It was less for animals given a TT at

week 10 compared to week 6. Thus, the intensity appears to follow the intensity of the cell mediated response.

8. Other cytokines
   Assays for IL-10 by the ELISA indicated a stronger response with PPD-B than PPD-A and no PPD-A boost post TT. Quantitative PCR for IL-13, IL-6 and IL-4 pos TT indicated an associated increase in IL-4 transcripts.

Conclusions

The TT detects infected animals 3 weeks after infection but is not maximal at this time. By 4 weeks it is maximal. It does not detect infected animals up to 2 weeks after exposure. Of the 30 animals tested twice one became non-responsive to a second test given at an interval of 28 days. However, this animal would have been identified as infected in the first test. This animal may represent a small percentage of animals that do become anergic. The implication is that in some circumstances infected animals might not be detected as a result of having been given a previous TT. This is likely to be a rare occurrence.

The IFNγ response develops post infection at the same time as the TT. It is evident 3 weeks post infection but not detected up to week 2.

The distinction of infected animals by contrasting the response to PPD-B and PPD-A needs to take account of the time post infection. Initially, infected animals produce a response with a more intense bias to PPD-A.

Of potential significance was the observation that the TT given to animals 4, 6 and 10 weeks post infection (but not up to week 3 see Figure 1) boosts the avian response more than the bovine PPD response. This may mask the diagnosis of infection if the IFNγ test is used in the week after skin testing.

The kinetics of the response to recombinant antigens is the same as that observed for PPD.

Future research

The current diagnostic test is far from perfect and there is a need to improve on its efficacy and reliability. The intention of vaccinating healthy animals in so-called hot spots, raises the need to distinguish between vaccinated and infected animals. A greater understanding of the immune mechanisms by which the skin test works is necessary in order to develop improved diagnostic tests. For instance, it may be possible to take advantage of the boosting of antibody production in infected animals following skin testing; it is known that non-infected animals, repeatedly skin tested animals are not sensitized to mycobacteria (Thom *et al* 2004). Detection of antibodies specific to mycobacteria may be particularly helpful in cases of inconclusive animals. A better understanding of the immune response to mycobacteria would also contribute to develop improved vaccination strategies and to the development of strategies that may permit the differentiation between vaccinated and infected animals.

Boosting of PPD-A responses by the comparative TT at seven days should be confirmed as this may have implications for policy.

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Publications generated from the project
M. L. Thom, J. C. Hope, M. McAulay, B. Villarreal-Ramos, T. J. Coffey, S. Stephens, H. M. Vordermeier, and C. J. Howard. The effect of tuberculin testing on the development of cell-mediated immune responses during *Mycobacterium bovis* infection. *In press.* Vet Immun Immunopathol

Review comment
This project had clearly defined objectives and focused on key issues related to the tuberculin test. The results provide Defra with reassurance in relation to the overall effectiveness of the test. It provided useful information on issues such as the interval between infection and reactivity to the test and the interval to full reactivity following a tuberculin test. The work was carried out competently, completely and represented good value for money. The finding that skin test induced anergy was rare and related only to one animal provides confidence in the continued use of this approach in the field (this animal would have been diagnosed as positive in the first test). No speculation was attempted as to why the single animal became anergic. However, the response of individual animals to each of the diagnostic procedures described is a function of a range of factors, both intrinsic and extrinsic, only some of which can be accounted for under the conditions of the project and very few of which can be considered to be attributable to “anergy” within as short a period as 19 weeks post-inoculation. Questions relating to the specificity and sensitivity of the skin test were

outside the scope of the work. Reviewers both before, and at the meeting itself, disagreed on the appropriateness of using a group of young animals in the experiments and no real consensus was reached on this issue. It was suggested that animals that have been naturally infected should be used to verify these findings.

Scores
Conclusions based on sound evidence: 3.0
Quality of science: 3.2
Overall rating: 2.9

Table 1 Pathology at necropsy.

Mean lesion score and standard deviation shown for the groups of animals.

Group	Mean pathology score	Mean no. tissues
1	16 ± 3	5 ± 1
2	10 ± 5	3 ± 1
3	12 ± 5	5 ± 2
4	8 ± 4	3 ± 1
5	11 ± 5	4 ± 2
6	15 ± 5	5 ± 1
7	15 ± 5	5 ± 1
8	0 ± 0	0 ± 0

Table 2 Summary of time to development of a response to the TT post infection.
Mean and (standard deviation) shown for animal groups at times of TT as indicated in Table 1. Responses to PPD-A and PPD-B shown as average increase in skin thickness (mm) 3 days after intradermal inoculation of 100 μl of PPD. R indicates animals would have been categorised as reactors.

Group	1st TT	PPD-A	PPD-B	Result	2nd TT	PPD-A	PPD-B	Result
1	wk 1	0 (1)	1 (1)		wk 10	9 (3)	34 (8)	R
2	wk 2	1 (1)	2 (1)		wk 10	6 (2)	31 (3)	R
3	wk 3	4 (3)	19 (16)	R	wk 10	8 (4)	38 (11)	R
4	wk 4	5 (3)	29 (9)	R	wk 10	4 (2)	24 (7)	R
5	wk 6	7 (1)	37 (10)	R	wk 10	5 (4)	23 (15)	R
6	wk 10	5 (3)	32 (3)	R	wk 19	5 (2)	29 (9)	R
7					wk 19	6 (3)	30 (10)	R
8					wk 19	1 (3)	0 (0)	

Table 3a Results of TT: Time course for animals becoming responsive after infection.

TT at:			**mm increase in skin thickness with**		
			PPD-A	PPD-B	Difference B-A
Week1	Group 1	481	1	1	0
		587	0	0	0
		878	0	0	0
		901	1	1	0
		920	-1	1	1
Week 2	Group 2	482	0	1	1
		567	2	2	0
		879	2	2	0
		902	0	1	1
		926	2	2	0
Week 3	Group 3	483	6	14	6
		575	0	8	8
		590	1	9	8
		910	7	46	39
		933	5	20	15
Week 4	Group 4	491	5	24	19
		576	9	27	16
		592	8	42	34
		886	3	35	32
		940	1	18	19
Week 6	Group 5	551	7	53	46
		578	6	38	32
		598	8	27	19
		887	6	36	30
		912	6	33	27

Table 3b Results of TT: Response for animals tested 10 weeks after infection.

		mm increase in skin thickness with		
		PPD-A	PPD-B	Difference B-A
Group 1	481	12	30	18
	587	9	30	21
	878	11	37	26
	901	6	47	41
	920	5	26	21
Group 2	482	5	32	27
	567	7	29	22
	879	5	34	29
	902	4	32	28
	926	8	26	18
Group 3	483	11	34	23
	575	6	35	29
	590	2	56	54
	910	9	38	29
	933	11	27	15
Group 4	491	7	26	19
	576	2	26	24
	592	5	31	26
	886	1	23	22
	940	4	13	9
Group 5	551	10	22	12
	578	6	36	30
	598	8	21	13
	887	0	0	0
	912	3	37	34
Group 6	555	7	33	26
	582	3	29	26
	599	9	34	25
	888	6	28	22
	915	1	36	35

Table 3c Results of TT: Response for animals tested 19 weeks after infection.

		mm increase in skin thickness with		
		PPD-A	PPD-B	Difference B-A
Group 6	555	7	25	18
	582	3	18	15
	599	8	42	34
	888	6	32	26
	915	3	26	23
Group 7	564	7	31	24
	583	4	13	9
	606	7	33	26
	891	11	31	20
	918	2	40	38
controls	487	0	0	0
Group 8	572	6	1	0
	610	0	0	0
	869	0	0	0
	945	0	0	0
Group 5	887	3	8	5

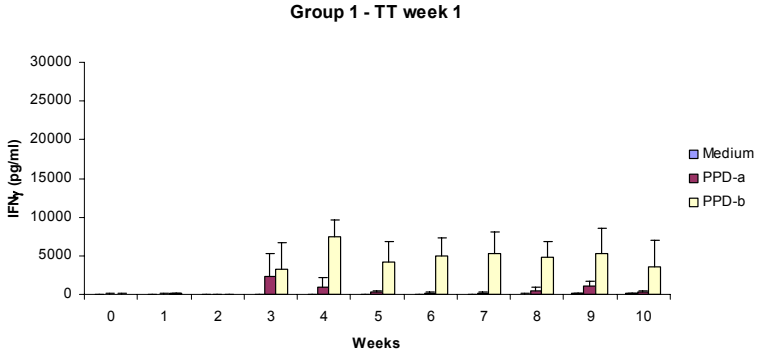
Table 4 Comparison of diagnosis with the commercial (CSL) ELISA and the quantitative ELISA.

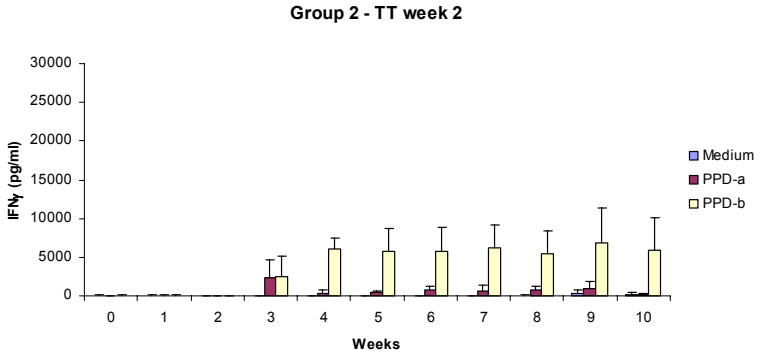
Cut-off		0.1 OD	0.05 OD	250 pg
Animal	Week	Buddle interpretation	EIA interpretation	IAH interpretation of ELISA
Group 2				
482	0	negative	negative	negative
567	0	negative	negative	negative
879	0	negative	negative	negative
902	0	negative	negative	negative
482	2	negative	negative	negative
567	2	negative	negative	negative
902	2	negative	negative	negative
482	3	positive	positive	positive
567	3	positive	positive	positive
879	3	negative	Avian	negative
902	3	negative	Avian	negative
Group 3				
483	0	negative	negative	negative
575	0	negative	negative	negative
590	0	negative	negative	negative
910	0	negative	negative	negative
933	0	negative	negative	negative
483	2	negative	negative	negative
575	2	negative	negative	negative
590	2	negative	negative	negative
910	2	negative	negative	negative
933	2	negative	negative	negative
483	3	positive	positive	positive
575	3	positive	positive	negative
590	3	positive	positive	positive
910	3	negative	positive	Avian
933	3	positive	positive	positive
483	4	positive	positive	positive
575	4	positive	positive	positive
590	4	positive	positive	positive
910	4	positive	positive	positive
933	4	positive	positive	positive
Group 4				
491	0	negative	negative	negative
576	0	negative	negative	Avian
592	0	negative	negative	negative
886	0	negative	negative	negative
940	0	negative	negative	negative
491	2	negative	negative	negative
576	2	negative	negative	Avian
592	2	negative	negative	negative
886	2	negative	negative	negative
940	2	negative	negative	negative
491	3	positive	positive	positive
576	3	positive	positive	positive
592	3	positive	positive	positive

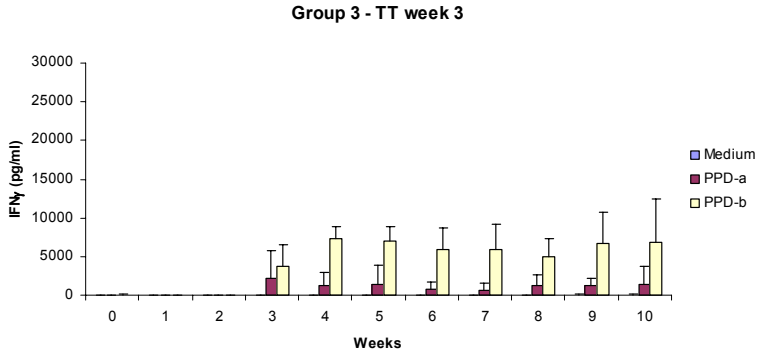
886	3	negative	Avian	Avian
940	3	positive	positive	positive
491	4	positive	positive	positive
576	4	positive	positive	positive
592	4	positive	positive	positive
886	4	positive	positive	positive
940	4	positive	positive	positive
Group 5				
551	0	negative	negative	negative
578	0	negative	negative	negative
598	0	negative	negative	Avian
887	0	negative	negative	negative
912	0	negative	negative	negative
551	2	negative	negative	negative
578	2	negative	negative	negative
598	2	negative	negative	negative
887	2	negative	negative	negative
912	2	negative	negative	negative
551	3	positive	positive	positive
578	3	negative	Avian	positive
598	3	negative	Avian	positive
887	3	negative	Avian	Avian
912	3	negative	Avian	Avian
551	4	positive	positive	positive
578	4	positive	positive	positive
598	4	positive	positive	positive
887	4	positive	positive	positive
912	4	positive	positive	positive
551	6	positive	positive	positive
578	6	positive	positive	positive
598	6	positive	positive	positive
887	6	positive	positive	positive
912	6	positive	positive	positive
551	7	positive	positive	positive
578	7	positive	positive	positive
598	7	positive	positive	positive
887	7	positive	positive	positive
912	7	negative	Avian	positive
551	8	positive	positive	positive
578	8	positive	positive	positive
598	8	positive	positive	positive
887	8	positive	positive	positive
912	8	positive	positive	positive
551	9	positive	positive	positive
578	9	positive	positive	positive
598	9	positive	positive	positive
887	9	positive	positive	positive
912	9	positive	positive	positive
Group 6				
555	0	negative	negative	negative
582	0	negative	negative	negative
599	0	negative	negative	negative
888	0	negative	negative	negative
915	0	negative	negative	negative

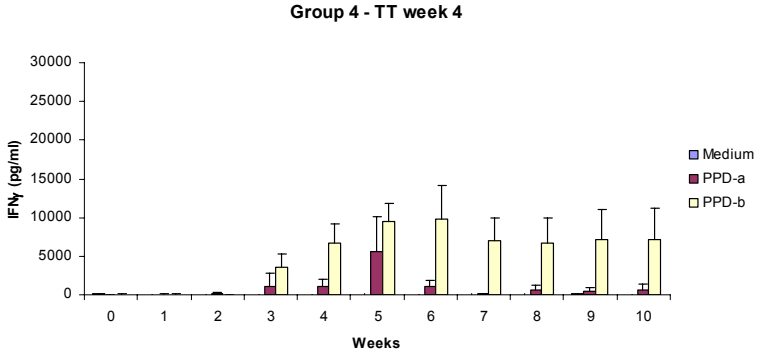
555	2	negative	negative	negative
582	2	negative	negative	negative
599	2	negative	negative	negative
888	2	negative	negative	negative
915	2	negative	negative	negative
555	3	negative	Avian	positive
582	3	positive	positive	Avian
599	3	positive	positive	positive
888	3	negative	Avian	Avian
915	3	negative	Avian	Avian
555	10	positive	positive	positive
582	10	positive	positive	positive
599	10	positive	positive	positive
888	10	positive	positive	positive
915	10	positive	positive	positive
555	11	negative	Avian	Avian
582	11	positive	positive	positive
599	11	negative	Avian	positive
888	11	positive	positive	positive
915	11	positive	positive	positive
555	12	positive	positive	positive
582	12	positive	positive	positive
599	12	positive	positive	positive
888	12	positive	positive	positive
915	12	positive	positive	positive
555	13	positive	positive	positive
582	13	positive	positive	positive
599	13	positive	positive	positive
888	13	positive	positive	positive
915	13	positive	positive	positive

Fig 1 IFNγ responses to PPD-B and PPD-A - means for groups as pg per ml ± sd.

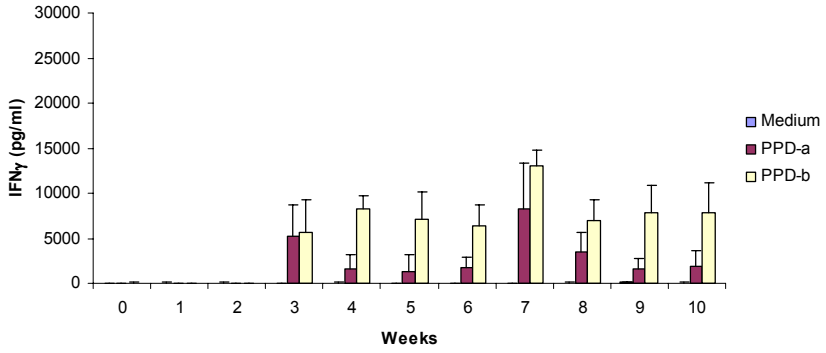




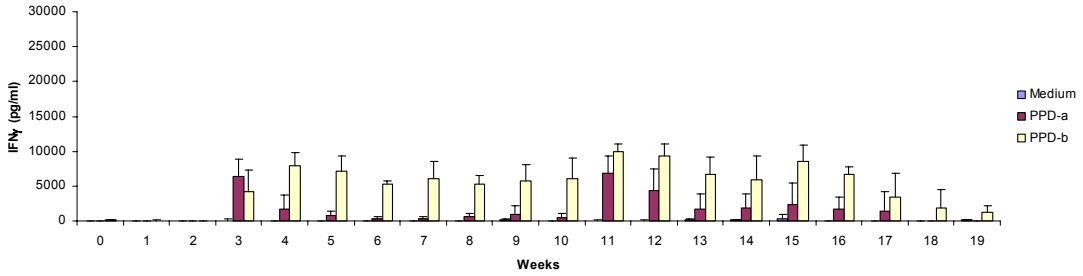




Group 5 - TT week 6



Group 6 - TT week 10



Group 7 - No TT

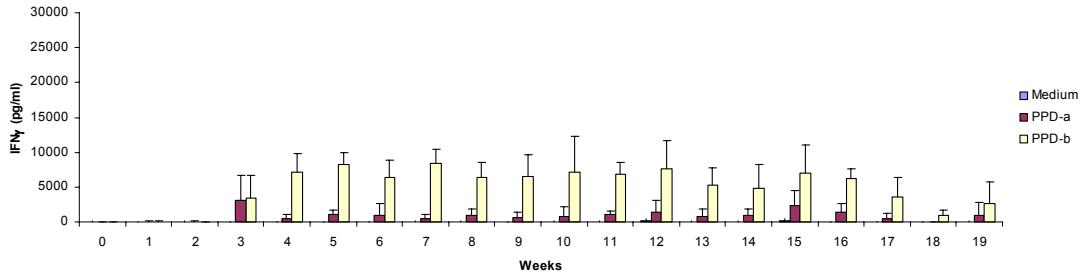
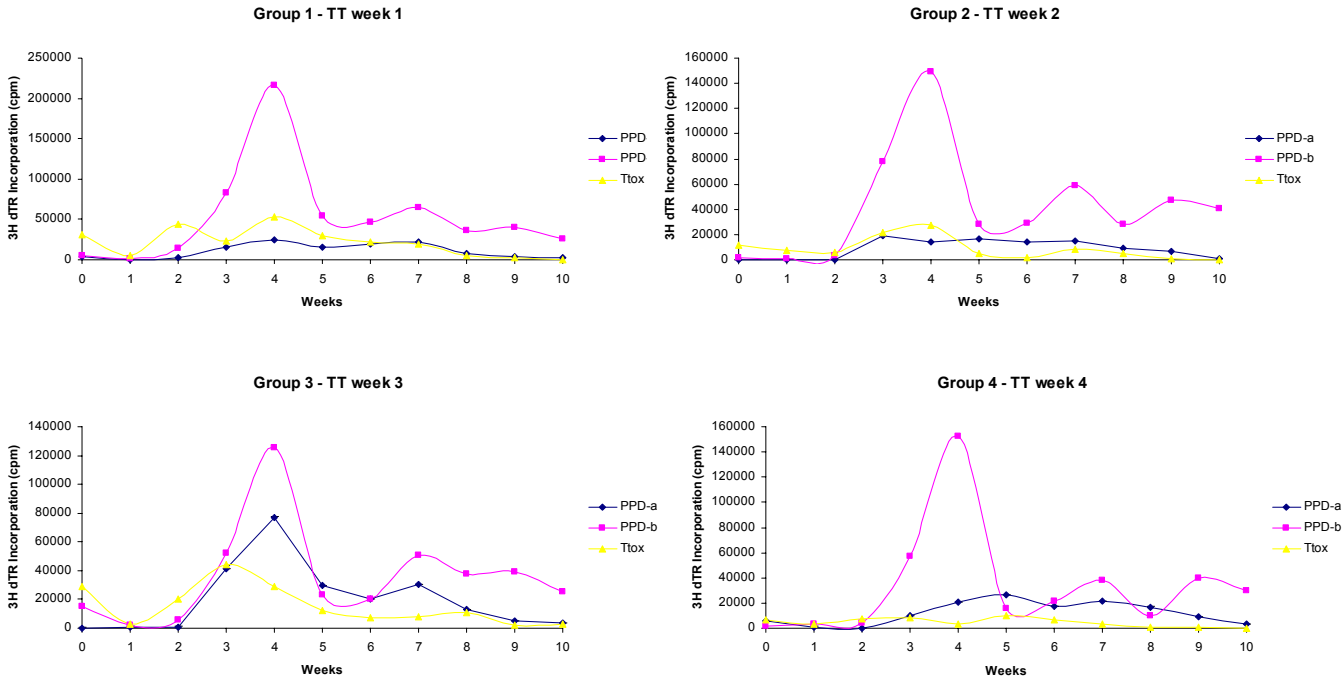


Fig 2 Proliferative response of calves to PPD-B, PPD-A.



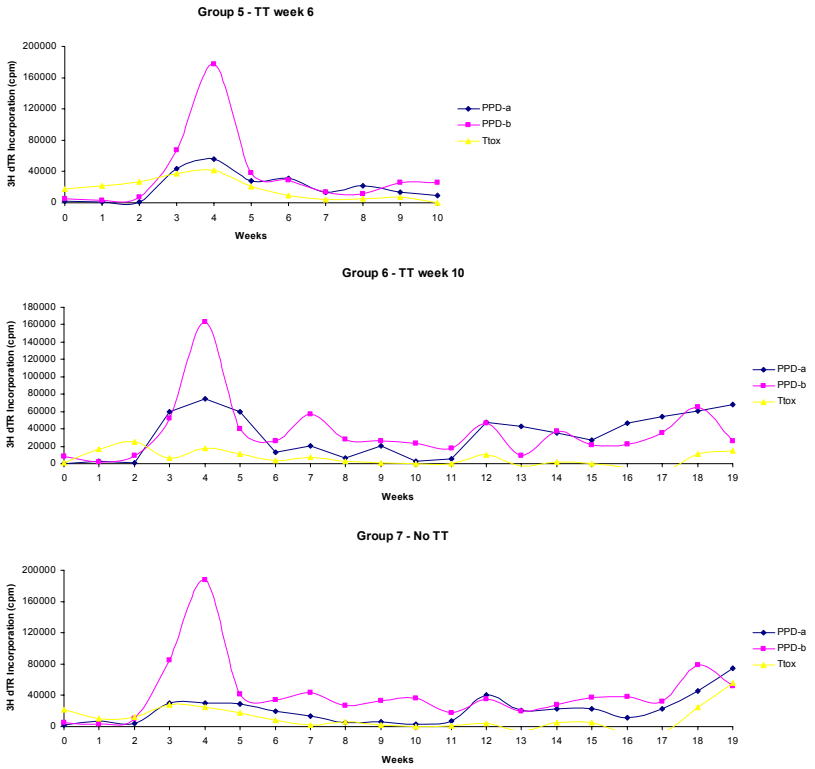


Fig 3 IFNγ response to recombinant antigens or peptides.

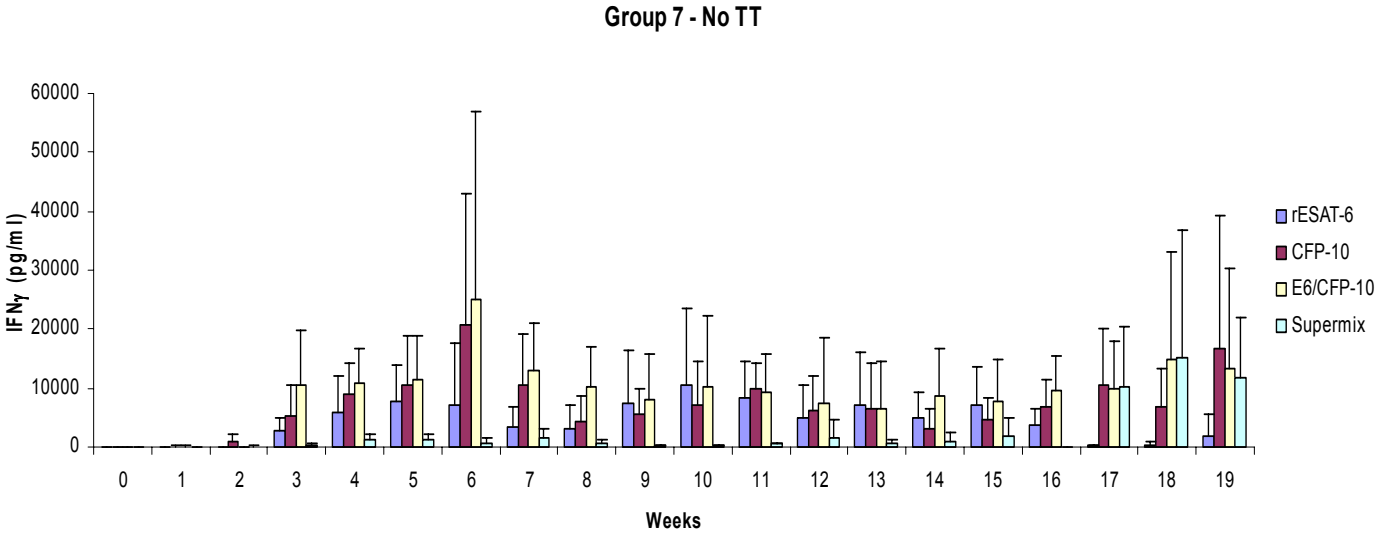
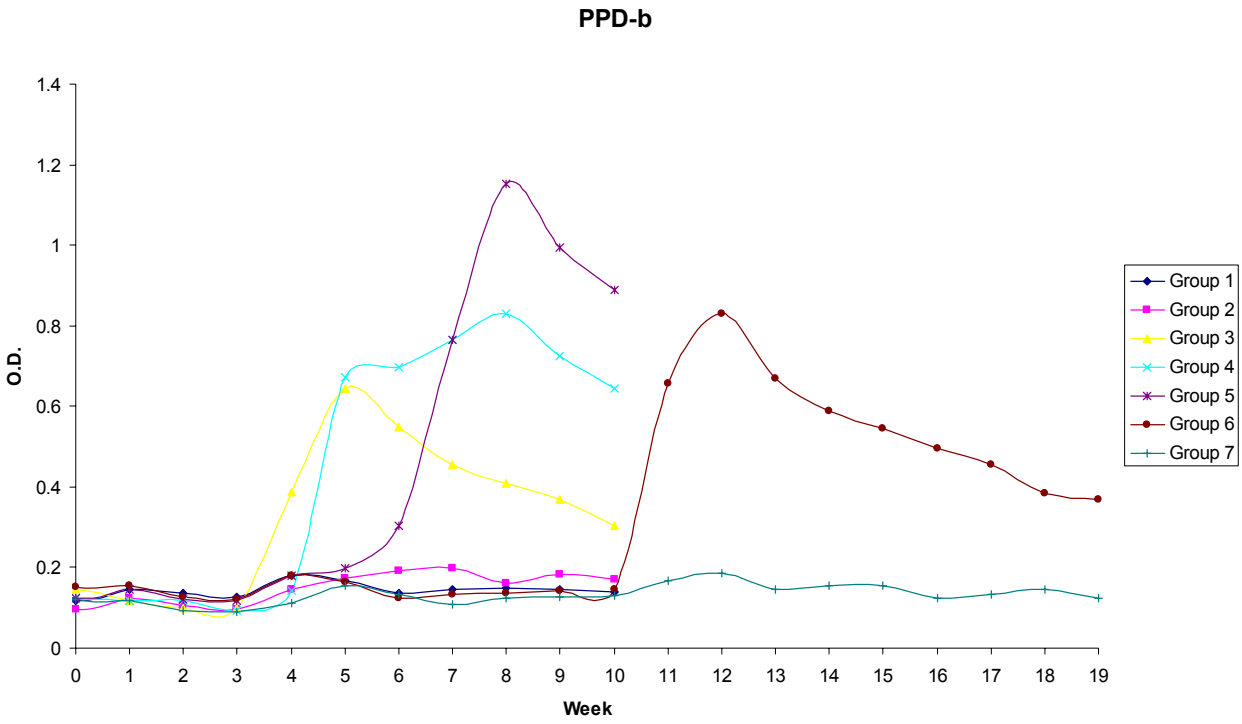


Fig 4 Antibody responses to PPD-B by ELISA.



SE3221 Volatile organic compound analysis for the rapid diagnosis of disease: TB in badgers and cattle as proof of principle

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/01/06
End date 31/12/08

Total cost £457,390

Abstract
Culture of *Mycobacterium bovis* from the live animal is an insensitive method for the diagnosis of tuberculosis in cattle and badgers. Current methods for the diagnosis of animal tuberculosis rely on immunological assays, which have to be underpinned by a fundamental understanding of the host immune response to infection, which in the case of tuberculosis, is incomplete.

Recently, significant progress has been made in developing tests for rapid diagnosis of disease based on the detection and analysis of volatiles present in clinical samples, such as blood, urine and breath. The advantages of this approach are that multiple markers of infection (not just immunological) are examined simultaneously without needing prior knowledge of the underlying biology.

We have recently obtained proof of principle that it is possible to differentiate badgers and cattle with tuberculosis from healthy controls by analysing the volatiles present in serum using an electronic nose (eNose) (Fend *et al* 2005).

A new tool, selective ion flow tube mass spectrometry (SIFT-MS) has been demonstrated to be able to monitor very low levels (a few ppb) of trace gases in breath, and several molecules have been shown to be associated with disease states in humans. Compared to the eNose, SIFT-MS is currently a larger and more expensive device, but unlike the eNose, is able to generate a mass/charge spectrum of the sensed sample; thereby allowing individual volatile compounds to be identified and quantified. Both approaches are complementary, and each can use the same clinical sample to generate a result within minutes.

Aims

- Build a close-fitting moulded device for the collection of all exhalations by mouth and nostrils from cattle and determine if bag samples of breath can be correlated reliably to fresh samples.
- Obtain samples from cattle and badgers for analysis and determine the infection risk associated with breath samples from *M. bovis* infected cows.
- Determine the accuracy of eNose and SIFT-MS for the detection of TB in cattle and badgers and identify the nature of at least two volatile organic compounds (VOC) associated with TB.

Relevance to Defra

A putative rapid and sensitive method of TB diagnosis would be beneficial in complementing (or ultimately replacing) existing immunodiagnostic tests for cattle and badgers. Test for cattle that are non-invasive and could be used repeatedly and short test intervals would greatly aid the control of bovine tuberculosis in herds with persistent outbreaks, and thus lead to a significant reduction in the costs associated with the TB control programme. For badgers, sensitive diagnosis is urgently needed to underpin surveillance of infection in badger populations and is required for the development of any future vaccination strategies for badgers.

Success of the project would provide proof of principle that pen-side diagnosis of infectious disease is possible using sensor technology and that if this were the case it could be applied to the detection of a number of infectious diseases of importance to Defra and the farmer.

Methods

Objective 01

A close-fitting moulded collection device that can be fitted over a cow's muzzle to collect all exhalations by mouth and nostril will be built. It will include connections to allow filtered air input, and to collect divided mouth and nostril outputs. The collection device is primarily to collect saturated gaseous samples but it will be designed to allow the collection of saliva and mucus. It will include sensors to monitor respiratory flow. The collection mask will be connected to a manifold that will permit direct sampling to a SIFT-MS, and to fill Tedlar bags and an eNose headspace collector. The bulk of the flow of breath from the cow will be led into a device for condensing liquid (EBC).

Following the drafting of a detailed specification for the sampling system, a mould will be made of a zone around the head of a (dead) cow and filled with quick setting foam and removed. After setting, the foam will be trimmed away to create a working shape around which glass fibre or resin based materials will be laid up incorporating valve seats and threaded links to connect to the inlet via one way valves and the manifold through another valve. A vacuum pump will be fitted in line with a restrictor to ensure that no back pressure is generated on the exhale phase. A rubber skirt will be fitted around the mask. To ensure correct timing of sampling particularly for the eNose, a flow sensor will be linked via an embedded microprocessor to ensure that valves in the manifold were switched at the correct time. The SIFT-MS draws its sample continuously via a capillary and so a continuous analysis will be made throughout the exhale cycle.

The sampling system will be tested by connecting it to a breath output simulator being developed as part of a separate study at Cranfield University by a visiting scholar. This will ensure that what is being measured relates to what is being exhaled and that any background volatiles from materials are measured.

Once the shape of the mould has been determined, multiple replicates will be moulded with medical grade inert materials that give off no volatile compounds and can be autoclaved. To avoid between-cow contamination the masks will be replaced between cows.

The SIFT-MS and the breath collection device will then be transported to Park Farm, Cambridge University. Healthy dairy cows at Cambridge (or elsewhere if appropriate) will be sampled using the breath collection system and breath compounds measured by SIFT-MS and bags filled with exhaled breath.

The direct results of cow-side sampling will be compared with bagged samples over a 30 day period. Bags of exhaled breath will be removed and stored. A sample of breath will be evacuated from the bag at intervals to determine whether there is a change of gaseous compounds measured in the bag. Changes could be caused by leakage or by endogeous chemical reactions between stored compounds. If bag samples are shown to be reliably correlated to fresh samples then the logistics of breath analysis in the project become simpler. Bags of breath (including those from *M. bovis* infected cows) can be brought to the analysis equipment rather than the equipment being taken to the cow.

Similarly, analysis of saliva, mucus, and exhaled breath condensate samples will determine the links, if any, between these and samples of exhaled breath. These, "secretions," will be analysed for specific markers in their own right or analysed as a surrogate for exhaled breath.

Objective 02
For the first two years of the project we shall obtain samples with which to evaluate eNose and SIFT-MS technologies. These shall include sera from badgers obtained from the Randomised Badger Culling Trial serum store, and a variety of clinical samples from cattle obtained through existing Defra-funded projects or from collaborators, including exhaled breath and its condensate (EBC) following completion of objective 1.

Samples will be obtained from the following sources:
1. Defra-funded work on-going at VLA;
2. Frank Griffin, Department of Microbiology, University of Otago, New Zealand;
3. Geoff de Lisle, AgResearch, Wallaceville Animal Research Centre, New Zealand;
4. Heike Köhler, Institute of Molecular Pathogenesis, Friedrich-Loeffler-Institut, Jena, Germany;
5. Defra-funded work on-going at IAH, Compton; and
6. Park Farm, Cambridge University.

Samples will be chilled as soon as possible after sampling and maintained frozen prior to analysing. Where possible, we shall attempt to collect clinical samples in parallel from individual animals. However, this will not always be feasible.

EBC from cattle experimentally-infected with *M. bovis* will be submitted to culture to determine if viable *M. bovis* are present in the sample. The principle aim is to determine the risk associated with handling breath samples from tuberculous cattle, although the data in itself will provide useful information on the excretion of *M. bovis* by infected cattle.

In order to evaluate the diagnostic potential of the eNose and SIFT-MS, results obtained with these methodologies will be compared with the standard immunological diagnostic tests for cattle and badgers. Therefore, the Brock Test ELISA (Goodger *et*

al 1994) will be performed on all badger sera analysed, and the BOVIGAM IFNγ ELISA (Wood & Jones, 2001) will be performed on all cattle, where possible as part of this objective.

Objective 03

We intend to build upon our initial results using serum (Fend *et al* 2005), extend these to samples other than serum (from cattle), and generate robust discriminatory models with which to classify samples as either from TB positive or negative animals.

Serum samples from badgers and cattle obtained through objective 2 will be analysed by both the eNose and SIFT-MS in order to generate 'odour profiles' for analysis.

- Serum samples will be tested from TB positive and negative cattle, including cattle infected with other mycobacteria or suffering from other respiratory infections that might confound the specificity of the test. The results obtained from cattle will be compared with results of regular BOVIGAM IFNγ tests performed with blood samples from the same animals.
- Serum samples from badgers will be analysed as for cattle, and the results compared with conventional serological methods of diagnosis.

On successful completion, analysis will be extended to the evaluation of other clinical samples from cattle (ie faeces, milk, nasal mucus, saliva, EBC, and possibly bagged breath). Analysis of samples will be performed as soon as possible after their collection to minimise the risk of sample changes or the introduction of artefacts. Samples will be transported quickly for analysis and then warmed to body temperature prior to and during analysis.

The sensitivity and specificity of the two methods of analysis for the different samples will be established using the largest number of samples available to us. We shall be limited by the availability of particular samples, eg milk, and the practical constraints in obtaining others, such as EBC and breath. We anticipate being able to obtain 200-300 serum samples from culture-confirmed tuberculous cattle and badgers, and at least this number of negative samples. The level of certainty will increase as the sample size used for training the model increases, and we may use analytical methods, such as neural networks.

The result of this objective will be the evaluation of both methodologies for the detection of TB in cattle and badgers. The relative accuracy of each method will be determined and appropriate mathematical analytical methods evaluated and refined.

The mass/charge spectra obtained by SIFT-MS will be analysed for the identification 'signature peaks' that will allow the identification of particular VOC associated with TB in comparison with healthy controls and cattle infected with other organisms. The key compounds will be identified through analysis of the SIFT-MS data using each of the three precursor ions (H_3O^+, NO^+ and O_2^+) for sera and other headspace samples, as appropriate.

Results
The majority of the data will be generated by two students employed through Cranfield University; conducting their work in pursuance of a PhD degree. One student will focus on the biological aspects of the project, and the other will focus on the mathematical and data analysis aspects. They will be closely supervised by senior scientists within the project and will be expected to work at Cranfield University, VLA, and other sites such as Cambridge University and the Animal Health Trust, as appropriate.

At the time of this report, one student has been offered one of the vacant posts and we are aiming to recruit the other soon. A second project team meeting is scheduled for the 20th January 2006. The plans for the breath sampling device in particular will be discussed with a view for the construction of the device to commence soon afterwards.

Conclusions
N/A

Future research
On completion of this objective we shall be able to state the accuracy of eNose and SIFT-MS for the detection of TB in cattle using a variety of clinical specimens. We shall be able to make assessment of the utility of each clinical sample for analysis, and make recommendations as to the potential future application of these technologies for the accurate and rapid detection of TB in animals.

It is likely that to realise fully the potential of the most promising approach, further research will be required. Further investigation, to (1) validate fully the methodology on a large panel of samples, and (2) develop the technology into a device suitable for on-farm or in-field use, is beyond the scope of this study. Nonetheless, this proposal will allow Defra to make decisions regarding the future utilisation and development of TB diagnosis based on either technology platform.

References cited in text
Fend, R., Geddes, R., Lesellier, S., Vordermeier, H.-M., Corner, L.A.L., Gormley, E., Costello, E., Hewinson, R.G., Woodman, A.C. & Chambers, M.A. (2005). The use of an electronic nose to detect *Mycobacterium bovis* infection in badgers and cattle. *Journal of Clinical Microbiology* **43**, 1745-51.

Goodger, J., Nolan, A., Russell, W.P., Dalley, D.J., Thorns, C.J., Stuart, F.A., Croston, P. & Newell, D.G. (1994). Serodiagnosis of *Mycobacterium bovis* infection in badgers: development of an indirect ELISA using a 25 kDa antigen. *Veterinary Record* **135**, 82-85.

Wood, P. R. & Jones, S.L. (2001). BOVIGAM: an *in vitro* cellular diagnostic test for bovine tuberculosis. *Tuberculosis (Edinburgh)* **81**, 147-155.

Review comment
This is a highly speculative project but Defra has set an objective to improve the diagnosis and detection of *M. bovis* in cattle and badgers and it is therefore legitimate and worthwhile to consider novel approaches. There would be potential advantages over existing tests and this work could make a contribution to the knowledge on *M. bovis* infections and their diagnosis in animals, particularly as there is no single

measurable immune parameter that is consistent throughout the course of *M. bovis* infections in cattle or badgers. It is therefore important to explore alternative supplementary indicators of infection and little effort to date has focussed on potential 'foot prints' of mycobacteria in clinical specimens. A good collaborative team is involved. However, volatile organic chemistry is a "minefield" and it is notoriously difficult to collect and interpret legitimate and secure samples in field conditions. Thus the potential number of confounding errors in the project could be larger than anticipated and although the pilot work has shown "consistent" differences between samples from diseased and control cattle, those differences could be due to anything other than *M. bovis*. Sooner or later the project will need to characterise the chemical difference that it suggests is the basis of the presence of *M. bovis.* At this point however, that is for future work.

Scores
Conclusions based on sound evidence: 2.0
Quality of science: 2.0
Overall rating: 2.3

SE3215 Development of immunological assays for the detection of *Mycobacterium bovis* infection in badgers

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/02
End date 31/03/05

Total cost £525,041

Abstract
The Krebs Report (Krebs *et al* 1997) into bovine tuberculosis in badgers and cattle stated that development of improved blood-based immunological tests for badgers could have significant impact on control studies and could be an important tool for epidemiological surveillance. Furthermore, the report recommended that a blood-based immunological test would be essential to monitor any badger vaccination programme and to provide support to the badger culling trial.

More recent research conducted at VLA has done much to further understanding of badger tuberculosis and provide reagents that enable more extensive investigation of badger immunology. As a result of this work, a lymphocyte transformation assay (LTA) is now available as a diagnostic test for badger tuberculosis (Dalley *et al* 1999), and was used to demonstrate the induction of a cellular immune response following BCG vaccination in a wild population of badgers in the Republic of Ireland (Southey *et al* 2001). The assay is suitable for research purposes but could not easily be used as a routine surveillance tool. Nonetheless, it demonstrated that badgers infected with *M. bovis* are competent to mount a cellular immune response, opening the door for the development of more suitable tests, such as the assay of IFNγ.

The objectives of this project were therefore aimed at implementing the recommendations made in the Krebs report regarding the development of improved blood-based immunological tests for badgers that could be used for surveillance, to monitor a badger vaccination programme, and provide support to the badger culling trial.

Aims
- Develop and optimise a whole-blood IFNγ assay for the detection of *M. bovis* infection in badgers and determine its accuracy using samples from wild badgers.
- Incorporate antigens recognised during *M. bovis* infection of badgers into validated serological assays.
- Develop an IFNγ ELISPOT assay and evaluate its ability to measure the magnitude of the immune response induced in badgers by BCG vaccination.

Relevance to Defra
Development of improved diagnostic tests for badger tuberculosis will be essential for accurate epidemiological surveillance of *M. bovis* infection in badgers and for future control strategies undertaken by Defra. A variety of sensitive and specific immunological tests will underpin research on the development of a badger vaccine, including studies using wild populations of badgers.

Methods
Development of a whole blood IFNγ ELISA for badgers

- *Identification of best antibody pair for IFNγ ELISA*. A number of antibodies that recognise badger IFNγ were produced or identified as part of Defra-funded ROAME project SE3213. These were screened to identify a suitable combination for the development of both a sandwich ELISA and an ELISPOT assay for the detection of badger IFNγ using flow-cytometry. The monoclonal antibodies (mAbs) from these lines were and supplied to BioVeris Europe, Witney, UK under contract to identify optimal pairs of mAbs for sandwich ELISA development. Half of each mAb was labelled by them with biotin and the other half with their proprietary BV-TAG™ label. Each biotin-labelled mAb was pre-incubated with streptavidin beads to which a positive control sample containing badger IFNγ (supernatants from badger lymphocytes stimulated with ConA mitogen) was added along with each TAG-labelled mAb in turn. Following two hours of incubation at RT, the analytes were read on a M-8 Analyser. In brief, the beads pass into a flow cell where they are captured magnetically onto the surface of an electrode, effectively separating the labeled products from unbound labels and other compounds in the assay matrix. An electrical potential is then applied to the electrode, exciting the BV-label near the surface of the electrode. Unbound labels, which are further from the electrode, are not excited. The emission of light is detected only from bound label, thereby reducing background and improving signal to noise ratios.
- *Preliminary screen of specific T-cell antigens recognised by tuberculous badgers*. Lymphocytes were isolated from badgers used in a collaborative study in the Republic of Ireland (see summary for SE3216 for further details) every three weeks for up to 21 weeks after experimental endobronchial infection with 10^4 cfu *M. bovis* and stimulated in the LTA assay (Dalley *et al* 1999) with PPD-B, PPD-A and specific proteins and peptides chosen for their immunogenicty and specificity in infected cattle and having little homology to Esat-6.
- *Optimisation of conditions for whole badger blood stimulation for the antigen-specific production of IFNγ and incorporation of specific antigens.* Whole-blood was placed undiluted into an equal volume of RPMI medium or diluted in RPMI medium before addition. Four different T-cell mitogens were included in the RPMI medium at different final concentrations to identify the most suitable mitogen for use in whole-blood. In addition, the optimum concentration of PPD-B was determined and specific antigens identified from the preliminary LTA screen (above) were incorporated.
- *Comparison of conventional ELISA and BioVeris assay for the detection of IFNγ*. The conventional badger IFNγ ELISA was compared with the BioVeris assay. Supernatant from ConA stimulated isolated lymphocyte cultures and plasma from PWM stimulated whole-blood, were titrated and analysed in both assays.

- *Identification of optimal conditions for handling whole blood from wild badgers.* Cultures of whole-blood were incubated in 24 well plates (with additional heparin) with PWM or left unstimulated. After overnight stimulation, the supernatant from these cultures was transferred to 96 well plates containing additional heparin and frozen to represent the conditions of the assay as applied to the Randomised Badger Culling Trial (RBCT). In addition, plasmin (fibrinolysin) was investigated as an alternative to heparin.

Validation of multi-antigen serological assays

- *Testing of sera from tuberculous badgers by multi-antigen ptint immunoassay (MAPIA)*. In collaboration with Chembio Diagnostic Systems, Inc., sera from 78 culture confirmed and 100 culture negative badgers were tested by MAPIA (Lyashchenko *et al* 2000) to identify novel antigen targets for serological reactivity in infected badgers.
- *Evaluation of antigen cocktails for improved serodiagnosis*. Based on the MAPIA data, an immunochromatographic test in cassette format was developed by Chembio for the rapid serodiagnosis of *M. bovis* infection. The cassette test used rMPB83 and TBF10 (a polyprotein fusion of Mtb8, CFP10, and the 38 kDa antigen) (Houghton *et al* 2002) printed onto membrane as a combination of the two antigens in one test line. Printing concentrations for each antigen were determined using sera from known antibody responders and negative controls. Once the test was optimised, its performance was evaluated using the same panel of sera used in the MAPIA. In a second collaboration with Lionex GmbH, Germany a similar approach was taken; this time using a cocktail of antigens in a conventional direct serum ELISA. ELISA plates were coated with a cocktail of the following recombinant antigens: MPB83, MPB70, CFP10, and the 16 kDa antigen (alpha-crystallin). Seventy-four serum samples from RBCT badgers (41 TB culture-confirmed) were evaluated in the new (Dachs TB) ELISA.
- *Validation of multi-antigen serological tests for badger TB*. The Chembio cassette test and the Lionex Dachs TB ELISA were validated according to a VLA test validation SOP using approximately 1400 sera from RBCT badgers. In addition to the cassette test, a dipstick format test was also produced by Chembio for comparison using the same combination of antigens as the cassette test. All three new tests were compared with data from the BROCK Test (Goodger *et al* 1994) performed at the same time on the same samples.

Development of an IFNγ ELISPOT assay and its ability to measure BCG vaccination of badgers

- *Identification of best antibody pair for IFNγ ELISPOT*. As a starting point for the development of an ELISPOT for badger IFNγ, we adopted the protocol used for cattle within the TB Research Group. Based on the approach taken to identify the best antibody pair for use in the badger IFNγ ELISA, 12 of the mAbs were screened as detection antibodies, paired with a rabbit polyclonal antiserum (Rb300) produced at VLA under SE3213 with specificity for badger IFNγ (Dalley *et al* 2004) or a mAb, using ConA to stimulate lymphocytes non-specifically in the ELISPOT plate.

- *Optimisation of the ELISPOT for use in BCG vaccinated or tuberculous badgers*. The ELISPOT was evaluated further using captive badgers, both at VLA and in Ireland, vaccinated with BCG (and unvaccinated controls) and then infected experimentally with *M. bovis* (in Ireland only). See summary for SE3216 for more details. The IFNγ ELISPOT was performed on all the badgers at regular intervals. Lymphocytes were stimulated with ConA, PPD-B, and CFP10. Two different concentrations of cells were used and two different mAbs were used as capture antibodies with Rb300 as detection.

Results

Identification of best antibody pairs for IFNγ ELISA and ELISPOT

- Thirty-two mouse monoclonal hybridomas generated from the immunisation of mice with DNA expressing badger IFNγ were assayed systemically for their ability to recognise native badger IFNγ in mitogen-stimulated badger lymphocytes using flow cytometry. From this screen, 13 hybridomas were selected for further development. The mAbs from these lines were supplied to BioVeris Europe, Witney, UK under contract to identify optimal pairs of mAbs for sandwich ELISA development using their proprietary electrochemiluminescent assay system. By this approach, each mAb was compared systematically with every other mAb in a checker-board arrangement. The assay was optimised in terms of labelled mAb concentrations. Further bead titrations were performed. Throughout these studies, Rb300 polyclonal was also included. Using Rb300 as the detection reagent revealed 11B9 to be the best capture mAb for use with the polyclonal. These results were confirmed in an ELISA for IFNγ using supernatant derived from ConA-stimulated badger lymphocytes. Further studies using the BioVeris assay used a combination of two mAbs, as found to be optimal in that system. The best combination of antibodies identified initially for the ELISPOT were 10H6-C1 mAb used as capture antibody with Rb300 as the detection.

Preliminary screen of specific T-cell antigens recognised by tuberculous badgers

- Lymphocytes isolated from badgers used in a collaborative study in the Republic of Ireland after experimental endobronchial infection with *M. bovis* were stimulated in the LTA assay (Dalley *et al* 1999) with the following antigens: PPD-B, PPD-A and MPB70; protein and peptides: Esat-6, CFP10, Rv3019c; and peptide pools: [redacted], [redacted] and [redacted]. The specific antigen CFP10 (whether as whole protein or peptides) was recognised as strongly as PPD-B, whereas Esat-6 was relatively poorly recognised. The other antigens were either poorly recognised, or not recognised at all at each time point.

Optimisation of conditions for whole badger blood stimulation for the antigen-specific production of IFNγ

- In order to make the IFNγ test practicable for use on larger numbers of blood samples, it was necessary to identify the optimal conditions for stimulation of heparinised whole-blood with antigen, thereby removing the need to isolate lymphocytes. Concanavalin A (ConA), staphylococcal enterotoxin B (SEB), and phytohaemagglutinin (PHA) were poor stimulators of IFNγ in badger whole-blood culture. In contrast, pokeweed mitogen was capable of stimulating good levels of

IFNγ (as measured by OD) at all concentrations tested. In all cases but two, the addition of undiluted blood into an equal volume of RPMI was found superior to blood additionally diluted. As a result of these studies, PWM was chosen as the mitogen for all subsequent studies with the whole-blood IFNγ ELISA.

- Previous work using experimentally infected badgers in Ireland had revealed a marked dose effect in the LTA over a range of PPD-B concentrations. However, comparing PPD-B in the whole-blood IFNγ ELISA at 30μg/ml and 5μg/ml revealed little significant difference in the overall response, except when the response was minimal, at which time the use of PPD-B at 30μg/ml was preferable. Based on these data, a concentration of 30μg/ml PPD-B was adopted for the IFNγ ELISA.

Incorporation of specific antigens into the IFNγ ELISA

- On the basis of which specific antigens were recognised by tuberculous badgers using the LTA (see above), CFP10 and Esat-6 were selected and used to stimulate lymphocytes isolated from badgers infected experimentally with *M. bovis*. Consistent with the results obtained in the LTA, lymphocytes from tuberculous badgers responded as well to CFP10 as they did to PPD-B, whereas the responses to Esat-6 were of a lower magnitude and appeared later in infection. The specificity of CFP10 and Esat-6 was confirmed in badgers vaccinated with BCG. Responses of BCG vaccinated badgers to 30μg/ml PPD-B in the IFNγ ELISA were low and dependent on the dose of BCG administered (see above), whereas no responses were seen to CFP10 or Esat-6 at any time point.

Comparison of conventional ELISA and BioVeris assay for the detection of IFNγ

- Both assays successfully detected IFNγ but the sensitivity appeared greater for the ELISA. Blood from 33 badgers from the RBCT (16 culture confirmed to have TB) were tested in both assays and the results obtained with each were found to correlate significantly ($r = 0.78$, $p < 0.0001$, Pearson correlation). However, further testing of RBCT samples was suspended when it became apparent that both the conditions of whole-blood stimulation used for the RBCT bloods were sub-optimal, and that blood taken *post mortem* was largely refractory to IFNγ production (see below).

Identification of optimal conditions for handling whole blood from wild badgers

- Clotting of whole-blood samples was a very rare event when using samples obtained from captive badgers at VLA. However, when the same protocol for antigen stimulation was adopted using blood samples from wild (RBCT) badgers at least 66 out of 822 samples (8%) were unusable in the ELISA due to clotting. In an experiment in Ireland using blood from badgers experimentally infected with *M. bovis*, a total of 169 out of 384 samples (44%) were clotted. Although the blood samples were taken from badgers directly into heparinised tubes, we investigated whether additional heparin at the 24 well and/or 96 well stages of the assay could prevent clotting. Clotting was observed in the 24 well plates where no additional heparin had been added (3/6 samples). A number of the remaining samples (24-3

= 21) that were transferred to the 96 well plate were subsequently found to be clotted after a freeze-thaw cycle. Interestingly, clotting was only observed in the samples that had no additional heparin at the 24 well stage, even though they were not clotted at the time of transfer. Even heparin at 125 IU/ml in the 96 well plate could not prevent the clotting. The effect of heparin on the OD values in the ELISA was determined. The presence of heparin beyond 25 IU/ml in the 24 well plate did reduce the amount of IFNγ produced during the stage of incubation with antigen but after incubation, additional heparin did not influence significantly the ability to detect IFNγ at the ELISA stage. Although plasmin could prevent clotting at a concentration of 5μg/ml, the best signal / background ratio was achieved with 75μg/ml. However, given the considerable cost of using plasmin at this concentration compared with heparin, the SOP for the stimulation of badger whole-blood was modified to include the addition of heparin to both the 24 well (25 IU/ml) and 96 well (16 IU/ml) plates.

Assessment of the performance of the IFNγ ELISA using samples gathered from the RBCT

- Using the modified standard opearating procedure (SOP) as above, a further 174 badgers from the RBCT (35 culture positive) were assayed using the IFNγ ELISA. Data were expressed as net OD (PPD-B minus US), the ratio of ODs for PPD-B and PPD-A, and the difference between PPD-B and PPD-A. Receiver Operator Characteristic (ROC) curves were generated for the analysis. Regardless of the way the data were expressed, the IFNγ ELISA was found to have a very poor performance. Closer inspection of the raw data revealed that only 48 of 171 (28%) of the badgers gave an OD>1 in the PWM positive control sample. This indicated that the condition of the blood at the time of the antigen stimulation was poor. If these 48 badgers were analysed separately, the performance of the test increased considerably. Expression of the data as net OD was found to be the most discriminative. The ELISA was most accurate (83.3%) at a cut-off of 0.0278, resulting in a sensitivity of 61.5% (95% CI, 31.6 – 86.2) and a specificity of 91.4% (95% CI, 77.0 – 98.2). These data demonstrated clearly that the condition of the blood at the time of antigen stimulation was critical to the performance of the IFNγ ELISA. Published studies on the production of IFNγ in blood cultures derived from cattle, identified that blood taken even shortly after death resulted in a significant reduction in the performance of the IFNγ test (Rothel *et al* 1992). The same appeared to be true for badgers, as the mean OD from PWM cultures of whole-blood obtained from eight captive badgers whilst under anaesthesia was 1.68 (SD = 0.29) compared with 0.97 (SD = 0.60) for samples obtained from 48 RBCT badgers *post mortem*. This difference was highly significant ($p < 0.0001$, Unpaired T Test with Welch correction).

Testing of sera from tuberculous badgers by MAPIA

- Sera from 78 culture confirmed and 100 culture negative badgers were tested by MAPIA. A range of antigens were recognised serologically by infected badgers, including *M. bovis* culture filtrate, MPB83, MPB70, CFP10, Esat-6, 16kDa antigen, and a number of fusion proteins. These data have since been published (Greenwald *et al* 2003).

Evaluation of antigen cocktails for improved serodiagnosis

- The sensitivity of the Chembio cassette test was found to be 53% with a specificity of 95% (Greenwald *et al* 2003). The results of the BROCK Test on the same sera were 47% sensitivity and 89% specificity. The sensitivity of the Dachs TB ELISA was found to be 61% with a specificity of 82% (Kampfer *et al* 2003). The results of the BROCK Test on the same sera were 46% sensitivity and 82% specificity. In both cases, the new serological tests based on the incorporation of additional antigens to MPB83, gave enhanced sensitivity compared with the BROCK Test.

Validation of multi-antigen serological tests for badger TB

- The results of this work were presented to Defra in Validation Report VDTC1087. At a specificity of 93-94%, the tests had the following sensitivities: 54% (BROCK); 57% (Dachs); 49% (cassette); 50% (dipstick). Half way through the validation exercise it became clear that the Dachs TB ELISA was underperforming compared with its initial assessment (Kampfer *et al* 2003). Testing was suspended for one month while the cause of the problem was identified by Lionex. A new conjugate was introduced but problems continued, resulting in only 265 sera being tested with an optimised version of the test. The cassette, dipstick and BROCK tests had a higher sensitivity (71-77%) among "super-excretor" (Smith *et al* 2001) (heavily infected) badgers. A total of 216 serum samples from the total of 1464 submitted to testing (14.8%) were either haemolysed or lipaemic. Grouping both together, the sensitivity of the cassette and dipstick tests on haemolysed/lipaemic samples was reduced compared with normal serum samples. However, whilst the sensitivity of the tests were lower when using haemolysed or lipaemic samples, the difference in the accuracy of the tests between both types of sample was statistically insignificant (cassette test, $p = 0.127$; dipstick test, $p = 0.456$, Fisher's Exact tests).
- *Repeatability.* The repeatability of the tests were compared using Kendall's coefficient of concordance to measure the extent of the overall agreement between five weekly test results by the same operator. For each test, nearly identical results were obtained on each occasion. Repeatability was further assessed for the BROCK Test and Dachs TB ELISA separately for the samples negative and positive by culture by calculating the intraclass correlation coefficient. By this there was some evidence that the BROCK Test was less repeatable than the Dachs TB ELISA.
- *Robustness*. Two different operators tested the same 19 sera on days four weeks apart. Using the kappa coefficient, all tests gave perfect agreement, with the exception of one test result for the cassette test. Further assessment using a British Standards Institute definition of reproducibility and the % coefficient of variation between pairs of OD's, revealed that the Dachs TB ELISA may give less variable results.
- *Overall performance*. An overall comparison of the performance of the four tests using the kappa coefficient, ROC analysis, and logistic regression analysis revealed the following:
  (i) For the culture negative samples, none of the tests differed significantly from the BROCK Test in the proportion of samples diagnosed negative, ie they did not differ in specificity. However all of the tests diagnosed correctly significantly fewer of the culture positive samples than the BROCK Test suggesting that all of the

three tests had a lower sensitivity than the Brock Test. Although the sensitivity of the Dachs ELISA was 57%, the sensitivity of the BROCK Test was higher on the 265 samples for which the Dachs ELISA results were obtained;
(ii) The cassette and dipstick tests both had higher kappa values than the Dachs TB ELISA indicating closer agreement with the BROCK Test results;
(iii) Comparison of both ELISA tests indicated little difference in the overall performance on the reduced number of shared samples, but the ROC curves showed a lower sensitivity for the Dachs TB ELISA if the cut-off was chosen to give a high specificity; and
(iv) The predicted mean values from a logistic regression analysis model showed similar specificities but a higher sensitivity for the BROCK Test than for the cassette or dipstick tests. Also, the Dachs TB ELISA had a slightly higher specificity and a significantly lower sensitivity than the other tests when the same 265 samples were compared.

Optimisation of the ELISPOT for use in BCG vaccinated or tuberculous badgers

- A consistent observation was that the use of $6x10^6$ cells per ml in the ELISPOT gave lower numbers of spots than the lower concentration of $2x10^6$ cells per ml. For infected badgers, high numbers of IFNγ producing cells could be detected using both PPD-B and CFP10. Although not statistically significant, the use of 11B9 as capture antibody appeared even better than 10H6-C1. On the basis of these results, 11B9 was used as the capture antibody for future studies (for consistency with the IFNγ ELISA) with $2x10^6$ cells per ml added to each well. Subsequent studies using infected badgers in Ireland demonstrated the following: (i) it was not possible to use whole heparinised blood in the ELISPOT, as the RBCs bound to the nitrocellulose matrix of the plates, resulting in high background noise; (ii) Rb299 polyclonal antisera (produced at the same time as Rb300 but from a different rabbit) was as good as Rb300 as the detection reagent in the ELISPOT. Assessment of the ELISPOT in an experimental vaccination-challenge study in Ireland showed that lymphocytes produced IFNγ in response to stimulation with PPD-B and CFP10 within two weeks of challenge with *M. bovis*. There was evidence of priming in the BCG-vaccinated group, since the responses of this group to PPD-B after challenge were greater than controls (significantly so, two weeks after challenge). This was supported by the lack of a difference in the response between the groups to CFP10 after challenge. The response to Esat-6 was weak compared with CFP10, which supported the results found in the IFNγ ELISA with badgers infected experimentally.

The detection of IFNγ by ELISPOT following BCG vaccination of badgers

- The IFNγ ELISPOT was used to measure the response of captive badgers vaccinated with BCG (see above). The ELISPOT was found to be a very sensitive measure of IFNγ in vaccinated badgers and was able to detect responses following boost with a lower dose of BCG.

Conclusions

This project succeeded in its objectives to develop an ELISA and ELISPOT assay for badger IFNγ. Using both tests, bovine tuberculin was demonstrated to stimulate the production of IFNγ in infected and BCG vaccinated badgers, and single antigens could be used to stimulate IFNγ production specifically in infected (but not vaccinated) badgers. Problems associated with blood clotting in the whole-blood

ELISA were addressed by the inclusion of heparin in the test. However, the test had a poor performance using bloods from culling trial badgers and it is speculated that this is a consequence of using blood samples obtained after death.

Compared with the ELISA, the ELISPOT revealed in BCG vaccinated badgers a greater (relative to unvaccinated animals) and more sustained IFNγ response, demonstrating that although badger lymphocytes primed by BCG vaccination produce little IFNγ, the number of IFNγ producing cells induced by vaccination is as great as observed in tuberculosis infection.

Additional studies demonstrated it was possible to improve the sensitivity of serodiagnosis of tuberculosis in badgers by incorporating multiple antigens as targets. However, full validation of the new tests revealed them to be no better than the existing BROCK Test. Although no more sensitive than the BROCK Test, the cassette test does have some advantages that merit its future use as a diagnostic test for badger tuberculosis. For example, the test is easier to perform and produces a result within 15 min, and as such it may be used animal-side.

Future research

A follow-on defra-funded project (SE3228) is addressing the evaluation of the IFNγ ELISA using blood obtained from wild (RBCT) badgers whilst under anaesthesia to determine whether this improves the performance of the test. At the same time, the BioVeris assay will be evaluated using the same samples to determine which is the more accurate test. Additionally under SE3228, blood samples from badgers culled in Ireland as part of on-going control operations will be stimulated in both the IFNγ ELISA and ELISPOT in order to compare both tests for their relative sensitivities, as well as determine the practicalities of performing the ELISPOT on relatively large numbers of animals. Data from this work will indicate which cell-mediated assay is suitable to support a field study of BCG vaccination, scheduled to commence in 2006.

The Chembio cassette test (named, 'Brock (TB) Stat-Pak') may be of value where a diagnosis of TB in badgers is required before the animal is returned to the wild. As such, the test could be used to support the field study of BCG vaccination in badgers, as well as open up new policy options based around a 'trap-test- cull or vaccinate' scenario. It would also be worthwhile evaluating the test for its ability to detect TB in badger carcasses, such as road traffic accident (RTA) animals.

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Review comment
This was a well-designed project with clear objectives and well regarded. Most of the objectives were achieved and the findings greatly facilitate efforts to develop a vaccine for badgers – one of the key measures for the control of TB in badgers and cattle. Progress on the development of a serological diagnostic test for badgers was disappointing. However, this was due to biological and technical constraints rather than deficiencies in the research project itself. Full validation of the new serological tests offered no sensitivity or specificity advantages over the existing "Brock" test. However, one new serological test could produce a result in 15 minutes and be performed trap side. The project also developed cellular assays for an ELISA and ELISPOT to detect IFNγ, and identified problems in relation to the use of the IFNγ test in wild badgers. This project has succeeded in providing the necessary immunological tools for the monitoring of TB infection in badgers and the evaluation of a badger vaccine. These tests will require further validation before they can be routinely used to study the immune response of badgers to experimental infection and/or immunization. This is being taken forward in SE3228 and the field experiment CB0115.

Scores
Conclusions based on sound evidence: 3.4
Quality of science: 3.4
Overall rating: 3.3

SE3223 Development of an oral BCG vaccine bait formulation for badgers

Organisation Veterinary Laboratories Agency
Weybridge

Start date 02/01/06
End date 01/01/09

Total cost £1,691,252

Abstract
The Godfray report (2005) on the Randomised Badger Culling Trial (RBCT) recommended that the formation of bTB policy by Defra is based on the assumption that badgers are involved in cattle TB as a wildlife reservoir.

Among the conclusions of the Independent Scientific Group (ISG) review of vaccine feasibility (2003) was the observation that large-scale vaccination of badgers would be necessary to have a significant impact on the disease in cattle, and that this would be logistically difficult to achieve without an oral vaccine.

BCG is currently the only vaccine candidate for badgers. A BCG vaccination study performed in badgers at VLA in 1988 demonstrated enhancement of cell-mediated immunity by vaccination together with prolonged survival of badgers infected intradermally with *Mycobacterium bovis* and delayed excretion of the organism. A recent unpublished study performed in the Republic of Ireland, to which VLA contributed (see summary for SE3216), demonstrated a protective effect of subcutaneous BCG vaccination against a more relevant intratracheal challenge with *M. bovis*.

For BCG to be a successful oral vaccine it will need to survive passage through the stomach. Following intratracheal challenge with *M. bovis*, possums vaccinated intraduodenally with BCG had a 100-fold reduction in bacterial burden in the lungs and increased lymphocyte responses to PPD-B compared with animals vaccinated intragastrically (Buddle *et al* 1997). This demonstrated that delivery of BCG to the intestinal tract could result in protective immunity and pointed to inactivation of BCG in the stomach as the reason for the failure of oral BCG vaccination.

Aims
- Lead vaccine formulations optimised and tested *in vitro*.
- Optimum bait formulation identified with which to deliver vaccine.
- Best vaccine formulation identified through protection studies in the guinea pig.
- Immunogenicity and safety of best vaccine formulation in bait evaluated in badgers.
- Safety of best vaccine formulation determined in cattle.

Relevance to Defra

The immediate benefit to emerge from this project will be the identification of a formulation of BCG suitable for delivery to wild badgers. This will include details of how to formulate BCG to protect it from degradation in the environment and the stomach, as well as optimisation of a bait for the delivery of the vaccine. These results will be of direct use to Defra in considering the viability of a policy for the wide-spread vaccination of badgers with BCG.

Methods

Lead vaccine formulations optimised and tested *in vitro*

Laboratory studies

- Four platform formulation technologies will be pursued in this project: (1) alginates; (2) lipids; (3) agarose/gelatine; and (4) Novasomes^{R}. With the exception of the latter, all have been demonstrated to protect BCG from in vitro conditions that mimic the GI tract.
- BCG will be supplied to each contractor for further development of the technologies. Aston University, UK will work on alginates, Immune Solutions Ltd (ISL), New Zealand on lipids, VLA on agaraose/gelatine, and Novavax, USA on Novasomes^{R}.
- A developmental protocol will be followed by each contractor with the specific aims of determining the optimum formulation(s) that preserve the maximum viability in simulated gastric fluid, as well as following storage in the formulation itself.

Further work on alginates will focus on three aspects: (a) maximising the incorporation of BCG in alginate beads; (b) increasing the survival time of BCG in alginate during storage (and application in the field); and (c) optimising the survival of BCG in simulated gastric fluid (SGF) and release into simulated intestinal fluid (SIF).
Potentially, less developmental work is required on the use of lipids, since these have already been proven to work in animal models (Aldwell *et al* 2003a,b). Nonetheless, these studies were based on the use of live laboratory cultures of BCG Pasteur, rather than other BCG strains. Initial work will focus on optimising the chosen strain of BCG lipid formulation. A further deliverable will be the evaluation of the raw materials and laboratory processes associated with the formulation of BCG in lipid for their suitability for eventual production of BCG-lipid to GMP. This will be in the form of a written report to VLA. Studies conducted in possums revealed performance differences between formulations differing in their lipid composition and possibly differences between batches of the same lipid. We shall examine new lipid formulations, as well as identify laboratory tests that can be applied to each batch of lipid for the purposes of quality control. These might include HPLC to define the precise lipid moieties present in the final formulation, melting temperature, etc. To address these issues, BCG will be incorporated into different lipid formulations at VLA, where they will be tested for BCG survival in SGF, as well as shelf-life and compatibility with bait. These formulations will be returned to ISL in New Zealand for QC profiling. Batches of the same lipid, but not containing BCG, will be supplied to VLA by ISL at regular intervals during the first two years of the project for the purposes of performing palatability studies both on captive badgers at VLA and wild badgers at Woodchester Park.
We shall build on the preliminary success obtained using gelatine and agarose to encapsulated BCG. The development of a BCG-agarose/gelatine formulation will

follow a similar strategy to that used by Aston University for alginates. Objectives will be to: (a) determine and optimise the survival time of BCG in agarose or gelatine during storage (and application in the field); and (b) optimise the survival of BCG in simulated gastric fluid (SGF) and release into simulated intestinal fluid (SIF).
Flavoured oral Novasomes^{R} have already been designed for use with both inactivated and live BCG by Novavax Inc. Various oils will be evaluated as the lipophilic component of the Novasome^{R} vesicles with a view to making them scent-attractive to badgers. Six different formulations will be produced for this project. All of the Novasome^{R} formulations will be prepared with PBS. The viscosity of the formulation will be adjusted appropriately to facilitate incorporation in the bait.
During the first year, each contractor will supply formulations to VLA devoid of BCG. These will be used by VLA and Central Science Laboratory (CSL) to perform palatability studies in bait. Information on the composition of baits and of the stability and palatability of the different formulations within the bait will be fed back to the contractors in order for them to refine their formulations. Baits (or their constituents) may be supplied to the contractors to assist them in their formulation development.

Animal studies

- Coincident with the initial phase of formulation development, we shall work on determining the appropriate model physiological conditions in which to evaluate the formulations. In order to do this, information on the badger GI tract would be highly beneficial. These data will be obtained for badgers in this project through deployment of a telemetric pH monitoring system (Bravo^{R} pH Monitoring System, Medtronic, USA, www.medtronic.com).
- This device will be used to obtain data on the pH environment of the badger GI tract, and by inference, the retention time in the stomach and the transit time in the intestine. A series of experiments will be performed at different times of the day, as the consumption of food during normal nocturnal activity will have a direct impact on pH, retention and transit times.

<u>Optimum bait formulation identified with which to deliver vaccine</u>

- Alongside the formulation studies, work will be undertaken on the development of a palatable bait that is compatible with the formulations emerging from the above work.
- Quantities of bait consumed can be quantified by including short-term biomarkers, such as fluorescein, already demonstrated to be fit-for-purpose by the VLA.
- Typical studies will include the stability of placebo formulations in baits, together with their palatability to badgers.
- Scent and flavour attractants may also be incorporated into the baits/formulations in order to determine the most attractive and palatable baits, whilst retaining their compatibility with the formulations themselves.
- Studies will be performed with wild badgers at Woodchester Park. CSL will use infra-red time-lapse video surveillance equipment to observe and record the behaviour of wild badgers at bait stations at night. Video evidence and the rate of bait disappearance will be used to measure the attractiveness and palatability of baits to wild badgers.
- Information on the composition of baits and of the stability and palatability of the different formulations within the bait will be fed back to the contractors in order for

them to refine their formulations. Baits (or their constituents) may be supplied to the contractors to assist them in their formulation development.

Bait uptake work with wild badgers

- During the second year, these baits will be used to deliver the most promising formulations. The formulations will be devoid of BCG but will contain a suitable biomarker.
- Candidate baits will be deployed at several badger setts in the CSL Woodchester Park study area. Uptake rates of at least two bait types will be tested, each at a minimum of three different badger social groups. The experimental design will involve repeating the bait deployment exercise in spring, summer, autumn, and winter. Baits will be replenished every day for up to 10 days. Each bait will contain the blood-borne biomarker iophenoxic acid (IPA). This organic iodine-based compound can be detected in blood serum of badgers for up to eight or nine weeks after consumption.
- Badger social groups used for bait deployment exercises will be routinely trapped throughout the year. A blood sample will be taken from all captured animals from these groups and their immediate neighbours. Blood serum samples will be subjected to laboratory analysis to assess rates of bait uptake by the identification of IPA (Jones *et al* 1997).

<u>Best vaccine formulation identified through protection studies in the guinea pig</u>

- The choice of which formulations to develop further will be determined by their efficacy in the guinea pig aerosol challenge model (Chambers *et al* 2001) at the Health Protection Agency, Porton Down.
- A total of six BCG Danish vaccine formulations will be tested in the second year.

Experiment 1

- Three formulations will be tested alongside the BCG-lipid formulation ISL. All four vaccines will be administered by oral gavage. An unvaccinated negative control group and a positive control group vaccinated subcutaneously with BCG Danish will also be included. Eight animals will be used per group.
- Eight weeks after vaccination, all animals will be challenged with 10-50 CFU *M. bovis* 2122/97 by the aerosol route.
- Five weeks after challenge, the animals will be killed humanely and lungs and spleen removed for bacteriology and histology.

By the conclusion of this first experiment, each contractor will have had additional opportunity to refine their formulations. The two best BCG formulations will be tested in another guinea pig experiment, but this time with the capacity for increasing the group size.

Experiment 2

- The best two formulations identified in Experiment 1 above will be tested. Both vaccines will be administered by oral gavage. An unvaccinated negative control group and a positive control group vaccinated subcutaneously with BCG Danish will also be included. Up to 10 animals will be used per group.

Immunogenicity and safety of best vaccine formulation in bait evaluated in badgers

- The purpose of conducting a safety study in the target species (badger) is to generate data that could be used to apply for an Animal Test Certificate, so that the vaccine could be evaluated in the field.
- For this it is essential that the vaccine under test is administered to immunologically naïve animals in a study conducted to GLP.
- A similar protocol will be adopted to that used to perform the parenteral BCG safety study (see summary for SE3216). The optimum oral BCG formulation will contain BCG Danish at the maximum possible dose. For the purposes of a safety study, this determines the maximum safe dose that can be used subsequently in field studies.
- Once vaccinated, the safety of the vaccine will be assessed by monitoring the badgers for significant changes to haematology, biochemistry, weight, temperature and behaviour compared to the base-line data already obtained. Blood sampling, temperatures, weighing, examination of the injection sites and examination of general condition will be undertaken prior to the first vaccination and regularly after each vaccination, for the remainder of the project. Samples will also be taken at these times to look for the excretion of BCG in tracheal aspirate, saliva, urine, and faeces using culture.
- A group of unvaccinated control badgers will serve as 'sentinel' animals. These will be assessed in the same way as the treatment groups. Should immunological responses to BCG be seen in the sentinel animals this would indicate spread of BCG from the vaccinated animals. Furthermore, changes in the physiological status of any vaccinated badger can be compared with sentinel animals. In this way it will be possible to discriminate an effect induced by vaccination from changes occurring at the population level (eg a concurrent infection affecting all animals in the study).
- Blood samples will also be tested immunologically to monitor the immunogenicity of the oral vaccine.
- Following the first vaccination, a repeat administration of the vaccine will be performed. This is also required for the ATC, and must comprise of the same vaccine delivered in the same way, but this time at the intended vaccine dose, which must be at least 10-fold lower than that used for the overdose.

Safety of best vaccine formulation determined in cattle

- In a comparable way to the badger study, a safety study with the same oral BCG formulation will be performed in cattle. Initially, the cattle will be offered the same bait alone as used for delivery to badgers. It is likely that cattle will avoid consuming the bait, in which case this is useful information to ascertain. Should the cattle actually consume the bait, the safety study will be performed subsequently using BCG formulated in badger bait. If the cattle avoid the bait, animals will be individually dosed with the BCG formulation, whilst under sedation.
- Two groups of five calves will receive BCG formulated as an overdose, and another group will receive BCG formulated at the intended vaccine dose.
- Eight weeks later one of the overdose groups will receive a second oral vaccination with BCG formulated at the intended vaccine dose.
- Six to eight weeks after the last vaccination, all calves will be skin tested using bovine and avian tuberculin, as used routinely for the diagnosis of bovine TB.

- Two to four weeks after the skin test result has been read, the calves will be killed and submitted to detailed post mortem examination.
- Blood samples will be taken twice prior to the first vaccination and at biweekly intervals throughout the experiment. These will be submitted to IFNγ ELISA.

Results
N/A

Conclusions
N/A

Future research
An important element of this project is that only formulations suitable for production on a larger scale to GMP will be chosen, thereby easing the progress to licensing and eventual evaluation and implementation in the field. However, further work will be required (likely through a private/public partnership) to realise this objective. As well as work relating to production, this will include safety studies in more diverse species, and efficacy studies in the target species.

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Review comment
The project aims to identify a suitable method for the mass oral delivery of BCG vaccine to badgers. Such a delivery system is essential if the large scale vaccination of badgers in the wild is to be undertaken in the future. Due to the recent start there are no results to date. However, preliminary investigations have suggested that each of the four chosen delivery systems have shown some promise (alginates, lipids, agarose/gelatine and Novasomes) and thus should be suitable for further evaluation.

It is still too early to assess whether any of the systems will be successful in a mass vaccination strategy or even palatable to badgers. It is also too early to say whether palatability will be an indicator of immunogenicity. Some felt that the science was not innovative but recognition was made that that could potentially improve the chances of success. It was felt that the potential benefits from a successful outcome and the high quality of the proposal fully justify the project. Reservations were expressed about the heavy reliance on external collaborators and the need for them to be as committed as the VLA to achieving milestones on schedule. At the end of this work programme the aim is for an oral vaccine to be ready for field delivery, with scientific and Good Manufacturing Practice issues addressed. This is a big step forward and a necessary forerunner to studies on the oral vaccination of badgers in the wild.

Scores
Conclusions based on sound evidence: 2.5
Quality of science: 2.6
Overall rating: 2.9

SE3216 Development and testing of vaccines against badger tuberculosis

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/02
End date 31/05/05

Total cost £479,634

Abstract
The purpose of this project was to support Defra in its aim to retain the option of badger vaccination. This was achieved by: (i) Supporting the development of an *Mycobacterium bovis* intratrachael challenge model for badgers in the Republic of Ireland through exchange of badger immunological reagents and protocols for their use; (ii) Evaluating the safety and immunogenicity of BCG vaccine in captive badgers; (iii) Performing BCG vaccination / *M. bovis* challenge experiments in collaboration with the Republic of Ireland in order to determine the efficacy of BCG vaccination in badgers; (iv) Developing suitable formulations for the oral delivery of BCG and their preliminary evaluation in a guinea pig vaccination / challenge model; and (v) Comparing the protective effect of BCG Pasteur and Danish strains of BCG as well as the testing promising subunit (protein) vaccines in a mouse *M. bovis* challenge model in order to demonstrate the suitability of BCG Danish as a vaccine for badgers and identify other possible candidate vaccines for badgers.

Aims
- To collaborate with a research group in the Republic of Ireland in developing systems for the experimental infection of badgers with *M. bovis.*
- To determine the safety and immunogenicity profile of BCG vaccine to captive badgers.
- Assist with experimental BCG vaccination/*M. bovis* challenge studies with badgers in the Republic of Ireland.
- To continune with the development of formulations of BCG vaccine suitable for oral delivery and their assessment for stability and efficacy in guinea pigs.
- To assess the most promising alternative to BCG vaccine for protective efficacy in a mouse *M. bovis* challenge model.

Relevance to Defra
The aim of this project was to enable Defra to retain the option of vaccinating badgers against *M. bovis* infection as a potential control strategy for bovine tuberculosis in GB. This was achieved through collaboration with the badger vaccination programme in the Republic of Ireland. The main benefits to Defra are:

- Access to a challenge model for efficacy testing of vaccines against TB in badgers;
- Information on the efficacy of BCG vaccination in badgers against experiment challenge with *M. bovis* and;

- Information on candidate vaccines which may serve as alternatives or adjuncts to BCG.

Such information will assist Defra in making informed decisions on the future prospect of badger vaccination, as well as having value to wildlife vaccination programmes and oral vaccination strategies, in general.

Methods

Experimental infection of badgers in Ireland

- *Defining a suitable dose for challenge*. Twelve badgers free of tuberculosis challenged with either high, medium, or low doses of *M. bovis* (10^4, 100, 10 CFU) via the intratracheal route, and one group left unchallenged. Blood samples obtained from each badger prior to challenge and at intervals thereafter. Serum and isolated lymphocytes subjected to immunological tests. All badgers subjected to detailed post mortem examination and tissue samples taken for bacteriology and histology.

- *Defining the intended challenge dose*. Twelve badgers infected intratracheally with 8000 CFU *M. bovis* and four left unchallenged. Three infected badgers killed at weeks 6, 12, 18, and 24 in order to describe the progression of experimental badger tuberculosis in terms of pathology and immunology. The four control badgers were killed at week 24.

- *Vaccination-challenge studies.* Having established a suitable experimental TB challenge model for captive badgers by the group in Ireland, two separate experiments were performed to evaluate the protective efficacy of BCG Pasteur administered via different routes. Badgers were vaccinated with BCG and challenged intratracheally with *M. bovis*. Three groups of badgers were included. Two groups vaccinated with BCG 12 weeks before challenge with *M. bovis*; one group SC, the remaining group via the intranasal and conjunctival routes. The remaining group was not vaccinated but challenged with *M. bovis* at same time as vaccinated groups (control). A number of immunological tests were applied to blood samples. All animals killed 12 weeks after challenge and submitted for culture and detailed post-mortem. A new group of 14 badgers were used to test the efficacy of an oral formulation of BCG. Seven badgers were vaccinated in the mouth and oesophagous with 10^8 CFU BCG Pasteur in a lipid matrix used previously to vaccinate mice and possums (Aldwell *et al* 2003, 2005). Badgers challenged approximately 13 weeks later with 10^4 CFU of *M.bovis*. Seven unvaccinated control animals challenged at the same time. Immunological responses were followed every 2-4 weeks. Post-mortem examination carried out 17 weeks after infection (30 weeks after vaccination) and pathology compared between animals. Samples taken for bacteriology.

Work with captive badgers

- *GLP safety study.* To expedite the licensing of BCG for badgers, a commercial source of BCG Danish was used. Study based on recommendations made by the Veterinary Medicines Directorate: to demonstrate vaccine has no detrimental effects if administered as an overdose and on more than one occasion to the same individual. BCG administered on the first vaccination as an ‘overdose’. One group of badgers inoculated IM and another group SC. A third group served

as unvaccinated controls. Fifteen weeks later badgers were given a second lower dose via the same route. The study ran for approximately thirty-four weeks. The IM and SC routes were used because formulations for the oral delivery of BCG were still at the experimental stage. At times before and after administration of BCG, samples were collected for physiological, microbiological, and immunological analyses.

Studies with preliminary formulations of oral BCG

- To prove principle that BCG tablet formulation could be used to generate an immune response on feeding, groups of mice were either fed pieces of the BCG tablets orally, control tablets orally, or injected with BCG intradermally. The serological response to BCG sonicate was measured at time of dosing, and days 14, 28, and 42.
- Developmental work on formulation of BCG for oral delivery continued with the use of gelatine and agarose as matrices for the incorporation of live BCG. Proof of principle using a BCG-gelatine vaccine formulation sought in the guinea pig *M. bovis* aerosol challenge model (Chambers *et al* 2001).

Testing vaccines in a mouse *M. bovis* challenge model

- *Comparing the protective efficacy of Pasteur and Danish strains of BCG.* To demonstrate equivalence between strains of BCG vaccine used to generate safety and efficacy data, a vaccination-challenge experiment was conducted in the mouse model (Chambers *et al* 2006). Mice were vaccinated with BCG Danish in the diluent provided, BCG Pasteur at the same dose (10^5 CFU) and in the same diluent, or inoculated with diluent alone (control).

- *Protective efficacy of protein subunit vaccines*. Six proteins evaluated in the mouse model. Mice immunised three times SC with proteins in MPL-TDM/DDA adjuvant. Two weeks following final immunisation, mice were challenged IV with 10^3 *M. bovis*. Bacterial loads determined 7 weeks later. The three most efficacious proteins were evaluated in a repeat experiment either individually or as a cocktail of all three proteins. Both lung and spleen taken for bacterial enumeration.

Results

All objectives were realised in full with the following outcomes.

In 2001 a facility to house badgers under appropriate biological containment was built in the Republic of Ireland (Badger Research and Observation Complex - BROC) to support a programme of badger vaccine development in Ireland. Through funding of this project it was possible to support a PhD student, employed by VLA and registered at University College Dublin, to undertake collaborative work in Ireland using the BROC facility. The first experiment was started in January 2002 with the objective of defining a suitable dose for challenge. The most consistent pathology and immunological responses were seen in badgers challenged with 10^4 CFU *M. bovis*. The pathology was considered typical of natural infection. The experimental infection of badgers allowed us to evaluate a number of immunological tests under development at the time. Encouraging results were obtained with a prototype interferon-gamma (IFNγ) ELISA. Data from the this experiment was then used to define the challenge dose (10^4 CFU *M. bovis*) for the second experiment, which

commenced in September 2002 and concluded in April 2003 with the aim of describing the progression of experimental badger tuberculosis in terms of pathology and immunology. The same samples were taken as the first experiment and similar tests carried out. Gross pathology revealed a gradual worsening of disease over time. Positive immune responses (cellular and humoral) were detected during the experiment.

Having established a suitable experimental TB challenge model for captive badgers in Ireland two separate experiments were performed in order to evaluate the protective efficacy of BCG Pasteur administered via different routes: subcutaneous (SC) or mucosally (experiment 1); and orally (experiment 2). By the six measures of severity of infection used in the first experiment: (a) the number of gross lesions; (b) the sum of gross lesion severity scores; (c) the number and (d) distribution of histopathological lesions; and (e) the number and (f) distribution of infection sites, the vaccinated badgers were better able to manage the experimental challenge than the control group, with the SC group generally less affected than the mucosal group. New immunohistochemical tools with which to study the granulomas of tuberculous badgers were added to those developed previously (Canfield *et al* 2002), and could be used to understand better the nature of the protective host response to TB in badgers. The immune responses were assessed as part of the study and revealed that measurement of IFNγ responses was a sensitive measure of exposure to *M. bovis*. Vaccination SC extended significantly the time to seroconversion following *M. bovis* infection, emphasising the association of seroconversion with more progressive disease seen in wild badgers (Chambers *et al* 2002).

A new group of badgers was brought into the BROC facility, Ireland in mid 2004 for the purposes of testing the efficacy of an oral formulation of BCG. One group of badgers were vaccinated in the mouth and oesophagous with BCG Pasteur in a lipid matrix used previously to successfully vaccinate mice and possums (Aldwell *et al* 2003, 2005), whilst another group remained unvaccinated. All badgers were subsequently challenged with *M. bovis*. Immunological responses in the vaccinated group compared with unvaccinated animals two weeks after challenge with *M. bovis* indicated the presence of immunological memory following oral vaccination with BCG. By two of the four of the measures of severity of infection for which data are available at the time of this report, that is, the number of gross lesions and severity of gross lesions, the vaccinated badgers were better able to manage the experimental challenge than the control group.

Any field trial of BCG in badgers in the UK will require the granting of an Animal Test Certificate (ATC), for which a GLP safety study is required before it may proceed. BCG strain Pasteur was recommended by the OIE as the strain of choice for the development of a veterinary TB vaccine. In support of this, there is a wealth of literature describing its efficaciousness in a variety of species experimentally challenged with *M. bovis*. Accordingly, the majority of work with BCG performed in mice and cattle at VLA has involved this strain. Similarly, the group at UCD responsible for the vaccination/challenge experiments performed in Ireland used the Pasteur strain of BCG for this reason. However, the only strain of BCG licensed for use in humans in the UK is the Danish strain. To expedite the licensing of BCG for badgers, this strain was used to conduct a GLP safety study, since it is produced to

GMP standards. No source of BCG Pasteur produced to this standard exists currently.

The GLP study commenced in July 2004 and was based on recommendations made by the Veterinary Medicines Directorate. Namely, to demonstrate that the vaccine has no detrimental effects if administered as an overdose and on more than one occasion to the same individual. This issue has not previously been addressed under controlled experimental conditions. The GLP safety study was completed in Spring 2005 and supported the conclusion that BCG vaccine produces no significant adverse effects to, nor is shed by, badgers when administered via the subcutaneous or intramuscular routes.

To demonstrate equivalence between the strains of BCG vaccine used to generate safety and efficacy data, we conducted a vaccination/challenge experiment in the mouse model developed at VLA. Both strains of BCG gave significant protection to challenge as measured by a reduction in the bacterial loads in the spleen and lung, and there was no significant difference in the level of protection conferred between strains. These data suggest that the protective efficacy of commercial BCG Danish is equivalent to the research stock of BCG Pasteur hitherto used, supporting the conclusion that BCG Danish is a suitable vaccine for use in badgers.

Work performed in collaboration with Aston University as part of Defra project SE3210 resulted in the production of tablet formulation containing live BCG that were stable at acid pH. In order to prove principle that such tablets could be used to generate an immune response on feeding, groups of mice were either fed pieces of the BCG tablets orally, control tablets orally, or injected with BCG intradermally. Significant seroconversion was observed in the group fed BCG tablets orally. This result suggests that, in principle, BCG can be delivered orally. Further formulation work continued in collaboration with Aston University under VLA Seedcorn project SC0017.

Through collaboration with the Health Protection Agency at Porton Down, further developmental work on the formulation of BCG for oral delivery continued; exploring the use of gelatine and agarose as matrices for the incorporation of live BCG. Matrices formed from gelatine and agarose were able to protect BCG from degradation in HCl, strongly suggesting these to be suitable matrices for the oral delivery of BCG. Further development of these formulations will contine as part of a new Defra-funded project on the development of oral BCG (starting in Jan 2006). Proof of principle using a BCG-gelatine vaccine formulation was sought using a guinea pig *M. bovis* aerosol challenge model. Animals were vaccinated either SC or orally. No animals died of TB during the experiment and changes in weight gain between control (unvaccinated) and vaccinated groups were similar. Protection against challenge was seen in all groups vaccinated with BCG, including those vaccinated orally. However, the challenge dose achieved was lower than intended and as a result the data should be viewed as inconclusive.

A minor component of this project was to identify other vaccine candidates that might serve as alternatives to BCG for the vaccination of badgers, in case the use of a live vaccine was considered unacceptable in wild animals. Six proteins were identified for evaluation in the mouse model. The three most efficacious vaccines were

evaluated in a repeat experiment either individually or as a cocktail of all three proteins. Both lungs and spleen were taken for bacterial enumeration. One protein induced significant protection in the spleen and lungs. Protection was not enhanced by administration as a cocktail of antigens.

Conclusions

Badger studies

- A reliable method for the experimental infection of badgers with *M. bovis* established in Ireland.
- Vaccinated badgers better able to manage experimental challenge than controls, with SC group better protected than mucosal group.
- BCG vaccine produces no significant adverse effects to badgers when administered via the SC or IM routes at the doses used and is not shed from badgers.

Studies with preliminary formulations of oral BCG

- In principle, BCG can be delivered orally in a solid matrix. Those formed from gelatine and agarose were able to protect BCG from degradation in HCl.
- Protection studies in guinea pigs were inconclusive.

Testing vaccines in a mouse *M. bovis* challenge model

- Both strains of BCG gave significant protection to challenge in mice with no significant difference between strains.
- One protein induced significant protection in the spleen and lungs. Protection not enhanced by administration as a cocktail of antigens.

Future research

- Based on these data, one recombinant protein has been chosen for further studies at VLA as a model protein subunit vaccine for cattle. However, even following three immunisations with the protein in mice, protection did not reach that achieved with a single dose of BCG. This is one of the reasons why future work on badger vaccine development should be focussed on BCG alone.

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Publications generated from the project

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Chambers, M.A., Gavier-Widen, D. & Hewinson, R.G. (2006). Histopathogenesis of experimental *Mycobacterium bovis* infection in mice. *Research in Veterinary Science* **80**, 62-70.

Review comment

The project rationale to assess relevant factors in relation to badger anti-tuberculosis vaccines was sound and has been delivered through excellent personnel, appropriately capitalising on collaborative links to maximise research facilities. This project of necessity, and for pragmatic reasons, has had to evaluate parameters specifically relating to the strain of BCG licensed for use in the UK and thus provides a basis to assist future studies with this strain, relevant to the UK and allowing comparisons with studies executed independently in other regions. The project has delivered its objectives on time and the results appear scientifically sound with appropriate conclusions drawn. Overall the project has reinforced the direction of the medium term work on badger vaccination and was undertaken constructively in collaboration with researchers in Ireland. Further development of oral delivery of BCG is needed (now on-going in SE3223). A number of formulations for BCG were described and warrant efficacy testing in badgers. As the vaccine is not expected to provide sterile immunity, some long term experiments on shedding of *M. bovis* following vaccination and challenge will be needed to estimate the extent of reduction of shedding brought about by vaccination over the lifetime of a badger. A short delay in onset of shedding will not be of much benefit in real life, but it is possible that vaccinated animals shed less as well as shedding later.

Scores

Conclusions based on sound evidence: 3.2
Quality of science: 3.1
Overall rating: 3.3

SE3228 A safety study of BCG vaccine in wild badgers – preparatory work

Organisation Veterinary Laboratories Agency
Weybridge

Start date 01/04/05
End date 31/12/05

Total cost £478,375

Abstract
Defra wish to have available a licensed vaccine that they could use to vaccinate badgers against bTB. There are defined steps to achieving a licence for an animal vaccine, including one for use in wildlife. One of these steps is to demonstrate that the vaccine is safe for the target species under both experimental and natural conditions (ie using captive and wild animals, respectively). The first requirement has been met by research undertaken by VLA and funded by Defra. The commercially available BCG Danish strain of the vaccine was demonstrated to be safe when administered to captive badgers (see summary report for SE3216). The next required stage is to demonstrate that the same is true using wild badgers. However, before such work can be begin a number of tasks need to be completed. These formed the basis of this proposal, and included: 1) submission of an Animal Test Certificate (ATC) application to the Veterinary Medicines Directorate (VMD); 2) further optimisation and evaluation of available badger blood tests for their ability to detect tuberculosis in wild badgers; 3) measuring the response of captive badgers to the dose and route of BCG chosen for the field study; and 4) a laboratory method to distinguish BCG vaccine from *Mycobacterium bovis* by culture.

Aims
- Submit application for an ATC to the VMD.
- Determine best combination of immunological tests to underpin vaccine field study.
- Determine IFNγ response of badgers to a single intrasmusclar inoculation of ~10^7 BCG.
- Write an SOP for discrimination of BCG from *M. bovis* in mixed culture.

Relevance to Defra
The last three aims of this project are required in order to inform the design of the field study, which is described in the ATC application. All four objectives of the project are required in order for Defra to sponsor the field study, as part of their intention to obtain a licence for the use of BCG in badgers.

In addition, identification of improved immunological tests will underpin future Defra policies on badgers and TB.

Methods

Submission of an ATC application to the VMD

The following key information is required for the submission:

1. Analytical information on the vaccine;
2. Safety of the vaccine (in the target species); and
3. Details of the trial.

Analytical information on the vaccine will be provided by the manufacturer, who produce the vaccine to GMP for use in humans. A veterinary consultant appointed by Defra will oversee the submission of the ATC.

Details on the safety of the vaccine in badgers is provided from a study performed in captive badgers under Defra-funded project SE3216. This study was performed to GLP standards. A report of this study will be audited by the Quality Assurance Unit of VLA and declared to have been performed in compliance with the 'Good Laboratory Practice Regulations 1999 (Statutory Instrument No. 3106)'.

The design of the trial is under development through a working committee, chaired by a representative from Defra TBD, and comprising of representatives from the Wildlife Unit of Defra, VLA, and Central Science Laboratory. Defra shall appoint a Study Investigator during the life of this project, who shall take responsibility for the writing the final trial design, using contributions from the committee members and with the advice of the veterinary consultant.

With these three pieces of information, the veterinary consultant will complete the application for the ATC.

Determination of the best combination of immunological tests to underpin a vaccine field study

As part of this project, we shall evaluate two new serological tests (Fluorescence Polarisation Assay, Diachemix, USA and SeraLyte test, PriTest, USA) as well as the three formats available for the detection of IFNγ: conventional ELISA (VLA), BioVeris M-series assay (BioVeris Europe), and a quantitative RT-PCR (VLA).

Fluorescent polarisation assay (FPA)

The principle behind the FPA is molecular rotation in solution. All molecules in solution rotate. The rate of rotation of a molecule is inversely proportional to its size. Very few molecules are fluorophores (naturally fluorescent). To make a non-fluorescent molecule fluorescent, a fluorophore must be attached to it (tracer molecule). By selecting a fluorophore, whose fluorescence lifetime (the time between absorbing a photon and emitting one) is on the same time scale as the rate of the molecule's rotation, FP can be employed to determine the tracer's size. Thus FP can be used to monitor a change in the size of a tracer and hence to detect its binding to a larger one, such as an antibody, in real time.

SeraLyte test

This assay combines the principle of a ELISA with a highly sensitive bioluminescence detection system and slides coated with a proprietary, nitrocellulose-based surface that avidly binds primary detection probes.

IFNγ ELISA

We have developed both ELISA (and ELISPOT) methods for measuring the IFNγ response of badger lymphocytes to antigen stimulation (see summary report for SE3215). Recent evaluation of the badger IFNγ ELISA using whole-blood obtained from RBCT badgers after death has yielded disappointing sensitivities. Data obtained both from infected and vaccinated badgers bled whilst under anaesthesia, strongly suggest that blood must be obtained from the live animal to be of use in the IFNγ ELISA. Similar results were obtained using an alternative platform for the IFNγ ELISA: BioVeris M-series test.

BioVeris M-series test

This assay combines the conventional sandwich ELISA format developed by VLA for the detection of badger IFNγ with BV™ technology; a process that uses labels designed to emit light when stimulated electrochemically (see SE3215).

Quantitative real-time (RT)-PCR

Recently, a Test Development Project (SB4018) was completed by the Technology Transfer Unit of VLA on the development of a quantitative RT-PCR-based method for the detection of badger IFNγ. The levels of IFNγ mRNA in PPD stimulated blood cells are calculated relative to non-stimulated control samples. An 18S rRNA assay is used to correct for differences in loading and quality of samples.

No further heparinised bloods will be taken from RBCT badgers after death. Instead, a subset of (up to 300) RBCT badgers will be anaesthetised and bled in the field to provide heparinised blood and cells for the evaluation of the IFNγ assays, and serum for the FPA and SeraLyte test conducted by the collaborating companies. These badgers will be submitted to a detailed post mortem protocol and subsequent extended culture according to the protocols used in Defra-funded project SB4004. Serum will also be submitted to the Brock Test and Brock (TB) Stat-Pak to provide a baseline against which to compare the new tests. Heparinised blood will be stimulated with PWM control, bovine PPD (PPD-B), avian PPD (PPD-A), and the *M. bovis* antigens CFP-10 (alone) and CFP-10/Esat-6 protein cocktail. The RBCT samples may be supplemented with samples obtained from Ireland.

<u>Determination of the IFNγ response of badgers to a single IM inoculation of ~10^7 BCG</u>

The GLP safety study dictates the upper dose that can be administered to wild badgers under the ATC; that is one tenth of the maximum dose used in the GLP study. This means that a dose no higher than $1.3x10^7$ CFU can be administered IM to wild badgers (see summary report for SE3216). Collaborative work performed in Ireland with captive badgers vaccinated SC with ~10^6 BCG Pasteur showed this to give no detectable peripheral IFNγ response (by ELISA or ELISPOT), although the badgers were found to exhibit vaccine-mediated protection on subsequent challenge with *M. bovis* (see SE3215 and SE3216). It is likely, therefore, that a similar dose of BCG Danish administered IM to wild badgers would not result in detectable IFNγ responses. It will be desirable to obtain evidence of a post-vaccination immune response in wild badgers in support of the claim for the licensed vaccine. Whether vaccination with a higher dose of BCG Danish (up to $1.3x10^7$ CFU) will result in

peripheral IFNγ responses is currently not known. For this reason, we shall vaccinate the two remaining naïve badgers from the GLP safety study IM with 1ml of reconstituted BCG Danish vaccine; representing a dose of 2-8x10^6 CFU. This dose is below the upper limit permitted for use in the field study and is 10x higher than the repeat dose used in the GLP study (as well as 10x the dose given to humans). It will also be convenient to administer, since it represents the entire contents of a vial of vaccine as supplied by the manufacturer. The reaction at the site of injection as well as the IFNγ response by ELISA and ELISPOT will be measured weekly for 9 weeks, or until the local reaction and IFNγ ELISA responses are undetectable.

Discrimination of BCG from *M. bovis* in mixed culture

As part of the field study, clinical samples (eg urine, tracheal aspirate, faeces, etc) from badgers will be obtained and submitted to culture in order to evaluate the TB status of the animal and see if BCG vaccine is being excreted from vaccinated animals. It is possible, therefore, that such clinical samples may contain both BCG and *M. bovis* if the samples are taken from vaccinated animals that are also infected with *M. bovis*. The World Health Organisation recommends (Grange *et al* 1996) the use of cycloserine to discriminate BCG from *M. bovis*, as it prevents the growth of *M. bovis* but permits the growth of BCG (Rist *et al* 1967). In these protocols, 20mg/L of cycloserine is used in Lőwenstein-Jensen medium. Our preferred medium for the quantitation of mycobacteria at VLA is Middlebrook 7H11 medium. Therefore, we shall determine whether 7H11 containing cycloserine is also suitable for supporting the growth of BCG, whilst resisting the growth of *M. bovis*. BCG Danish vaccine and a laboratory stock of *M. bovis* will be plated over a dilution series onto 7H11 containing cycloserine over a range of concentrations from 0 to 40mg/L. In this way, the optimum concentration of cycloserine for use will be determined, as well as quantification of any inhibitory effects of cycloserine on the growth of BCG. Clinical samples obtained from the GLP study badgers before BCG vaccination and stored frozen since, will be used to establish the protocol for the differentiation of BCG from *M. bovis* in mixed culture. First, different clinical samples (saliva, tracheal aspirate, urine, and faeces) will be seeded with known quantities of BCG or *M. bovis* and then plated onto 7H11 +/- cycloserine. In this way the sensitivity for detection of BCG will be determined, as well as the inhibitory effect of cycloserine on *M. bovis* confirmed for the different clinical samples. In the next stage of work, clinical samples will be seeded with both BCG and *M. bovis*, and the samples plated onto 7H11 +/- cycloserine. In this way, the ability to differentiate BCG from *M. bovis* in co-infected clinical samples will be determined. On completion of the experimental work, the optimised protocol will be produced as a Standard Operating Procedure that can be followed by whichever laboratory staff are tasked with the culture work from the field study.

Should cycloserine be found not to permit the discrimination of BCG from *M. bovis*, a PCR-based approach will be adopted as a fall-back measure. Discrimination between BCG and *M. bovis* by molecular means will be based on the use of PCR primers flanking and within the RD1 region (absent from BCG) but present in *M. bovis*.

Results

Submission of an ATC application to the VMD

The GLP safety study of BCG in badgers was completed as part of SE3216. The Study Report was audited by the Quality Assurance Unit of VLA in October 2005, supporting the claim that the study was performed in compliance with the GLP Regulations. The Study Report formed part of the completed ATC application submitted to the VMD by the veterinary consultant in November 2005. The following month, the VMD asked for further information. The additional information was provided by the veterinary consultant in January 2006. At the time of this report we are expecting the application to be considered by the Veterinary Products Committee (VPC) when they meet later in January.

Determination of the best combination of immunological tests to underpin a vaccine field study

Optimisation of the FPA and SeraLyte test

Sera from RBCT badgers were supplied to Diachemix and PriTest for the purposes of test optimisation. Up to 148 samples were provided for which detailed PME and extended culture results were already available from SB4004. Two dilutions of serum were used (1:5 and 1:10) for the FPA. The performance of the FPA was better using sera diluted 1:10. Using 67 culture negative and 66 culture positive samples, the FPA achieved a maximal accuracy of 72% (sens = 61%, spec = 84%). In contrast, using 78 culture negative and 70 culture positive samples, the SeraLyte achieved a maximal accuracy of 79% (sens = 67%, spec = 89%). Eighty sera (78 culture negative and 70 culture positive) were tested additionally with the Brock Test. At a high specificity of 94% typical for the Brock Test, the Brock Test was 46% sensitive, whist the SeraLyte was 55% and the FPA only 27%.

Blinded comparison of all tests

Approximately 250 RBCT badgers were anaesthetised and bled in the field to provide heparinised blood and cells for the evaluation of the IFNγ assays, and serum for the FPA and SeraLyte test. These badgers have been submitted to a detailed post mortem protocol and subsequent extended culture and at present culture results are known for 195 of them. Sera from 245 of the badgers have been tested by the collaborating companies in the FPA and SeraLyte, and at VLA in the Brock Test and Brock (TB) Stat-Pak. Heparinised blood from the same animals was stimulated with PWM control, PPD-B, PPD-A, CFP-10 (alone) and CFP-10/Esat-6 protein cocktail. The plasma from these samples has been tested in the IFNγ ELISA and BioVeris M-series assay. The cells were processed for RNA extraction and the quantitative RT-PCR performed. Once the remaining culture results have been obtained the data will be analysed by qualified statisticians at both VLA and CSL. The RBCT samples have been supplemented with 149 plasma samples obtained from culling operation in Ireland. These have been tested at VLA in both the IFNγ ELISA and BioVeris M-series assay. In addition, these animals were tested in the IFNγ ELISPOT assay in Ireland. It is unlikely, however, that culture results will be available for the Irish badgers until mid-2006.

Determination of the IFNγ response of badgers to a single IM inoculation of ~10^7 BCG

Two naïve badgers received 1.5x10^6 CFU and 2.8x10^6 CFU BCG Danish in the lumbar muscle. The sites of inoculation were examined weekly for signs of swelling or other local reaction. None were observed for the nine week duration of the experiment. IFNγ responses were lower when measured by ELISA than by ELISPOT. One badger was positive by ELISA only five weeks after vaccination, whereas the other was positive at weeks 3, 5, and 6. The ELISPOT responses followed a similar kinetic in each of the badgers, being positive at weeks 2, 5, and 9 after vaccination.

Discrimination of BCG from *M. bovis* in mixed culture

Identification of a suitable cycloserine concentration

Serial dilutions of *M. bovis* 2122/97 and BCG Danish were made and plated on 7H11 medium containing pyruvate, or 10, 20, and 40mg/L cycloserine. Plates were examined 3, 4, and 6 weeks after incubation at 37^{o}C. After 3 and 4 weeks, *M. bovis* had grown on all plates but those containing the highest concentration of cycloserine, despite the fact that 20mg/L is the recommended concentration of cycloserine (for incorporation into Lőwenstein-Jensen medium) (Grange *et al* 1996). However, by 6 weeks, growth of *M. bovis* was also evident on plates containing 40mg/L cycloserine, probably due to degradation of the antibiotic in the agar; since the antibiotic is relatively labile at 37^{o}C (Clark, 1967). As expected, by 3 weeks of incubation BCG had grown on all plates, including those containing 40mg/L cycloserine. Importantly, there was no evidence of an inhibitory effect of cycloserine on BCG growth, as the counts obtained at 40mg/L were equivalent to those obtained on plates without cycloserine.

Use of cycloserine with 'spiked' clinical samples

Samples of saliva, tracheal aspirate, urine, and faeces obtained from badgers prior to BCG vaccination were seeded with known quantities of *M. bovis* 2122/97 and BCG Danish (stocks at 2-8x10^6 CFU/ml). BCG diluted 1:100 grew from all clinical samples on 7H11 within 14 days, but at this time point, only BCG at 1:10 had grown on 7H11 + cycloserine. However, by day 22 there was growth from all clinical samples spiked with BCG down to 1:10^{-4} and no difference in the number of colonies recorded on 7H11 with or without cycloserine. The limit of detection in each sample was 100 CFU/ml. By comparison, *M. bovis* diluted 1:10^{-4} had grown from all clinical samples within 14 days of culture on 7H11, and by 28 days, positive cultures were obtained from all clinical samples spiked with *M. bovis* down to 1:10^{-5}. Where cycloserine was present in the media, by 14 days *M. bovis* had not grown from any clinical sample spiked with even undiluted *M. bovis* (at approx. 10^6 CFU/ml). Unfortunately, by day 22 when detection of BCG on 7H11 + cycloserine was optimal, there was also growth of *M. bovis* in the presence of cycloserine. Therefore in this experiment, although 40mg/L could inhibit the growth of *M. bovis* for 14 days whilst still allowing BCG to grow, by day 22 it was possible for growth to be due to either BCG or *M. bovis*. In the first experiment, cycloserine at 40mg/L had inhibited *M. bovis* for up to four weeks. This suggests that a still higher dose of cycloserine is needed to resist *M. bovis* growth consistently for three weeks or longer.

The next stage of the experimental work – to repeat with clinical samples spiked with both *M. bovis* and BCG in the same sample – is on-going at the time of this report. *M. bovis* was added to tracheal aspirate, urine, and faeces at approx. 10^6 CFU/ml. BCG

at 20-80 CFU/ml was added to the same samples and thoroughly mixed before plating on 7H11 without cycloserine, or with cycloserine at 40, 60, and 80 mg/L. Based on the previous experiments, we anticipate growth of BCG but not *M. bovis* after 14 days incubation on 7H11 + 40mg/L cycloserine. However, after 21 days we may see further growth due to 'escape' of *M. bovis* from cycloserine inhibition. It is hoped that this will be delayed, or prevented altogether, at the higher concentrations of cycloserine, whilst neither reducing the sensitivity of BCG detection nor slowing its growth. The plates will be examined weekly. Individual colonies appearing on the plates containing cycloserine will be spoligotyped. This will continue weekly until *M. bovis* first appears by spoligotype. It is anticipated this experiment will be completed by mid-February and an SOP suitable for the field experiment written by the end of that month.

Conclusions

All objectives are on target to met by the end of February 2006.

- All information required by the VMD for the ATC application has been supplied and we await the outcome of the VPC meeting in January.
- All testing for the comparison of new immunological tests for badger TB has been completed. At the time of this report 50 extended culture results were pending, at which point the data will be analysed by statisticians at VLA and CSL. The results will be written into a report that will be considered by the TB Vaccine Studies Data Group (TBVSDG). They will make recommendations to Defra as to which immunological tests will be used in the vaccine field study.
- The human BCG vaccine (Danish strain) is immunogenic to badgers when administered IM as a full dose of one vial per animal. Although only two animals were available, there was no evidence of an adverse reaction to vaccination, in either the youngest or oldest badger. Post-vaccination immune responses were best detected with the use of the ELISPOT, and this assay should be considered to support the field experiment.
- Evidence has been obtained that cycloserine can be used to inhibit to *M. bovis*, whilst permitting the growth of BCG. However, the optimal dose of cycloserine to use is currently being determined in order to define the culture conditions for maximal BCG sensitivity without the interference of *M. bovis* co-growth.

Future research

The results from this project will be taken forward in the badger vaccine field study.

- Further data may be required by the VPC before the VMD grant the ATC.
- The immunological tests recommended for use in the study by the TBVSDG will require support from Defra in the form of equipment purchase, staff training, technology transfer, and validation, before the field study begins. The extent of this depends on the tests that are recommended.
- A dose of one vial of BCG Danish vaccine administered IM has already be chosen for the field study on the basis of work on badgers in this project and in SE3216.
- An SOP for the use of cycloserine in media by the VLA TB Diagnostic Section will be written in time for the start of the field study.

References cited in text

Clark, J. (1967). Stability of antituberculous drugs in Lőwenstein-Jensen medium. *The Journal of Medical Laboratory Technology* **24**, 212-213.

Grange, J.M., Yates, M.D. & de Kantor, I.N. (1996). Guidelines for speciation within the *Mycobacterium tuberculosis* complex, 2nd edition, Geneva, Switzerland. Ref: WHO/EMC/ZOO/96.4.

Rist, N., Canetti, G., Boisvert, H. & Le Lirzin, M. (1967). L'antibiogramme du BCG. Valeur diagnostique de la résistance à la cycloserine. *Revue de tuberculose et de pneumonologie*. **31**, 1060-1065.

Review comment

This project is a direct follow on to SE3216, which established some of the basic elements in captive badgers required for evaluating a vaccine in wild badgers. The aims of this project were essential in pursuing the overall objective of obtaining a Marketing Authorisation (MA) for a licensed BCG product for use in badgers. The safety study provided data which are obligatory for an Animal Test Certificate (ATC) application, and subsequently an MA application. The development of a relevant panel of immunological assays will aid in the assessment of vaccine efficacy in the field in that it will enable parameters other than bacteriological to be monitored. Demonstration of efficacy in field trials is often difficult and immunological read outs will give a meaningful range of tools to judge vaccine efficacy and to compare the response of badgers in the wild with those used in experimental efficacy studies. The project has been preparatory work for an extended project on badger vaccination via a field study/experiment. The badger field vaccination study has become prominent on the agenda for a variety of reasons and it is one which will prove difficult as it requires science to keep pace with policy requirements, which is not always possible. Nevertheless a great deal will be learnt from such a project which should provide Defra with the knowledge and expertise to implement oral badger vaccination should it become a reality in the next decade. The further field study utilising the ATC will principally collect safety data but efficacy data where possible will also be collected.

Scores

Conclusions based on sound evidence: 3.0
Quality of science: 3.0
Overall rating: 3.0

CB0115 Field trial to assess the safety and efficacy of Bacille Calmette Guerin (BCG) vaccine administered parenterally to badgers

Aston Down

Start date 01/01/06
End date 31/03/10

Abstract
This project aims to collect data on the safety and efficacy of Bacille Calmette Guerin (BCG) vaccine given by intramuscular injection to badgers in a field setting.

We will survey and bait mark (ie feed inert coloured beads at setts then record the sites at which the undigested baits are deposited in the faeces) the study area to estimate badger numbers, locations and social group boundaries. Badgers will then be caught in cage traps and transported to a local sampling facility where they will be anaesthetised, individually identified and sampled before release. We will use test results to estimate the prevalence of infection then randomly allocate social groups to receive either vaccination or no treatment.

The area will be re-trapped, badger re-sampled and those allocated to vaccination will receive an intramuscular dose of BCG. Safety data will be collected by detailed monitoring for the 24 hours post vaccination. If our data indicate that the vaccine is safe, we will continue annual surveying, bait-marking, sampling & vaccination for up to 3 additional years to assess the effectiveness of the vaccine.

We will also *post mortem* any badgers found dead in the study area to confirm their TB status and the extent and nature of any TB lesions.

The study will provide safety and efficacy data for use to support an application for a marketing authorisation to use BCG for the control of bTB in badgers.

Aims
- Collect and analyse safety data for the use of intramuscular BCG vaccine in badgers.
- Collect preliminary efficacy data for the use of intramuscular BCG vaccine in badgers.
- Collect and analyse efficacy data for the use of intramuscular BCG vaccine in badgers.

Relevance to Defra
This project will be of direct benefit to Defra in demonstrating whether BCG vaccine is safe for wild badgers. The project also intends to gather data related to the efficacy of the vaccine. These data could form the basis of an application by Defra for marketing authorisation for the licensed use of BCG vaccine for badgers. Data on the efficacy of the vaccine (even if of low statistical power) will be of considerable value to Defra for informing any future policy related to badger vaccination.

Methods
Occupiers within an approximately 50 km^2 study area in Gloucestershire have been recruited voluntarily to the study.

In the study area standard methods will be used to record badger signs and these data together with those from bait marking (ie feeding a palatable bait laced with a different colour/type of indigestible plastic marker at individual setts then observing the subsequent distribution of markers in badger latrines to indicate where territorial boundaries lie) will be used to provide an estimate of badger numbers and identify social group boundaries.
The treatment area will be divided into 3 geographically separate zones of approximately equal size which will each be trapped (using standard metal box traps baited with peanuts) over a one week period. On first capture each animal will be microchipped with a unique identification number (also be tattooed on the ventral abdomen), a range of morphometric data (eg weight, length, age, sex, toothwear) recorded, blood for immunology and samples for microbacterial culture (ie tracheal aspirate, urine, faeces and bite wound swabs) collected.

These baseline immunological data will be used by the TB Vaccine Studies Data Group (TBVSDG) to identify social groups as high, medium or low prevalence. Social groups will then be randomised to treatment (BCG vaccination) or control (no intervention) based on TB prevalence but also ensuring a similar distribution of treatment/control groups across the study area. All areas will be re-trapped, new animals enrolled, existing study animals re-sampled and animals from the treatment group vaccinated by the intramuscular route with commercial BCG Danish reconstituted in the Sauton diluent supplied. A minimum of 12 badgers from each of the vaccinate and control groups will be monitored for body temperature over 24 hours. Two weeks later, at least 12 vaccinated animals and a similar number of controls will be re-trapped, re-sampled and examined for adverse reactions. Additional safety data will be collected by means of *post mortem* examination of animals notified as found dead in the study area; examinations will comprise gross *post mortem*, culture of specified pooled lymph nodes and visibly lesioned organs, histological examination of specified lymph nodes, visibly lesioned organs and vaccination sites.

All results will be made available to the TBVSDG who will remain blind to vaccination status until their initial analysis has been completed. If the vaccine appears safe, the study will continue for up to 3 further years in which we will re-survey, bait mark, trap, sample and vaccinate annually. We will also continue the found dead survey for the duration of the efficacy study. All results will be made available to the TBVSDG who will complete an interim efficacy analysis at the end of year 3 and advise on continuation for a further year or cessation of the study.

The final year of the study will not include re-vaccination.

Results
N/A

Conclusions
N/A

Thursday 6th July 2006

Ecology/Husbandry

Thursday 6th July 2006

Venue: The Diskus, Transport House, 128 Theobald's Road, London WC1X 8TN

09:00 – 09:30	Registration and Coffee		
09:30 – 09:40	Introduction		Chairman- *Alastair Macmillan*
	Code	**Title**	**Speaker(s)**
09:40 – 10:05	SE3002	Ecological correlates of tuberculosis incidence in cattle	Warwick
10:05 – 10:30	SE3009	The risk to cattle from *Mycobacterium bovis* infection in wildlife species other than badgers	Oxford
10:30 – 10:55	SE3010	The risk to cattle from wildlife species other than badgers in areas of high herd breakdown risk	CSL
10:55 – 11:20	SE3032	The long term intensive ecological and epidemiological investigation of a badger population naturally infected with *Mycobacterium bovis*	CSL
11:20 – 11:40	Coffee		
11:40 – 12:05	SE3107	Develop innovative methods to estimate badger population density	CSL
12:05 – 12:30	SE3035	Estimating badger density in RBCT proactive and control areas	CSL
12:30 – 13:00	SE3108	An integrated study of perturbation, population estimation, modelling and risk	CSL/Oxford
13:00 – 13:45	Lunch		
13:45 – 14:10	SE3110	A molecular genetic analysis of badger social structure and bovine tuberculosis	CSL
14:20 – 14:35	ZF0531	The ecological consequences of removing badgers from an ecosystem	CSL
14:35 – 15:00	SE3029	An investigation of potential badger/cattle interactions and how cattle husbandry methods may limit these	CSL
15:00 – 15:25	SE3119	An experiment to assess the cost-effectiveness of farm husbandry manipulations to reduce risks associated with farmyard contact between badgers and cattle	CSL
15:25 – 16:30	Coffee		
15:45 – 18:30	Closed Session – confidential project specific issues. Proposed way forward of the research area		

SE3002 Ecological correlates of tuberculosis incidence in cattle

Organisation Department of Biological Sciences
University of Warwick

Start date 16/08/99
End date 16/12/03

Total cost £436,784

Abstract
Badger density, habitat characteristics and herd management practises were measured on 292 farms located along 10 geographical gradients of cattle bTB incidence in the UK. On 60 farms, diagnosis of *M. bovis* excreted into the environment was performed by PCR testing badger sett and latrine samples. Farms with evidence of badger activity, and with cattle access to setts, had an increased likelihood of breakdown, whereas badger density was not related to herd incidence at any spatial scale tested (farm, local or regional). Farm habitat complexity was associated with increased bTB incidence, independent of badger density. Environmental *M. bovis* was detected in badger setts on 78% of farms, and the proportion of samples PCR positive increased with per head incidence, but not with herd incidence, badger density, or habitat type. Herd size, feeding maize silage, and purchase of adult cattle were all identified as risk factors, whereas feeding hay, and cattle stocking densities >3/ha, were observed to be protective.

Aims
- To assess the relationship between bTB and badger density.
- To assess the relationships between bTB and farm environment characteristics.
- To investigate the prevalence of environmental *M. bovis* on farms.

Relevance to Defra
BovineTB is persistent in geographically defined hotspots for reasons unknown. bTB management requires an understanding of the relationships between bTB incidence and environmental and farm management characteristics that contribute to its persistence. In particular, understanding the nature of the relationship between badger density, *M. bovis* excretion rates, and bTB incidence is necessary to improve the sensitivity of any future badger control policy, and to identify possible practises to reduce the risk of bovine infection.

Methods
20-58 farms were monitored along each of 10 (20km long × 3km wide) transects purposively selected to maximise herd incidence gradients running from high (bTB hotspot) to adjacent low incidence areas. Badger densities were estimated by ground survey between 2000 and 2003 using 4 non-invasive surrogate markers including (i) active sett density per farm, (ii) main sett density within 1km of farms, (iii) density of active holes at main setts within 1km of farms, and (iv) badger latrine density per linear distance surveyed per farm. Habitat variables were generated by direct observation and/or extraction from geographic maps. Farm management practises were provided by farmers for the retrospective 5 year period (1995-1999) to first

interview. Outcome variables included herd breakdown occurrence (0/1) and herd incidence calculated for the appropriate periods using Vetnet testing records. Replicate soil samples from badger setts on 60 farms, and faeces from badger latrines on 12 farms, were tested by PCR for presence of *M. bovis* in 2003.

Results

Measures of badger density on 292 farms varied between study regions. Controlling for testing interval, herd size, and regional clustering, the odds of herd breakdown were marginally increased on farms with evidence of badger activity (OR=2.04, 95% C.I. 0.99-4.21, *P*=0.054), and amongst herds that had access to >2 (range up to 14) active setts (OR=2.22, 95% C.I. 1.13-4.37, *P*=0.021). Mean herd incidence was also marginally higher on farms with evidence of badger activity, but only on farms containing woodland and scrub, shown to be favoured habitats by badgers for sett building (b=0.99, z=2.16, *P*=0.031). Compared to farms of exclusively grassland, habitat compositions that included woodland were associated with increased odds of herd breakdown and increased herd incidence independently of badger density: there was no evidence that herd incidence or breakdown occurrence was linearly or non-linearly related to badger density at any of the spatial scales examined (farm, within, and between regional transects).

Molecular screening for *M. bovis* in badger setts and latrines on farms revealed 47 (78%) of the 60 farms to be PCR positive; 43% of setts and 29% of latrines were positive per contaminated farm. 16S rRNA sequences were demonstrated in 3 of 12 positive setts tested indicating the presence of viable cells. The fraction of samples PCR positive per farm was positively related to per head incidence (*b*=0.1.27, *z*=2.01, *P*=0.044, r^2=0.131), but not to herd incidence, badger density or to habitat type.

In multivariate analysis, increased odds of herd breakdown were demonstrated for farms with evidence of badger activity (OR=2.79, 95% C.I. 1.05-7.42, *P*=0.039), farms comprising mixed habitats including woodland (OR=2.81, 95% C.I. 1.18-6.66, *P*=0.012), and arable land and woodland (OR=3.46, 95% C.I. 1.55-7.72, *P*=0.002) (pseudo r^2 = 0.154). Increased herd incidence was associated with mixed habitats including arable and woodland (*b*=0.749, *z*=4.17, *P*<0.001, r^2 = 0.224), but not with any measure of badger activity or sett accessibility.

In multivariate analyses of farm management variables to explain single herd breakdown occurrence, increased odds were shown for farms that fed maize silage to cattle (OR=4.76, 95% 1.72-13.13, *P*=0.003), and bought in adult cattle (OR=3.77, 95% 1.23-11.54, *P*=0.020) (pseudo r^2 = 0.096). Multiple breakdowns were associated with medium (>80-160) (OR=15.82; *P*=0.001) and large (>160) (OR=11.51; *P*=0.004) herd sizes, whereas feeding hay and high stocking densities (>3/ha) (OR=0.29-0.33; *P*≤0.040) were protective (pseudo r^2 = 0.186).

Conclusions

In our sample, there was some evidence that farms on which there was badger activity had an increased likelihood of bTB breakdown compared to farms without badger activity, adding to existing evidence of an association between badger and cattle bTB (Krebs *et al* 1997; Griffin *et al* 2005; Donnelly *et al* 2005). However, badger density showed neither a linear or non-linear relationship with bTB incidence, likely due to spatio-temporal heterogeneities in badger infection and *M*. *bovis* excretion rates (Delahay *et al* 2000; Olea-Popelka *et al* 2003). The prevalence of *M*. *bovis* detected in the environment was substantially higher than expected, and tested cells appeared intact and viable. The proportion of replicate environmental samples positive by PCR was positively related to per head bTB incidence. One implication is that any future policy of focal badger culling targeted at infected groups may be more efficacious (and certainly more ethical) than blanket culling aimed to reduce badger density *per se*.

Farm habitat complexity (including habitat compositions preferred for sett building) appears to be intrinsically associated with increased bTB incidence, but independently of badger density. Farm biosecurity eg reduced feeding of maize silage, prevention of cattle access to active setts and latrines, and monitoring bTB status of purchased cattle, are management practises that could (Phillips *et al* 2000), or are currently, being addressed.

Future research

- To evaluate non-invasive diagnostics of badger (group) infection.
- To advance studies on the relationships between badger density, badger infection and excreting incidence in different habitats.
- To evaluate the significance of environmental *M*. *bovis* to bTB persistence in cattle and wildlife.

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Publications generated from the project

Courtenay *et al*. Badger density is a poor predictor of bovine tuberculosis breakdown incidence on UK farms. Submitted.

Courtenay *et al*. Husbandary practises as risk factors for single and multiple tuberculous incidents on UK cattle farms. Submitted.

Courtenay O *et al*. *Mycobacterium bovis* in the environment: bovine tuberculosis management is missing a potentially important reservoir. Submitted.

Review comment

This was a highly ambitious project, in which milestones were not met due to the outbreak of Foot and Mouth Disease and predictive models should have been constructed but were not due to the loss of the main modeller and the absence of positive field results. In multivariate statistics it is good practice to suggest rigorous criteria for the inclusion of factors ($P<0.02$ rather than the usual 0.05) to avoid spurious ascription of significance however, here a very 'generous' $P<0.1$ was used. Consequently, the results are peppered with inconsistencies such as factors 'significant' (sic) but only at one out of many sites. The calculation of badger density was as accurate as was feasible but follow-up on PCR findings with specialised mycobacterial culture would have been appropriate. Further limitations posed by the nature and composition of bTB data on herds in the areas studied were considerable. These appeared to relate to results of tuberculin tests conducted at intervals covering a wide span and appeared to treat tests consequential to index tests as separate entities. Consolidation of tests on individual herds, and on their contiguous herds, as representation of an outbreak period would have provided a clearer picture when subjected to advanced statistical analysis. For many parameters reported, there was insufficient information to draw firm conclusions for guiding future action. Clear patterns were not established in relating bTB incidence to either badger density or habitat characteristics. Overall the project failed to deliver useful outputs.

Scores

Conclusions based on sound evidence: 2.2
Quality of science: 1.5
Overall rating: 1.7

SE3009 The risk to cattle from *Mycobacterium bovis* infection in wildlife species other than badgers

Organisation Wildlife Conservation Research Unit
University of Oxford

Start date 01/05/99
End date 30/09/04

Total cost £1,214,788

Abstract
We studied the importance of common farmland wildlife in bovine tuberculosis epidemiology using a systematic survey of wild mammals (*n*=4,393) on 12 dairy farms. The work primarily used live-sampling, and the animals were a representative fraction of those present. Cultures were prepared from 10,397 samples (faeces, urine and tracheal aspirate). One of the 1307 bank voles (*Clethrionomys glareolus*), and three of the 43 badgers (*Meles meles*), yielded positive *M. bovis* isolates. This was the first isolation for a bank vole, and the strain was of the type found in cattle and badgers on the same farm. However, the mean prevalence of infectious individuals among common farmland wildlife is extremely low (the upper 95% CI <=2.0 for all abundant species). Mathematical models illustrate that the disease is unlikely to be maintained at such low levels. We conclude that these animals are relatively unimportant as reservoirs of bTB, having insufficient within-species (or within-group) transmission to sustain the infection, though occasional spill-overs from cattle or badgers may occur. Ongoing diagnostic work using direct PCR supports our findings of extremely low prevalence, with just seven further isolates being made from four farms (2 bank voles, 1 rat, 4 wood mice).

We explored further the associations between risk of bTB in dairy cattle and possible predictors, including habitat and potential wildlife reservoirs, in an unmatched case-control study of 120 herds. Information theoretic approaches identified predictors explaining the greatest variation in bTB breakdowns. Management of farmland in ways favourable to wildlife was associated with reduced bTB risk.

Aims

- To estimate robustly the prevalence of individuals infectious for *M. bovis* in potential wildlife reservoirs other than badgers.

- To investigate the epidemiology and molecular type of bTB in wildlife, informed by the collection of data on the ecology of potential *M. bovis* reservoir species.

- To quantify the risk to cattle associated with bTB infection in wildlife using modelling and risk analysis.

Relevance to Defra

The need for information on the potential risk to cattle from *M. bovis* infection in wildlife other than badgers has been repeatedly emphasised in reviews of bTB control strategies. A particular problem has been the lack of systematic sampling. This makes extrapolation of the data difficult. There has also previously been an emphasis on 'game' and 'pest' species, at the expense of many abundant farmland mammals with considerable opportunity to interact with cattle and badgers. The current project directly addressed the need for robust estimates of the prevalence of wild animals infectious for *M. bovis*. It also quantified the likely risk posed by wildlife to cattle using both associative and deterministic modelling. An important additional output, derived from the risk analysis, has been the identification of potentially modifiable habitat variables as being important to the risk of bTB in cattle.

Methods

Our project was designed to yield data that could readily be extrapolated. We took a farm-scale approach, undertaking a cross-sectional survey of a representative sample of wild animals present on each holding. This enabled us to compare directly the strains of bTB found in wildlife with those identified in cattle at the same farm. The farms themselves were selected using a formally-defined strategy, which was based around the random selection of dairy farms with a history of bTB in cattle. Our study therefore avoided the sampling biases inherent in previous research. A total of 8 'case' farms and four control farms with no recent history of bTB were studied. They were located in the following geographical areas: Derbyshire, Carmarthenshire, Gwent and N. Somerset.

By agreement with our Defra project officer, deer were excluded from the study at the outset. This was because they were a primary focus of a parallel study on bTB in wildlife being conducted by the Central Science Laboratory (CSL), and their low numbers on any individual farm would preclude the calculation of sensible estimates of infection prevalence. We adopted a two-phase sampling strategy. In the first phase we aimed to survey a representative sample of all terrestrial species present on the first 7 farms. For the remaining farms, following consultation with Defra and CSL, we focused on obtaining representative samples of small mammals (<30g), rabbits, squirrels and rats. All farm dogs and cats were also sampled. Badgers were trapped throughout the study, except during the 'closed' season (Jan-May inclusive, as defined by English Nature and the Countryside Council for Wales). Representative sampling of badgers was therefore possible on 4 case and 2 control farms.

The project largely used a live-sampling approach. This permitted us to survey a large and representative sample of wild animals present on each farm, as well as domestic dogs and cats. A live-sampling approach is suitable for determining the prevalence of individuals infectious for *M. bovis* (ie excreting bacilli) and which therefore pose a direct risk to cattle. It is not suitable for estimating the underlying prevalence of all infected individuals. Identifying all infected individuals is relevant during operations to control bTB in wildlife populations, whereas the prevalence of infectious individuals is the key parameter required to estimate risk to cattle – the purpose of this project. We also collected carcasses from road traffic accidents, pest control operations and game-bags. These carcasses were all obtained from within 5km of the study farms. Urine, faeces and tracheal aspirates were obtained from

each animal that was live-sampled. In addition, key lymph nodes and tissues were taken from carcasses during *post-mortem* examinations.

To maximise the likelihood of isolating all strains of *M. bovis* present, we used a range of media (solid, liquid and egg- and agar-based), since different strains have different media preferences. Our protocol therefore differed from that routinely used for the isolation of *M. bovis* from badgers. All specimens were seeded onto three types of media: modified Middlebrook 7H11 (2 slopes), acidified Löwenstein-Jensen medium with pyruvate (2 slopes), and BBL Mycobacteria Growth Indicator Tubes (MGIT 960) (1 tube). Initial diagnosis was by morphological means and by Ziehl-Neelsen staining. Confirmatory testing of presumptive positives was by growth onto selective media by the TB Reference Laboratory, Weybridge and/or confirmation by polymerase chain reaction (PCR). Four PCR-based methods were used. These were PCR for IS1081, a multi-copy element generally present in 6 copies in members of the *Mycobacterium tuberculosis* (MTB) complex; PCRs to detect and distinguish between *M. microti* and classical *M. bovis* on the basis of deletion regions (known as RD7 and RD4); and Spacer-OLIGOnucleotide TYPING – 'spoligotyping'.

DNA was also extracted directly from duplicate stored samples using the Qiagen Blood and Tissue DNA extraction kit (Beswick *et al* 1999). PCRs for the Hsp65 were used (specific for MTB complex), with the identity of putative *M. bovis* isolates being confirmed by sequencing. These analyses are ongoing at the Health and Safety Laboratories, Sheffield.

For the additional study analysing the risk of bTB in cattle in relation to habitat factors and potential wildlife reservoirs, an unmatched case-control design was used. Cases and controls were defined as in the main study. Thirty cases and 30 controls were randomly selected from all eligible herds in two geographical areas (ie 120 herds analysed). The potential predictors examined using logistic regression included a wide range of habitat variables, with an emphasis on boundary characteristics. Indices of badger density, herd size, and proximity to other recently infected herds were also included, these having been associated with bTB risk, as was farm area. Estimates of small mammal abundance were made on the basis of the extent, quality and configuration of hedgerows, using predictive models developed in the main study. Details of boundaries of farm ownership are not publicly available. So, for the purpose of estimating habitat characteristics within farms, each was assumed to be a circular area of 100 ha – the median reported to Defra's Rural Payment's agency by the study farms – and centred on the herd's registered grid co-ordinates. Dairy enterprises tend to have compact configurations, and to be centred around farm buildings, because of the need for milking. Nevertheless, these assumptions will inevitably introduce some errors. Therefore, the observed relationships between bTB and habitat features will be underestimates. Detailed information about badger distribution, density and bTB status is also unavailable. Badger road traffic accident (RTA) records, available at a 1km resolution, were therefore used as indices of population density. No data on the infection status of the RTA badgers was available.

The fit of different logistic regression models was assessed using an information-theoretic approach. In this, a series of relationships (models) between the herd breakdowns variable, and the habitat predictors is formulated. Competing models with different combinations of predictor variables are compared and ranked according

to their ability to explain the observed phenomenon. The Akaike Information Statistic - which provides an inverse measure of model fit - was used to compare models. A second, derived, measure (Akaike weight) was used to show the probability, given the data, of each model being the best out all of those considered. The relative importance of *individual* variables is indicated by their Predictor Weights. The overall objective of the analysis was to include those variables accounting for some variation in the herd breakdowns, and so develop an approximating model that lost as little information as possible about the real-world system (Anderson *et al* 2000). Where several variables are believed to explain a given process, the approach is less likely than traditional hypothesis-testing methods to generate spurious findings (Burnham & Anderson 2002).

Since many predictors could plausibly contribute to herd breakdowns we fitted multiple models with permutations of the predictor variables. To keep the number of possible combinations within reasonable limits, the models were built in stages. First, the habitat data alone were used. In addition to the summary variables for land cover, deciduous woodland area and grazed grassland area (variable 'grazed/mown turf') were included separately because of their associations with badger density (Reason *et al* 1993). Further sets of models were then produced using the variables featured in the top-ranking models and also factors considered *a priori* likely to be associated with bTB risk: badgers, county and topography; and agricultural data including herd size, stocking density and proximity to other bTB cases.

Results

In total, 4,180 animals of 16 species were live-sampled (Table 1). The numbers of each species trapped were similar on case and control farms (in *t*-tests for differences between farm-types, $P>0.5$ for all species except squirrels, where $t=1.474$, $df=10$, $P=0.171$). Most species were trapped on every farm. For those which were not, the distribution of species occurrence was either similar, or was higher on case farms (Table 1). Faecal samples were obtained for 88% ($n=3683$). Excluding the shrews ($n=291$), which we largely did not attempt to sample under anaesthetic, urine samples were obtained for 77% ($n=3003$), and tracheal or gastric aspirates for 88% ($n=3455$). Across all species, three types of clinical samples were obtained from 65% ($n=2736$), and at least two types from 86% ($n=3577$). The ease with which clinical samples were obtained varied across species (Table 1). Sampling was most complete for mice and voles, and these were also the animals trapped most frequently on study farms. *Post-mortem* samples were obtained for a further 213 animals (5 wood mice, 1 house mouse, 9 bank voles, 1 field vole, 17 rats, 45 common shrews, 1 water shrew, 14 rabbits, 5 squirrels, 1 polecat, 100 foxes, 8 badgers, 1 hedgehog, 3 moles, and 2 fallow deer). *Post mortem* samples were also obtained from 149 (4%) of the animals previously live sampled.

Contamination rates for cultures were higher on the egg-based media, and were lower for urine than for faeces or tracheal aspirates (the latter were not decontaminated). The contamination rates (%) for LJp slopes, 7HII slopes, and MGIT 960 tubes were: urine <1, <1, 13; faeces 24, <1 20; tracheal aspirates 40; 5; 15. Very few animals (<1%) had contamination on all cultures.

One bank vole and three badgers were found to be infectious for *M. bovis*. The isolates were made on MGIT 960 medium (bank vole), LJp medium (n=2 badgers)

and 7H11 medium (n=1). The specimen types were tracheal aspirates (bank vole and n=1 badger) and pus (n=2 badgers). Table 2 shows the 95% confidence intervals for the prevalence of infectious individuals for all the species sampled. Inclusion of finite population corrections (Cochran, 1977) did not materially alter the 95% confidence intervals with the following exceptions (raw 95% CIs): house mouse (0, 5.0), rat (0, 4.4), rabbit (0, 1.6), grey squirrel (0, 1.8), dog (0, 8.0), cat (0, 4.0).

One of the 8 badgers, but none of the other animals, examined *post mortem* was infected with *M. bovis* (spoligotype SB0129). This diagnosis was made on MGIT and LJp media. The prevalences of infection (%) (raw 95% Confidence Intervals) for badgers was 6.7 (2.5, 15.0). Genotyping with a range of PCR methods specific for either the MTB complex or for *M. bovis* provided consistent confirmation of *M. bovis* for all the cases. No isolation of *M. microti* was made using either the live- or *post-mortem* samples.

M. bovis was isolated from badgers on 2 of the 4 case farms, and from no badgers on the two control farms where representative trapping was possible. The *M. bovis* isolate from the bank vole (1/4) was of the same spoligotype (SB0673) as isolates from cattle and two badgers at the same farm. The spoligotypes of the isolates from badgers were all of the same type as those previously identified in cattle at the same farm (4/4; Fisher's exact test to compare badgers and bank voles p=0.143, note power of comparison is constrained by small sample size). The spoligotypes for the badger isolates were SB0129 at farm 1 (n = 2 badgers, including dead specimen) and SB0673 at farm 2 (n = 2 live-sampled badgers).

The results of the mathematical modelling are shown in Table 3. Since the lower values of the prevalence confidence intervals are all zero we cannot predict exactly what transmission rates and basic reproductive rates will be. We simply know that we must have $R_0 < 1$ and hence some upper limit on β. The values of the transmission parameter, β, were all low. Depending on the prevalence, and the values chosen for the population density parameter, β ranged from 0.002-0.007. Even with the upper bound of the confidence intervals for prevalence of infectious individuals, the transmission rates and basic reproductive rates would have to be extremely small to be compatible with the prevalences we observed in the field. By comparison, the R_0 values for badgers ranged from 1.025 to 1.229 (based on the upper and lower confidence limits of the prevalence estimates). This is similar to the estimates of R_0=1.1 to 1.2 obtained from other, larger, field studies of bovine TB in the badger (Smith 2001).

In the additional risk analysis, all the top-ranking models included distance to the next nearest infected herd (range 0.3-8.7 km) and herd size (Table 4). Of the badger variables tested, only the number of badger road-kill reports within 1km was an important predictor. Indices of small mammal abundance were not present in the top-ranking models. The estimated odds ratios for all the variables appearing in the most parsimonious models were robust: with the exception of the variable 'gaps', the estimates from univariate analyses were virtually unaltered by the addition of herd size and nearest bTB case (Table 5), or other explanatory variables to the models.

Hedgerow characteristics appeared in 19 of the 21 top-ranking models (Table 4). Key parameters were the number of wildlife strips (ungrazed buffer strips adjacent to

field boundaries from which cattle are excluded, usually by fencing), the number of hedgerow gaps, and the score for hedgerow abundance (summarised in variable 'Hedgepc2'). High Hedgepc2 scores typified 'hedge-poor' farmland with few hedgerows and large field sizes, such as result from industrial post-war management. Taking for illustration the hedgerow parameters of two contrasting farms in this study, a 'hedge-poor' farm with a hedge density of 5.3km/100ha, mean hedge length of 186m and a mean connectivity score of 2.9 would be expected on average to have 1.6 times greater risk of bTB (95% Confidence Interval 1.0, 2.4) than a 'hedge-rich' farm with a hedge density of 13.4km/100ha, mean hedge length of 177m and a connectivity score of 3.7, after controlling for the other factors (herd size and distance to next bTB case) in the top-ranking model. Comparisons of the predictor weights (Table 4) show that the hedgerow parameter was about 2.5 times more important than the badger abundance index, and 28 times more important than SDI in explaining bTB incidence.

Conclusions

The same strain of *M. bovis* can infect bank voles, badgers and cattle on the same farm. However, the prevalence of infectious individuals is extremely low in this, and in other abundant farmland wildlife. Mathematical modelling suggests it is unlikely that the wildlife species we studied represent a significant reservoir of infection for cattle, though occasional spill-overs from badgers and cattle may occur. Our additional case-control study supports the view that small mammals are unlikely to contribute to the risk of bTB in cattle. Rather, the risk of bTB is associated with farmland habitat management. Our analyses consistently showed that managing boundary features in ways generally considered favourable to wildlife conservation (and encouraged by agri-environment schemes) are associated with a decreased risk of bTB in cattle.

Future research

We suggest that future research should explore further the associations between habitat and local risk of bTB in cattle. Important variables to be studied would be the behaviour of badgers and cattle under varying ecological circumstances, and also indices of susceptibility to infection. Ideally, intervention studies would be conducted to test experimentally the impact of boundary structures on bTB risk. It is increasingly recognised that identifying the conditions associated with the transfer of disease from reservoir hosts, and managing these conditions, can offer a valuable alternative strategy to simply reducing the population density of the reservoir.

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Publications generated from the project

Mathews, F., Macdonald, D.W., Taylor, G.M., Gelling, M., Norman, R.A., Honess, P.E., Foster, R., Gower, C.M., Varley, S., Harris, A., Palmer, S., Hewinson, G., Webster, J.P (2006) Bovine tuberculosis (*Mycobacterium bovis*) in British farmland wildlife: the importance to agriculture. *Proceedings of the Royal Society.* DOI 10.1089/rspb.2005.3298 (available online)

Macdonald, D.W., Riordan, P., Mathews, F. Biological hurdles to the control of TB in cattle: a test of two hypotheses to explain the failure of control. *Biological Conservation* (*in press*).

Mathews, F., Lovett, L., Rushton, S., Macdonald, D.W. Bovine tuberculosis in cattle: reduced risk on wildlife-friendly farms. *Biology Letters* (subm.)

Gelling, M., Macdonald, D.W., Mathews, F. Are hedgerows the route to biodiversity? Small mammals' use of hedgerows in pastoral farmland. *Landscape Ecology* (subm)

McLaren, GW., Mathews, F., Fell, R., Gelling, M., Macdonald DW. 2004. Body weight responses as a measure of stress: a practical test. *Animal Welfare*. **13**: 337-341.

Mathews, F., Honess, P., Wolfensohn, S. 2002. The use of inhalation anaesthesia for wild mammals in the field. *Veterinary Record* 785-787.

Review comment

The project had a high degree of difficulty due to the limitations imposed by the need to use live sampling, as opposed to post-mortem examination, for the assessment of current infectivity. Retrospectively it was felt that it might have been more logical to acquire the results from SE3010, where analyses were performed post-mortem, and then if prevalences justify further research into this question to have funded research into the frequency of infectivity in non-badger hosts. The low reliability of live testing meant that the results produced by this experiment were not robust and prevented some of the aims from being achieved. Additionally the modelling of risks was not possible due to the very low infection rates found. The most useful results appear to be that risk of transmission from most species evaluated seems to be minimal. In addition, there are indications that herd size and reduction in density of hedgerows increase TB incidence. However, further research is required to test, evaluate and explain these observations.

Scores

Conclusions based on sound evidence: 1.5
Quality of science: 1.2
Overall rating: 1.2

Table 1 Numbers of animals trapped and clinical samples obtained by live-sampling. (Notes: *n* refers to numbers in each category. TA is tracheal aspirate. TA column includes 30/41 cats and 20/20 dogs that had throat swabs rather than TA at request of owners. Due to licensing restrictions, badgers sampled on 6/12 farms only. Polecats and foxes sampled on first 8/12 farms. Most species were trapped on all farms. For those which were not (either due to licensing restrictions or failure of capture), the following numbers (%) were obtained on control farms: yellow-necked mouse 40 (15); house mouse 15 (26); pygmy shrew 1 (13); water shrew 5 (45); rabbit 48 (24); polecat 1 (14); fox 3 (33); badger 14 (33).

Species	animals (*n*)	clinical samples (*n [%]*)		
		TA	Urine	Faeces
Yellow-necked mouse (*Apodemus flavicollis*)	268	254 [94.8]	216 [80.6]	255 [95.1]
Wood mouse (*Apodemus sylvaticus*)	1338	1204 [90.0]	1110 [83.0]	1277 [95.4]
House mouse (*Mus musculus*)	58	51 [87.9]	31 [53.4]	51 [87.9]
Bank vole (*Clethrionomys glareolus*)	1307	1131 [86.5]	1130 [86.5]	1221 [93.4]
Field vole (*Microtus agrestis*)	330	259 [78.5]	302 [91.5]	317 [96.1]
Rat (*Rattus norvegicus*)	76	73 [96.1]	39 [51.3]	68 [89.5]
Common shrew (*Sorex araneus*)	272	3 [1.1]	29 [10.7]	208 [76.5]
Pygmy shrew (*Sorex minutus*)	8	2 [25.0]	2 [25.0]	6 [75.0]
Water shrew (*Neomys fodiens*)	11	0 [0]	7 [63.6]	10 [90.9]
Rabbit (*Oryctolagus cuniculus*)	202	196 [97.0]	109 [54.0]	145 [71.8]
Grey squirrel (*Sciurus carolinensis*)	189	179 [94.7]	26 [13.8]	95 [50.3]
Polecat (*Mustela putorius*)	7	4 [57.1]	3 [42.9]	0 [0.0]
Fox (*Vulpes vulpes*)	9	9 [100]	3 [33.0]	8 [88.9]
Badger (*Meles meles*)	43	32 [74.4]	25 [58.1]	16 [37.2]
Dog (*Cannis cannis*)	21	21 [100]	0 [0.0]	1 [5.0]
Cat (*Felis domesticus*)	42	42 [100]	9 [21.4]	5 [11.9]
TOTAL	4180	3460	3041	3683

Table 2 Prevalence of infectious individuals and confidence intervals.
(Notes: raw confidence intervals computed using Wilson's method for small proportions).

Species	Prevalence infectious animals % (*n/n*)	Raw 95% confidence intervals
Yellow-necked mouse (*Apodemus flavicollis*)	0 (0/268)	0, 1.4
Wood mouse (*Apodemus sylvaticus*)	0 (0/1338)	0, 0.3
House mouse (*Mus musculus*)	0 (0/58)	0, 6.2
Bank vole (*Clethrionomys glareolus*)	0.1 (1/1307)	0.0, 0.4
Field vole (*Microtus agrestis*)	0 (0/330)	0, 1.2
Rat (*Rattus norvegicus*)	0 (0/76)	0, 4.8
Common shrew (*Sorex araneus*)	0 (0/272)	0, 1.4
Pygmy shrew (*Sorex minutus*)	0 (0/8)	0, 32.4
Water shrew (*Neomys fodiens*)	0 (0/11)	0, 25.9
Rabbit (*Oryctolagus cuniculus*)	0 (0/202)	0, 1.9
Grey squirrel (*Sciurus carolinensis*)	0 (0/189)	0, 2.0
Polecat (*Mustela putorius*)	0 (0/7)	0, 35.4
Fox (*Vulpes vulpes*)	0 (0/9)	0, 30.0
Badger (*Meles meles*)	7.0 (3/43)	2.4, 18.6
Dog (*Cannis cannis*)	0 (0/21)	0, 15.5
Cat (*Felis domesticus*)	0 (0/42)	0, 8.4

Table 3 Selected input parameter values and basic reproductive rate, R_0, estimated from the mathematical model.

(Notes: R_0 =1/1-prevalence, where prevalence is the upper confidence interval for prevalence of individuals infectious for *M. bovis* as given in Table 3. The lower confidence intervals for prevalence always gave estimates for R_0 that were <1.0).

Species	Densities (measured in field)			Demographic parameters (from literature)		R_0 (computed from model)
	Mean	95% Confidence interval	Maximum	Birth rate/ day (a)	Death rate/day (b)	
Yellow-necked mice/100m	2.8	1.8, 3.8	22	0.0274	0.001825	1.0142
Wood mice/100m	7.2	6.4, 8.0	29	0.0274	0.001825	1.0030
Bank voles/100m	6.4	5.7, 7.1	29	0.0274	0.001825	1.0040
Field voles/100m	1.4	1.0, 1.7	12	0.0274	0.001825	1.0122
Common shrews/100m	0.9	0.6, 1.2	12	0.00216	0.008120	1.0142
All small rodents/100m	16.9	15.5, 18.4	49	0.0274	0.001825	1.0030
rats/0.25ha	7.5	3.1, 11.9	23	0.03596	0.002740	1.0504
rabbits/ha	17.8	6.7, 28.8	49	0.0288	0.002740	1.0194
squirrels/ha	13.0	7.6, 24.4	46	0.0123	0.002740	1.0204

Table 4 Akaike Information Statistics for logistic regression models relating bTB incidence in cattle herds to agricultural, badger and habitat predictors. The overall % correct classification ranges from 68.3 to 75.8 (mean 70.1% correct presence and 74.4% correct absence).

Model	AIC_c[a]	ΔAIC_c[a]	w[b]	w_i/w_j[c]	R^2[d]
Herdsize[e], nearcase,[f] hedgepc2[g]	138.90	0.00	0.085	1.00	0.34
Herdsize, nearcase, hedgepc2, head,[h] badgers[i]	139.00	0.09	0.081	1.05	0.38
Herdsize, nearcase, hedgepc2, head	139.00	0.09	0.081	1.05	0.36
Herdsize, nearcase, hedgepc2, turfedge[j]	139.60	0.70	0.060	1.42	0.36
Herdsize, nearcase, hedgepc2, turfedge, head	139.90	1.00	0.051	1.68	0.37
Herdsize, nearcase, hedgepc2, gaps,[k] head,	139.93	1.03	0.051	1.68	0.39
Herdsize, nearcase, hedgepc2, turfedge, head,	138.96	1.06	0.050	1.70	0.39
Herdsize, nearcase, hedgpec2, gaps, head	139.99	1.09	0.049	1.72	0.37
Herdsize, nearcase, hedgepc2, badgers	140.05	1.15	0.048	1.78	0.35
Herdsize, nearcase, hedgepc2, gaps	140.27	1.37	0.043	1.98	0.35
Herdsize, nearcase, head	140.29	1.39	0.042	2.00	0.33
Herdsize, nearcase, hedgepc2, width[l]	140.33	1.43	0.042	2.04	0.35
Herdsize, nearcase	140.41	1.51	0.040	2.12	0.31
Herdsize, nearcase, turfedge	140.45	1.55	0.039	2.17	0.33
Herdsize, nearcase, hedgepc2, density[m]	140.57	1.67	0.037	2.30	0.35
Herdsize, nearcase, head, badgers	140.59	1.69	0.036	2.33	0.35
Herdsize, nearcase, hedgepc2, turfedge,	140.75	1.85	0.034	2.52	0.37
Herdsize, nearcase, hedgepc2, head, width,	140.78	1.88	0.033	2.56	0.38
Herdsize, nearcase, hedgepc2, SDI[n]	140.79	1.89	0.033	2.57	0.35
Herdsize, nearcase, hedgepc2, coverpc1[o]	140.80	1.90	0.033	2.58	0.35
Herdsize, nearcase, hedgepc2, head, width	140.84	1.94	0.032	2.63	0.36

[a] Akaike's Information Criterion adjusted for small sample sizes. ΔAIC_c indicates the amount of support for the model relative to the top-ranking one (higher values show less support).
[b] Akaike weight. Another index of the strength of evidence for each model. It is the ratio of the ΔAIC_c of the target model relative to all the other models and is the probability of the model being correct, given the data.
[c] Evidence ratio. The ratio of the Akaike weight of candidate model to that of top-ranking model. It shows the extent to which the 'top' model is better than the model in question.
[d] Nagelkerke's R-square.
[e] Number of cattle in herd.
[f] Distance to next nearest case of bTB (km).
[g] Second principal component describing hedgerow abundance.
[h] Mean number of wildlife strips per hedgerow.
[i] Number of badger road-kill records within 1km of farm grid-reference.
[j] Length of edge of mown or grazed turf (km).
[k] Mean number of gaps in hedgerow per 100m.
[l] Mean hedgerow width (m).
[m] Stocking density of cattle (number/ha).
[n] Shannon's diversity index.
[o] Coverpc1, principal component 1 describing landcover.

Table 5 Predictor weights for variables appearing in the most parsimonious models (Δ AIC_c<2), together with odds ratios from logistic regression of bTB risk.

Variable	Predictor weight	Number of models in which variable appears	Univariate Odds ratio	95% Confidence interval for odds ratio[a]	Change in 2 log likelihood (R^2)[a,b]	Odds ratio from multivariate model[c]
Herdsize	1.00	21	1.01	1.01, 1.02	-	-
Nearcase	1.00	21	0.72	0.53, 0.98	-	-
Hedgepc2	0.84	17	1.61	1.07, 2.44	3.65 (0.03)	1.56
Head	0.51	10	0.01	0.00, 2.0	2.26 (0.02)	0.01
Badgers	0.33	7	1.14	0.94, 1.39	0.80 (0.02)	1.11
Turfedge	0.23	5	0.92	0.83, 1.03	2.10 (0.02)	0.91
Gaps	0.14	3	4.08	0.78, 21.35	1.21 (0.01)	2.56
Width	0.11	3	0.91	0.77, 1.08	0.69 (0.01)	0.92
Density	0.04	1	0.95	0.80, 1.12	0.63 (0.01)	0.93
SDI	0.03	1	2.34	0.51, 10.81	0.54 (0.01)	1.93
Coverpc1	0.03	1	1.00	1.00, 1.00	0.12 (0.00)	1.00

[a] From univariate logistic regression.
[b] Compared with model which includes herd size and nearcase only.
[c] From logistic regression models also containing herd size and nearcase.

SE3010 The risk to cattle from wildlife species other than badgers in areas of high herd breakdown risk

Organisation Central Science Laboratory
York

Start date 01/01/2000
End date 29/04/2004

Total cost £754,511

Abstract
The main objective of this study was to assess the risk to cattle from wild mammals other than badgers, by collecting information on the distribution, frequency and pathology of *M. bovis* infection in species in the cattle TB hotspots of south-west England. Carcasses (n=4714) underwent systematic post mortem examination, culture of tissues and spoligotyping of isolates. Infection was confirmed in foxes, stoat, polecat, common shrew, yellow-necked mouse, wood mouse, field vole, grey squirrel, and roe, red, fallow and muntjac deer.

Infected cases occurred in Worcestershire, Herefordshire, Gloucestershire, Wiltshire, Somerset, Devon and Cornwall, but were clustered where most carcasses were collected. Comparison of smaller areas showed substantial variation in prevalence.

Lesions were found in a fox, a stoat and a muntjac, and in a high proportion of positive fallow, red and roe deer. In fallow, red and roe deer the principal sites of infection were the lungs and associated lymph nodes. Isolation of common *M. bovis* spoligotypes from several species is consistent with inter-species transmission, and were similar in frequency of occurrence to those in cattle and badgers.

Information on host prevalence, pathology, ecology, density and distribution was integrated in an assessment of the risk to cattle. Most species exhibited a relatively low risk to cattle. The highest risks were associated with fallow and red deer. Given these results, the paucity of data on interactions between deer and cattle and their rapidly expanding numbers and distribution in southern England, deer may be a potential source of infection for cattle.

Aims
- Survey for *M. bovis* infection in a range of wildlife species by post mortem examination and culture.
- Describe *M. bovis* prevalence, severity of infection and sources of excretion in target species by post mortem examination.
- Quantify the potential relative risks to cattle associated with *M. bovis* infection in several wildlife species by integration of ecological and epidemiological data.

Relevance to Defra
Bovine tuberculosis is a serious disease of cattle and the incidence of herd breakdowns in the UK has increased in recent years. Although the badger is implicated in the spread of infection to cattle, the potential role of other wild mammals is poorly understood. Prior to the present study only limited research had been

undertaken on the prevalence of *M. bovis* infection in other mammals. This work identified infection in a range of species and suggested that prevalence levels were generally low [1], but non-systematic sampling and small sample sizes may have biased some studies. Furthermore, prevalence levels alone are unlikely to provide sufficient information to assess the potential risks of transmission to cattle, as this will also be influenced by host ecology, and the pathology and epidemiology of the disease in each species.

Methods

The collection of carcass was split between two phases. During the initial extensive phase (March 2000 – July 2002) carcasses were collected from throughout the south-west of England, but with priority given to triplet areas of the Randomised Badger Culling Trial (RBCT)[2]. During the intensive phase (July 2002 – Sept 2003) carcass collection was concentrated in the vicinity of selected triplet areas in contrasting geographical areas (Herefordshire, East Cornwall and Cotswolds).

Collection of carcasses required the establishment of a network of local contacts throughout south-west England as sources of supply (eg gamekeepers, stalkers, farmers, pest control operatives, Defra's Wildlife Unit, mammal researchers, veterinary clinics, wildlife rescue centres etc). Collected carcasses were subjected to a systematic *post mortem* examination, including the identification of visible lesions. Tissues from all carcasses were subjected to mycobacterial culture and suspicious lesions were cultured separately. All isolates were spoligotyped to confirm they were *M. bovis* and to identify strain types.

In the original project proposal it was anticipated that live sampling of wild mammals would take place during the intensive phase of work. However, following wide consultation it was decided that the priority should be to increase the sensitivity of detection of infected hosts, and the optimum strategy for achieving this was to increase sample sizes. Hence resources previously earmarked for live trapping and sampling were redeployed to allow more carcasses to be collected.

In order to integrate information on prevalence, pathology, abundance and ecology of host species, a semi-quantitative risk assessment was carried out.

Results

A total of 4714 were subjected to examination and mycobacterial culture of tissue samples. Infection was confirmed in foxes, stoat, polecat, common shrew, yellow-necked mouse, wood mouse, field vole, grey squirrel, roe deer, red deer, fallow deer and muntjac (see Table 1). The prevalence of infection was significantly higher in adult fallow and roe deer, and in female foxes. Sample sizes varied widely between species and consequently so did confidence limits associated with prevalence estimates. Small sample sizes for species in which no positive cases were observed (eg house mouse, sika deer), provide very unreliable evidence for the absence of infection. No evidence of infection was found in hedgehogs, rabbits, bank voles and brown rats, despite relatively large sample sizes.

Prevalence estimates for deer may have been underestimated because the majority of submitted samples were incomplete carcasses, and subsequent analyses showed

that the likelihood of detecting lesions, and of confirming *M. bovis* infection by culture and spoligotyping was significantly lower than for complete carcasses.

Infected cases were found in Worcestershire, Herefordshire, Gloucestershire, Wiltshire, Somerset, Devon and Cornwall, although they tended to be clustered in Gloucestershire and Herefordshire where most carcasses were collected. Although carcass collections were targeted at, or adjacent to, the treatment areas of the randomised badger culling trial, sample sizes varied substantially between areas. Hence, differences in prevalence between triplet areas were only carried out for foxes, red, roe and fallow deer, and the only species in which significant variation was detected was the latter. Investigation of the prevalence of infection in smaller areas showed substantial variation, for example a prevalence of 2.7% was found for roe deer in part of north Gloucestershire but infection was absent from those collected from the Mendips in Somerset.

No confirmed lesions were found in small mammals, grey squirrel or polecat. Only a single fox and a stoat exhibited gross pathology, both with macroscopic lesions in the mesenteric lymph nodes. This site of infection is consistent with infection by ingestion and raises the possibility of excreted bacilli in faeces. Lesions were only found in a single muntjac, but were present in a high proportion of positive fallow, red and roe deer. In the latter species the principal sites of infection were in the lungs and the associated lymph nodes, consistent with infection by inhalation and the potential for onward transmission.

The isolation of common *M. bovis* spoligotypes from a variety of wild mammal species is consistent with inter-species transmission. Comparison with cattle and badgers showed a similar frequency of occurrence of spoligotypes with that found in wild mammals.

Information on the prevalence of infection, pathology, and the ecology, density and distribution of wild mammals was integrated in a semi-quantitative risk assessment for the likelihood of transmission to cattle. The lowest risk was presented by the grey squirrel, with intermediate levels of risk associated with small mammals, fox, stoat, polecat, muntjac and roe deer, and the highest risks being posed by red and fallow deer (see Table 2).

Conclusions

Although sample sizes for some species were inadequate to rule out their potential involvement, it seems unlikely that most of those in which infection was not detected, pose a significant risk to cattle. In most of the species in which infection was confirmed, the likely risks to cattle appear relatively low. The roles of polecats, stoats and foxes are also unclear, although the relative scarcity of the former and infrequency of pathology in all three suggests that they are unlikely to represent a high risk to cattle. The present study did however identify pathology and levels of infection in fallow and red deer, and in some locations in roe deer, that indicate a potential risk of disease transmission to cattle. However, substantially higher levels of infection have been observed in badgers, which can also excrete potentially large numbers of bacilli and will forage on pasture and in buildings used by cattle. Nevertheless, in the light of the results presented here, the paucity of data on interactions between deer and cattle and their rapidly expanding numbers and

distribution in southern England, it seems prudent to consider deer as a potential, although probably localised, source of infection for cattle.

Future research
Given the identification of infection in some species of small mammals, their abundance and wide dsitribution, further investigation of the pathology of infection in small mammals is required before there is sufficient evidence to rule them out.

The high relative risk scores associated with deer suggest that their potential for transmission to cattle should be further investigated. CSL is currently undertaking a more comprehensive risk assesment of the potential role of deer (SE3036 funded by Defra). However, the collection of further information on pathology in deer, contact rates with cattle, and more reliable estimates of deer abundance and distribution would greatly benefit this assessment.

References cited in text
[1.] Delahay, R. J., de Leeuw, A. N. S., Barlow, A. M., Clifton-Hadley, R. S. & Cheeseman, C. L. (2002). The status of *Mycobacterium bovis* infection in British wild mammals: a review. *Veterinary Journal* **163**, 1-16.

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Review comment
The project was well thought out and was executed to as high a standard as was achievable in a field based study of this magnitude. The high quality pathodiagnostic and mycobacteriological components gave credence to the overall conclusions regarding the likely tuberculosis status of the various populations, within the constraints of the number of animals and the locations studied. The findings indicate that other animals do become infected with TB, particularly deer, and they pose a potential risk. Additionally, the results show that apart from some deer species, mammals other than badgers are unlikely to constitute significant sources of infection when epidemiological and ecological factors are taken into account. Systematic sampling were not used, therefore the samples collected are not representative of the species population. For example samples that were suspected of having disease

were excluded. In addition they often relied on badgers from the Road Traffic Accident surveys and gamekeepers. These limitations should be born in mind when interpreting prevalence estimates. There were not any meaningful comparisons of geographical variation in incidence of disease in wild mammals due to the sample size obtained. Further investigation on the role of deer species as vectors and information on the rate of spread of deer, particularly in Wales are greatly needed.

Scores
Conclusions based on sound evidence: 3.0
Quality of science: 2.8
Overall rating: 2.7

Table 1 Estimates of the prevalence of *M. bovis* confirmed cases (and 95% confidence intervals) for wild mammals collected and examined during the study.

		Prevalence of culture positive cases confirmed by spoligotype				
					95% Confidence Interval	
Species	**Number Collected**	**Negative**	**Positive**	**Prevalence**	**Min**	**Max**
Fox	**756**	732	24	**3.17**	2.04	4.69
Otter	**21**	21	0	**0.00**	0.00	13.29
Mink	**51**	51	0	**0.00**	0.00	5.70
Stoat	**78**	75	3	**3.85**	0.80	10.83
Feral Ferret	**16**	16	0	**0.00**	0.00	17.07
Weasel	**50**	50	0	**0.00**	0.00	5.82
Polecat	**24**	23	1	**4.17**	0.11	21.12
Feral Cat	**1**	1	0	**0.00**	0.00	95.00
Mole	**83**	83	0	**0.00**	0.00	3.54
Hedgehog	**102**	102	0	**0.00**	0.00	2.89
Water Shrew	**4**	4	0	**0.00**	0.00	52.71
Common Shrew	**41**	40	1	**2.44**	0.06	12.86
Pygmy Shrew	**42**	42	0	**0.00**	0.00	6.88
Rabbit	**347**	347	0	**0.00**	0.00	0.86
Brown Hare	**15**	15	0	**0.00**	0.00	18.10
Yellow-Necked Mouse	**36**	35	1	**2.78**	0.07	14.53
Wood Mouse	**333**	331	2	**0.60**	0.07	2.15
Water Vole	**5**	5	0	**0.00**	0.00	45.07
Bank Vole	**157**	157	0	**0.00**	0.00	1.89
Field Vole	**67**	66	1	**1.49**	0.04	8.04
House Mouse	**5**	5	0	**0.00**	0.00	45.07

Brown Rat	**317**	317	0	**0.00**	0.00	0.94
Grey Squirrel	**450**	448	2	**0.44**	0.05	1.60
Red squirrel	**1**	1	0	**0.00**	0.00	95.00
Feral goat	**1**	1	0	**0.00**	0.00	95.00
Roe deer	**885**	876	9	**1.02**	0.47	1.92
Red deer	**196**	194	2	**1.02**	0.12	3.64
Sika deer	**3**	3	0	**0.00**	0.00	63.16
Fallow deer	**504**	482	22	**4.37**	2.76	6.53
Muntjac	**58**	55	3	**5.17**	1.08	14.38
Feral sheep	**5**	5	0	**0.00**	0.00	45.07
Feral wild boar	**7**	7	0	**0.00**	0.00	34.82

Table 2 A semi-quantitative risk assessment showing the potential risk of disease transmission to cattle, relative to the badger, from those mammal species in which infection was detected during the study. Prevalence was derived from confirmed positive cases in Table 1. Badger prevalence and associated confidence interval was taken from RTA data collected from 1994 to 1999 (R. Clifton-Hadley, unpublished data). The likelihood of excretion, and contact with cattle, are the mean values of the combined risk distributions. Biomass is the product of the relevant density and bodyweight calculated from published sources. The final risk value is the median, (and inter-quartile range) based on 10,000 simulations.

	Prevalence (min, max)	Mean Likelihood of excretion	Mean Likelihood of contact with cattle	Biomass kg/km^2	Final median risk score (inter-quartile range)
Grey squirrel	0.44 (0.05, 1.60)	0.13	0.22	275	**0.002 (0.001 - 0.006)**
Yellow necked mouse	2.78 (0.07, 14.53)	0.13	0.19	14	**0.001 (0.000 - 0.003)**
Wood mouse	0.60 (0.07, 2.15)	0.13	0.23	40	**0.001 (0.000 – 0.001)**
Field vole	1.49 (0.04, 8.04)	0.13	0.23	680	**0.034 (0.011 – 0.092)**
Common shrew	2.44 (0.06, 12.86)	0.13	0.19	45	**0.003 (0.000 – 0.009)**
Stoat	3.85 (0.80, 10.83)	0.17	0.13	2	**0.000 (0.000 – 0.000)**
Polecat	4.17 (0.11, 21.12)	0.17	0.23	1	**0.000 (0.000 – 0.000**
Fox	3.17 (2.04, 4.69)	0.27	0.42	12	**0.004 (0.000 – 0.009)**
Red deer	1.02 (0.12, 3.64)	0.92	0.6	1158	**0.834 (0.266 – 2.135)**
Fallow deer	4.37(2.76, 6.53)	0.96	0.71	722	**1.624 (0.808 – 3.123)**
Roe deer	1.02 (0.47, 1.92)	1.00	0.63	462	**0.262 (0.122 – 0.494)**
Muntjac	5.17 (1.08, 14.38)	0.67	0.42	275	**0.329 (0.119 – 0.812)**
Badger	10.94 (9.76, 12.21)	1.00	1.00	54	**1.000**

SE3032 The long-term intensive ecological and epidemiological investigation of badger populations naturally infected with *Mycobacterium bovis*

Organisation Central Science Laboratory
York

Start date 01/04/04
End date 01/04/06

Total cost £1,192,509

Abstract
This project represents the continuation of data collection from an intensively studied wild badger population at Woodchester Park, and the initiation of studies at a new site in Gloucestershire that has been subjected to culling as part of the Randomised Badger Culling Trial (RBCT). Bait marking was used to establish the configuration of badger social group ranges, and an intensive live-capture regime provided demographic and epidemiological data. The behaviour of badgers in response to culling operations was also monitored by radio-tracking adult animals in the perturbation study site.

Aims
The basic scientific objectives were to continue to collect ecological and epidemiological data from the Woodchester Park badger population, consistent with that obtained in previous years[1,2,3], and begin data collection in a further study area established on the edge of an RBCT proactive cull treatment area[4] (I2). Specifically this involved,

(a) Woodchester Park Study
- Obtaining data on the spatial configuration of social group ranges by bait-marking.
- Collecting data on the size, structure and infection status of the population by capture-mark-recapture and clinical sampling.
- Collation of collected data onto epidemiological and spatial databases.

(b) Study of a perturbed population in I2
- Obtaining data on the efficiency of badger culling operations (by comparison with bait-marking, trapping, radio-tracking and collection of faecal DNA from badger latrines).
- Obtaining data on the spatial configuration of social group ranges by bait-marking.
- Collecting data on the size, structure and infection status of the population by capture-mark-recapture and clinical sampling.
- Collecting data on the behaviour of individual badgers in response to culling operations (by radio-tracking).

Relevance to Defra

The development of sustainable policies to control *M. bovis* transmission from badgers to cattle is only possible through a deeper understanding of the ecology and dynamics of disease in badger populations, and interactions with cattle. Since 1975 CSL has conducted intensive long-term monitoring of a high-density badger population at Woodchester Park. This has provided data on spatial and temporal epidemiological patterns, ecological, demographic and behavioural processes that have enhanced our understanding of badger ecology, management and the epidemiology of *M. bovis* infection[1,2,3,5]. Continued monitoring of this population provides Defra with a strategic resource to explore a range of potential future policy options. The initiation of similar research on a population subjected to culling allows the collection of data on culling efficiency and its demographic and epidemiological effects. Culling badger populations can have counter-productive effects on the control of TB in cattle[6,7], although further studies are required in order to fully understand the management implications.

Methods

The configuration of badger social group ranges was determined by bait-marking in the spring of 2004 and 2005 in Woodchester Park, and in 2005 only in I2. Feeding of bait containing indigestible plastic markers took place at active badger setts and was followed by a survey for latrines. The results were used to produce digital maps of social group territories using a geographic information system (ArcInfo version 7.2.1, ESRI, Redlands, California, USA).

Live capture of badgers in both study areas took place all year round except between February and May to avoid the capture of very young cubs, lactating and heavily pregnant females. Each badger social group was trapped once in each season, using steel mesh box traps baited with peanuts and set after 4-8 days of pre-baiting. Traps were located on or near to badger 'runs' at the active setts in each territory. Trapped badgers were anaesthetised prior to examination, and if previously unmarked were given a unique identifying tattoo. The location, sex, body weight, condition, reproductive status and age class were recorded for all captured animals. Clinical samples (ie sputum, faeces, urine pus from abscesses, bite wound swabs and blood) were obtained from captured badgers. Any badgers found dead within the study areas were subjected to *post mortem* examination and tissues were cultured to assess TB status.

The infection status of badgers was determined by the bacterial culture of clinical samples and a serological test (ELISA) for the presence of antibodies to *M. bovis*. Suspect *M. bovis* culture isolates were confirmed by spoligotyping.

All adult badgers caught in I2 were fitted with radio-collars. Cubs captured in their year of birth were not collared for welfare reasons, as collars fitted to cubs may not allow sufficient space for natural growth. Collared animals were radio-tracked to determine home range size, movement patterns and sett use.

During trapping at both sites a hair sample was taken from each animal from which a genetic profile was obtained (costed under SE3110). Also, in I2 all known badger latrines were visited on 3 consecutive days per week (Sept – Nov, 2004), to collect fresh faecal material. Samples were stored in ethanol and sent to the University of

Sheffield for DNA extraction and genetic profiling to attempt to identify untrapped animals[8].

Results

(a) Woodchester Park

The configuration of badger social groups in the Woodchester Park study area was determined by bait marking in the spring of 2004 and 2005.

Live capture of badgers in 2004 and 2005 provided trapping data from 644 individual capture events, comprising 291 different individuals (126 males and 165 females). Of these, 159 were cubs, 198 were adults caught in previous years and 9 were adults that had not previously been caught.

Results of the culture of clinical samples indicate that the prevalence of culture positive badgers was approximately 9.1% in 2004, and 4.7% for 2005 (although this is a preliminary figure as not all culture results are available yet). In addition, 51 badger carcasses were recovered from the study area over the two years, and submitted for *post mortem* examination for tuberculous lesions and the microbiological culture of tissue samples. Of these, 29 had died as a result of collisions with cars (of which 3 were positive) and 22 were found dead elsewhere (5 of which were positive).

During 2004 and 2005 the estimated annual prevalence of ELISA positive badgers (ie antibody positive) was approximately 19.4 % and 35.4 % respectively.

(b) I2

Feeding took place at 17 active badger setts in the study area in 2005, and the results show mean group ranges in the culled area to be less well defined than in the unculled area suggesting a more fluid social structure in the culled population.

A total of 87 and 61 individual capture events took place in 2004 and 2005 respectively. In 2004 these comprised 52 different individuals, all caught for the first time, and included 28 males and 24 females, of which 36 were cubs. In 2005, 52 different individuals were also captured, 6 of which were adults caught for the first time, and 31 were cubs. Of the adults, 2 were males and 4 were females.

Results for 2004 and 2005 indicate the prevalence of culture positive badgers to be approximately 8%. In addition, during 2004 and 2005, five badger carcasses were recovered from the study area and submitted for *post mortem* examination for tuberculous lesions and the microbiological culture of tissue samples. All had died as a result of collisions with cars, and two were found to be positive for TB. During 2004 and 2005 the estimated annual prevalence of ELISA positive badgers (ie antibody positive) was approximately 37% and 44% respectively.

Fifteen animals were fitted with radio-collars prior to the 2004 cull. Reliable home range estimates were obtained for 12 of these before culling began. One animal was killed by a car, one collar was shed and another failed. An additional 18 animals were fitted with collars in 2005 and we are continuing to collect home range and movement data on the 15 animals that are currently known to be alive. Losses are attributed to animals captured as part of the culling trial and collar malfunction or loss. To date

2,538 location fixes have been collected during 707 hours of radio-tracking at night, in addition to 1,726 day time locations. Preliminary results reveal that home ranges of badgers living in the culled area are approximately 40% larger than ranges of those outside the culled area.

A total of 194 faecal samples were collected from latrines in I2. DNA extraction was carried out on all samples but due to time constraints and staff shortages at University of Sheffield, genotypes were only obtained from the first 102 samples. Amplifiable DNA was obtained from 101 (99%) of the samples, however, consensus genotypes for three loci (the minimum number required to separate siblings) were only obtained for 28. Preliminary analysis has identified a minimum of 14 individual badgers from the part of the study area within the proactive culling area, prior to the 2004 cull. Six (43%) of the 14 individuals identified from the faecal DNA matched the genotypes of six (55%) of 11 animals culled during 2004. We are awaiting the results from genotyping of hair samples taken from captured badgers to enable us to proceed further with these analyses.

Conclusions

Demographic and epidemiological data from Woodchester Park has contributed to the only long-term data series on *M. bovis* infection in a wild badger population. The long-term trends continue to show substantial temporal variation in the incidence of infection but no simple linear relationship with population density (Figure 1).

Preliminary results from the Perturbation Study Area in I2 indicate that the density of badgers is considerably lower than at Woodchester Park and that density in the culled part of the study area is correspondingly lower than in the unculled area. Additional information collected on sett activity also indicates that the perturbed population living in the culled area is more trap-shy than the less disturbed population. Bait-marking suggests that previous culling operations may have affected the spatial configuration of badgers in the culled area. Radio-tracking suggests that individuals living in the culled area are also ranging further than badgers in the unculled area.

Both populations continue to be monitored in 2006 during which time we will conduct a comprehensive epidemiological analysis of the Woodchester population and a summary of the behavioural response of badgers in the culled population in I2.

Future research

The continued population monitoring and live-sampling of badgers at Woodchester Park and in the I2 Perturbation Study Area provide unique opportunities for further collateral research. This includes the field testing of a wide variety of research and management techniques (eg new diagnostic techniques for detection of *M. bovis*, validation of methods for estimating badger abundance). In addition, the substantial database provides opportunities for statistical investigations into epidemiological and ecological phenomena. In these respects the study areas, database and project infrastructure offer a unique strategic resource. In the case of the Woodchester Park study area this has the extra benefit of long-term data which allows investigation of ecological and epidemiological phenomena which may operate over many years.

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Publications generated from the project

Carpenter, P. J., Pope, L. C., Greig, C., Dawson, D. A., Rogers, L. M., Erven, K., Wilson, G. J., Delahay, R. J., Cheeseman, C. L. & Burke, T. (2005). Mating system of the Eurasian badger, *Meles meles*, in a high density population. *Molecular Ecology* **14**, 273-284.

Delahay, R. J., Walker, N., Forrester, G. J., Harmsen, B., Riordan, P., Macdonald, D. W., Newman, C. & Cheeseman, C. L. (in press). Demographic correlates of bite wounding in European badgers (*Meles meles* L.) in stable and perturbed populations. *Animal Behaviour*.

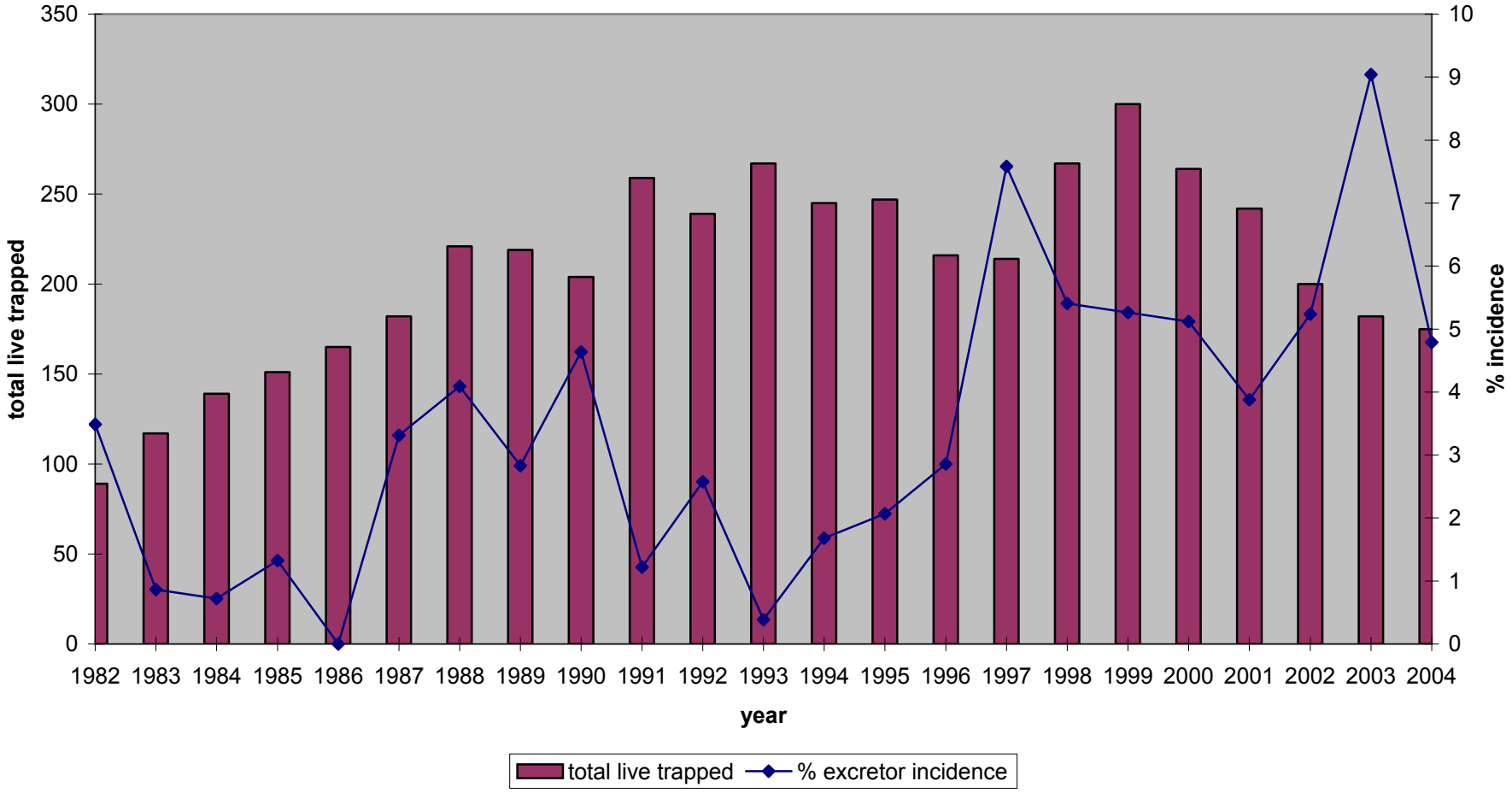
Review comment

This project comprised of two elements. The on-going collection and collation of Woodchester Park data and a perturbation aspect conducted in area I2 of the RBCT. The Woodchester Park element was a risk free project guaranteed to deliver its objectives. The stated objectives are to collect data and to collate those data. Extensive and intensive data sets are of immense value in ecology, however, it was felt that this value was not fully realised by analysis and interpretation. However, this sound and detailed long-term study will help to provide background data on badger

biology with relevance to TB. The second element is of critical importance in order to understand the role that interference in badgers has on breakdown rates in cattle. The work thus far has been successful. However, the limited data presented regarding areas adjacent to the “culled” area require extension before any useful assessment can be considered. The lack of replicated sites suggests the power is weak. Possible integration of the data with the MacDonald study (SE3108) could provide more power and insight. In future, the long-term Woodchester Park study would appear to benefit from greater precision in terms of identifying required outcomes relating to policy requirements. In addition, there is a need to ensure that the work contributes to parallel work elsewhere. Otherwise, there are dangers of having considerable understanding of population dynamics and disease transmission at one site that may have little relevance in other locations.

Scores
Conclusions based on sound evidence: 2.1
Quality of science: 2.2
Overall rating: 2.1

Figure 1 Incidence of *M. bovis* culture positive badgers and population size at Woodchester Park.



SE3107 Develop innovative methods to estimate badger population density

Organisation Central Science Laboratory
York

Start date 01/04/99
End date 31/03/05

Total cost £1,150,521

Abstract
Badgers are nocturnal and fossorial, and therefore notoriously difficult to count. Prior to this project, the only reliable method for estimating badger populations was live capture-mark-recapture. This is laborious and expensive, and therefore can only be carried out over a limited area. Less intensive methods are required as tools for the management of badgers, particularly in the context of the management of bovine Tuberculosis. In this project, methods for estimating and monitoring badger population size were developed and tested. At the outset, a review of candidate methods was carried out. Four candidates were identified and tested. Work was carried out at Woodchester Park, where the badger population is well described, and in the Randomised Badger Culling Trial (RBCT) areas. Two field sign index methods were tested: sett activity surveys and faecal accumulation rate surveys. Additionally, two direct count methods were tested: faecal DNA recovery and spotlighting. All four methods provide different types information on badger populations, and all may be useful in different situations. However, we found that the direct count methods, ie spotlighting and DNA recovery from the field provide more useful information on badger populations, and should be used where resources allow. Full details of the projects findings are available in the SID5 final project report, and associated peer reviewed papers.

Aims
- Identify candidate techniques for assessing badger density from previous work and develop technology for remote monitoring techniques.
- Collect data using candidate techniques, and Mark Recapture, on a population of badgers.
- Compare estimates from candidate techniques with baseline estimates based on trapping data. Calibrate the results of index methods against known populations.
- Validate methods in low density and reduced density populations.
- Monitor badger density and recolonisation in the badger removal area in the Krebs experiment, or any replacement strategy as appropriate.

Relevance to Defra

Reliable methods to estimate and monitor badger populations are required in a number of aspects of bTB research and management. For example, if badger population reduction is used as a management strategy, reliable monitoring methods are required in order to determine the effectiveness of culling operations, measured in terms of reduction in population size. Alternatively, in any vaccine research and implementation, it would be necessary to estimate badger population size in order to effectively plan the application and delivery programmes.

Methods

1. Review of candidates

   The first stage of the project was to carry out a review of the potential candidate techniques for estimating and monitoring badger abundance. We focused primarily on techniques that did not involve live capture. A full review of methods used on carnivore species in general was carried out (Wilson & Delahay, 2001). Four methods were identified as being potentially suitable for estimating and monitoring badger numbers across areas equivalent in size to those used in the RBCT. These were, sett activity surveys, faecal accumulation rate counts, faecal DNA recovery and nocturnal spotlighting surveys. Sett activity surveys and faecal accumulation counts fall into the class of indirect, index methods ie where the size of the badger population is assumed to be related to the field sign counts. Spotlighting surveys are a direct observation approach, ie the animals themselves are actually seen and counted. We also classed faecal DNA recovery as a direct method, as an individual genotype is derived from each faecal sample, which identifies the individual that deposited it. Therefore, it is analogous to catching and marking individuals. Both of these direct approaches are classed as incomplete counts, as only a proportion of the individuals present are seen or identified.

2. Sett Activity Surveys

   Trials at Woodchester

   Badger setts vary widely in appearance, from a short underground tunnel with one entrance hole and a small spoil heap outside, to extensive tunnel systems with substantial amounts of excavated soil outside many entrance holes. Setts that are in use by a number of badgers can usually be distinguished from those that are disused or rarely used, on the basis of the presence of characteristic signs of activity. If the intensity of these signs is related to the number of resident badgers then this could provide a relatively simple tool for estimating badger abundance at a local level. We developed an objective method for scoring certain measurable sett activity characteristics, and investigated the relationships between these and the number of resident badgers.

   A scoring system for recording activity at badger setts was developed, based on aspects of the physical appearance of setts that field staff used in making subjective assessments of the likely number of resident badgers. The presence and absence of signs of trampling, signs of recent digging / bedding, and badger footprints at each sett entrance, plus the number of latrines and fresh droppings were recorded. The number of entrance holes (EH's) at setts with each of these activity signs were investigated with respect to season, and the number of known residents, in order to assess their value as predictors of the number of sett residents. The activity signs were also used to calculate summary activity scores

for setts, and again correlated with the number of badgers estimated to be resident. This was tested at Woodchester by scoring setts immediately prior to trapping, over the course of the 2000-2001 field season. Badger trapping was carried out such that each social group in the study site was trapped once in May-June, August–September, October-November and January-February, relating to spring, summer, autumn and winter respectively.

Sett activity surveys in RBCT areas
Sett activity data was collected as above from a large random sample of setts in 4 triplet areas from 2002 to 2004 inclusive. Additionally, for the purposes of a separately funded piece of work for Defra/ISG, we visited a sample of main setts in all proactive areas of the RBCT one month after culling had taken place, and made subjective estimates of the numbers of badgers present. For the purposes of the present study, we also took the opportunity during these visits to collect data on sett activity using the methods described above.

3. Faecal accumulation rate counts at sett latrines
Badger latrines near setts appear to be important for intra-group communication. The deposition of droppings at hinterland latrines may also serve as a defence of the sett against interlopers (Roper *et al* 1993), a strategy that would be best served where a high proportion of the resident individuals made deposits. Given their widespread use by badgers, monitoring latrines was considered as a potentially useful approach to estimating badger numbers.

Trials at Woodchester
The method was tested at Woodchester Park, where the population is well described. Badger social group territories were delineated by baitmarking (Delahay *et al* 2000). We selected eleven social groups distributed throughout the study area. Social group size was estimated by live-trapping badgers and employing a Minimum Number Alive (MNA) method (Rogers *et al* 1997). All latrines within 30 m of each sett within the selected group territories were located. At the first visit, all droppings were marked with a sprinkling of white builders chalk dust. This was used because it is easily visible, persists during wet weather and is odourless. At each subsequent visit, the number of droppings deposited overnight were counted, and the latrine re-marked with chalk dust. Counts were carried out for 10 consecutive days, and an index of faecal accumulation rate (FAR) was calculated. Data was collected in each season in two different years, although the study period ran from autumn 1999 to autumn 2002 due to the delay caused by the foot and mouth disease outbreak.

Faecal counts in RBCT areas
Latrine counts were carried out at 10 active main setts in proactive area D3, one month before the initial proactive cull in autumn 2002, and at the same time of year in 2003. The 10 setts were selected to be aggregated in one area near the centre of the trial area. Defra carried out an initial and follow-up cull in the period between the two surveys. The FAR was estimated at each sett using the above method.

4. DNA recovery from faeces

Recent developments in molecular techniques have made it possible to obtain a genetic fingerprint using DNA from faecal samples collected in the field, thus providing a method of sampling animals without trapping or observing them (Kohn & Wayne 1997; Palsboll 1999). This infomation can be used to estimate population size in several ways by considering each sampled fingerprint as analogous to an animal capture. By carrying out repeated, temporally distinct sampling visits, a dataset of genotypes can be collated and treated as 'mark-recapture' data (eg Banks *et al* 2002). Alternatively, by plotting a non-linear regression curve through a plot of the number of droppings sampled against the cumulative number of new genotypes, an asymptote is reached at the estimated population size (Kohn *et al* 1999).

Trials at Woodchester

A suite of 39 DNA microsatellite markers for badgers has been produced using blood and hair samples taken from animals trapped at Woodchester Park (Carpenter *et al* 2005). We hypothesized that systematic sampling and genotyping of droppings deposited at communal latrines near badger setts would enable us to estimate the number of resident individuals. The pilot study was carried out at Woodchester Park, where the long term intensive live trapping study provides robust independent estimates of badger numbers for comparison, plus complementary data on individual life-histories. Three social groups were targeted: 'Parkmill', 'Nettle' and 'Kennel'. A preliminary study was carried out to identify the optimum methods for storing samples and extracting DNA from badger faecal samples (see Frantz *et al* 2004). Latrines close to setts within three social groups were located and fresh samples identified daily for 10 days as described in the latrine count index method above. A small sample of each fresh dropping was taken, and a genetic profile determined as described in Frantz *et al* (2004). Population size was estimated from the set of genotypes using non-linear regression. Hair samples from badgers previously trapped in the target groups were used to provide genetic fingerprints as a reference baseline against which to compare the faecal genotypes. The trap records provided a baseline population size estimate. Remote video surveillance at the target setts provided further information on resident badgers during the 10-day sample collection period. Full details of this study can be found in Wilson *et al* (2003).

Faecal DNA recovery in RBCT areas

A faecal DNA study was carried out in the I2 proactive treatment area of the RBCT. We wished to gather as much information as possible on latrine use patterns by badgers, across the study area, in addition to comparing genotypes derived from the faeces with those from badgers removed from the area in the subsequent cull. Therefore, a full survey of a 30km^2 area within the I2 proactive area was surveyed for latrines, including boundary, hinterland, and sett latrines. This was carried out in August / September 2004. Faecal collection was carried out as above, prior to the 2004 proactive culling operation.

5. Spotlighting surveys using distance sampling

Distance sampling, based on perpendicular distances from line or point transects to observations, has been widely used in recent years to estimate the abundance of vertebrates (Buckland, Goudie, & Borchers, 2000; Buckland *et al* 2001). Distance sampling produces density estimates by using the distances to observations of the target species from a line or point transect to produce a detection function. This is then used to calculate a value for the probability of detection, which when combined with the actual number of animals seen gives an estimate of density. Distance sampling was trialled at Woodchester Park, and the population estimate compared to that derived from the ongoing live-trapping programme. A full description of this study is given in Hounsome *et al* (2005).

A section of the Woodchester Park study site was selected. The badger social group territorial arrangement was identified during spring baitmarking survey (Delahay *et al* 2000), therefore the number of badger social groups encompassed by the chosen area was known. This allowed estimation of the number of badgers present in this area using mark-recapture models, to compare with the spotlighting estimate.

Nineteen line transects were established across the study area. Line transects were established along random bearings across fields, so that sampling was random with respect to the expected distribution of the population. A compass was used to follow each bearing and the end points of the line transects were marked using highly reflective tape. The 19 line transects measured 5.43 km in total length and these were repeatedly sampled by surveyors until the minimum of 80 observations which is generally required for reliable modelling of the detection function, was achieved (Buckland *et al* 2001). Surveying was carried out in pairs, comprising an 'observer' and a 'recorder'. Both surveyors carried a 1.2 million candle power spotlight fitted with a red filter and binoculars (8 x 42). The distance and bearing to every badger seen was recorded using a compass and laser rangefinder.

The density of badgers was estimated using the program DISTANCE 4.0 (Thomas *et al* 2003). This was corrected for the proportion of time that badgers spend in open habitats, estimated from radiotracking data at Woodchester. Abundance was calculated by multiplying density by the study area size, and compared to the baseline, trapping-derived estimate.

Results

1. Sett Activity Surveys

The mean number of sett entrance holes (EH's) per sett exhibiting each type of activity was calculated for each season (Table 1.1). Significant seasonal differences were found only for the total number of EHs per sett that had fresh excavations, bedding and footprints. Both excavations and bedding were most frequently observed in winter and spring, and footprints were least evident in the summer. However, these observations did not follow the same seasonal trend as the number of badgers trapped. For example, the highest counts of EHs with footprints per sett occurred in the winter, coinciding with the lowest numbers of badgers trapped.

Linear regression was used to investigate relationships between individual activity scores and badger numbers within season, and to determine whether they could be used to predict the number of badgers trapped. The regressions were carried out separately for each season. The scores for several activity types and the summary activity indices were significantly positively associated with badger numbers in spring and summer (Table 1.2) but not in autumn and winter. The 'weighted' summary index (see Final Report for details of how the indices were calculated) explained slightly more of the variation in badger numbers in both spring and summer than any of the individual activity types. However, the low r^2 values and high standard deviations around the regression line indicated that the individual activity types and summary indices were poor predictors of badger numbers. In autumn and winter, there were no significant correlations between the index values and badgers trapped.

Stepwise linear regression was used to determine if in each season a subset of activity types could be selected that could reliably predict badger numbers. The variables included in the model selection process were the number of EHs associated with each activity type and the number of droppings per sett. In spring, the number of EHs with a 'compact' floor only was selected in the final model (r^2=0.15, n=49, p<0.01). In summer, the numbers of EHs with 'compact' floors and footprints were selected (r^2=0.37, n=55, p<0.01). However, the predictive power of both models was low.

Random sett surveys in RBCT areas

A total of 359 setts were surveyed in the proactive, reactive and control areas of triplets E, I, H and G in 2002. In 2003 and 2004, the surveys were repeated in the proactive and control areas only. The setts were randomly selected from the sett survey data on the Defra trial database. Approximately 30 setts in each trial area were surveyed. The weighted activity index was calculated for each sett.

The mean sett activity scores declined in all four proactive triplet areas (Table 1.3). The decline in mean sett activity score ranged from approximately 15% to 57%. Given that at Woodchester there was an association between mean sett score and the number of resident badgers in at least two seasons, this would appear to indicate a progressive decline in badger numbers in proactive areas over the 3-year period. However, it should also be noted that the mean sett scores in the survey-only ('control') areas also declined in 3 of the 4 areas, although to a lesser extent than the proactive areas. The results are therefore difficult to interpret and should be viewed with caution. These results take no consideration of the timing of the culls with respect to the time of our surveys, nor do they take weather factors into account, which was also shown at Woodchester to affect mean sett activity scores.

A sample of ten main setts was surveyed for activity in the proactive areas of the RBCT one month after each culling operation. There was a marked overall decline in sett activity scores at main setts in proactive areas over the period of study (Table 1.4). The year of highest mean sett activity was 2002. Mean main sett activity declined to its lowest overall level in 2004 (excluding 2001 when we had data from triplet H only).

2. Faecal accumulation rate counts at sett latrines

In the first year, the mean number of active latrines per sett was 2.4, 2.7, 2.3 and 1.9 in autumn winter, spring and summer respectively, with a maximum of 6. The number of droppings deposited on any given night also varied, both between latrines and at the same latrines on different days. The mean number of deposits at each sett per day, or faecal accumulation rate (FAR), was 2.5 $\pm$ 2.6, with a range of 0 to 11. The mean FARs for all latrines combined in autumn, winter, spring and summer were 20.3, 20.7, 20.1 and 16.9 respectively. The seasonal differences in these values were not significant. Badger social group size (derived from MNA) was significantly correlated to faecal accumulation rate in autumn, winter and spring of Year 1 (Table 2.1.). The period over which latrine counts were carried out coincided with a substantial decline in badger numbers at Woodchester Park (ie from autumn 1999 to autumn 2002). This gave us an opportunity to explore whether the relationship between social group size, and FAR held over different densities at the same site. The estimated total number of badgers across all social groups studied at Woodchester Park, and the associated mean total FAR scores are given in Table 2.2. In the first season of study, the estimated number of badgers present across all groups studied was 87. By autumn 2002, the estimated MNA had declined to 48 individuals. Faecal accumulation rates declined also, although the pattern of change was not exactly consistent with that for badger numbers. For example, the highest faecal accumulation rate was in the second winter season, which had one of the lowest estimates of badger abundance.

A Generalised Linear Model was used to relate badger social group size and FAR. The final model is given as ***group size = exp(0.99 + 0.71 x [log(FAR+1]*)**. The noise inherent in the relationship meant that precision was limited. To illustrate this, Table 2.3 shows typical FAR values, and the group sizes they predict, with upper and lower 80% confidence intervals.

Faecal counts in RBCT areas

The FAR at each selected sett was estimated using the method described above. Average FAR for the ten setts ranged from 0.2 to 5.0 droppings/day in 2002. This was consistent with the range (0 – 7.2 droppings/day) observed at Woodchester. These FAR rates declined significantly in 2003, with a maximum FAR of only 2.0 droppings/day. Three setts had no active latrines present near them, and appeared inactive, and access permission to one further sett was withdrawn. The average number of droppings/day per sett was 2.6 in 2002. At Woodchester this value was 2.5. In 2003, mean FAR per active sett had declined to 0.9 droppings/day. Mean FAR across all setts monitored in 2002, including those where no active latrines remained, was 0.6 droppings/day. This is a 77% decline in FAR at the sample of D3 setts, before and after culling, implying that badger social group size at the setts monitored was considerably smaller on average after the two culls.

Table 2.4. shows the FAR's at each sett monitored and the group size that would be predicted by the relationship derived at Woodchester. The actual numbers of badgers removed from the setts in the subsequent cull are also given.

3. DNA recovery from faeces

Badger DNA was extracted from 47 (89%) of the 53 droppings deposited in the monitored latrines over the collection period. The analysis revealed that the collected faecal samples represented 20 different individuals. Comparison with the reference set of genotypes derived from the live-trapped badgers showed that 16 of these corresponded with known resident badgers (Table 3.1). A further four faecal samples produced genetic profiles that did not match any of those from trapped badgers, and a further 14 that had been trapped were not detected by faecal sampling (Table 3.1). Despite not being represented in the latrines, eight of these 14 were confirmed by video surveillance to have been resident during the faecal sampling period. The remaining six badgers that were trapped but did not use the latrines were not positively identified on video.

The sex ratio of those badgers that had visited latrines and whose identity was known from trapping records was 1:1 (8 males, 8 females), as it was for those badgers which had been trapped at the setts in question but had not deposited faeces at latrines (7 males, 7 females). Of those badgers for which age data were available (ie those which had been originally trapped as cubs) there was no significant difference in the mean ages of those that did and did not deposit faeces at latrines, and the latrines were used by badgers ranging from cubs of that year to a nine year old animal. Individual badgers deposited faeces at latrines on between one and six separate nights during the 10-day collection period.

A combination of trapping records and genotypes was considered to provide the most complete baseline estimate of group size (Table 3.1).

Rarefaction analysis of faecal genotypes produced estimates of group size for 'Parkmill' and 'Nettle' which were comparable with the baseline estimates (Table 3.1). Unfortunately, analysis of data from the 'Kennel' social group did not produce consistent results as 20% of the regression curves reached asymptotes with unrealistically high values, indicating that too few samples were collected. When the data for all three social groups were combined, the analysis again produced a median asymptote (Figure 3.1.) that was in close agreement with both the baseline trap-derived estimates of group size, and the estimates based on trapping and genotypes (Table 3.1).

Faecal DNA collection in RBCT areas

A total of 194 fresh faecal samples were collected and sent to Sheffield University Molecular Genetics Facility for analysis. They processed 102 of these samples. For reasons that remain unclear, consensus genotypes could only be achieved for 13 samples. This represented a success rate of only 13%. Of these 13 samples, 9 individuals were identified. Of these 9 individuals, 6 matched the genotypes of badger trapped by Defra. The success rate was too low to investigate patterns of latrine use, and to make any attempt to estimate badger numbers.

This illustrates very clearly that using faecal DNA in such studies is not straightforward, and appears to be extremely sensitive indeed to laboratory conditions and protocol. Different laboratory staff processed the samples from Woodchester and I2. The genotyping success rates were radically different, and

until the reasons for this are clarified, initiating a larger scale study using this method carries a significant risk of failure.

4. Spotlighting surveys using distance sampling
   Using mark-recapture calculations on the live-trapping data, the baseline estimate of badger abundance in the area where spotlighting took place was 68 (% coefficient of variation = 7.4). The total study area was 2.41 km², therefore estimated density was 28.2 badgers km^{-2}. This was used as the baseline against which to compare the population estimate from spotlighting.

   Using the spotlight data, badger density in open habitat was estimated to be 24.2 badgers km^{-2} (% Coefficient of Variation = 21.9). The area of open habitat available within the study area was 1.543 km^2 producing an abundance estimate of 37 badgers (95% CI = 24 to 58, Table 4.1). This estimate takes no account of the proportion of time spent by badgers in open land where the surveying took place. Examination of radio-tracking data indicated that on average the proportion of time badgers spent in open habitats was 0.485 $\pm$ 0.07. After carrying out this correction, the subsequent estimate of abundance for the study area was 77 badgers (95% CI = 45 to 131, Table 4.1).

Conclusions

1. Sett activity was broadly related to the number of sett residents, as would be expected. However, the appearance of activity at setts was also related to several factors independent of badger numbers. It was not possible to infer actual badger densities from sett activity surveys. However, the decline in sett activity values measured in certain trial areas over time suggested that marked changes in badger population size over large areas could be indexed using sett activity surveys. Of the methods tested, this is the easiest and cheapest to carry out. It requires no specialist equipment, and only one visit to each sett. Therefore we suggest that this method could be used either a) over large areas (RBCT areas or larger) where the population changes are likely to be significant (eg due to culling and then recolonisation), and where the management requirement is merely to provide evidence as to whether the population has changed, or b) as a secondary method to compliment abundance estimates or trends derived using a more reliable method.

2. Faecal accumulation rates at latrines near to setts were related to the number of badgers in the social groups. Confidence intervals around predictions of group size from FAR surveys were relatively wide, however the precision was sufficient such that the FAR index derived before and after culling in proactive area D3 of the RBCT indicated that a real decline in badger numbers had occurred. This method is based on estimates of social group size. Therefore, this approach should be used to monitor sites over time where information is held on the number and configuration of groups, or could be determined by baitmarking. This method has the advantage over sett activity surveys in that estimates of badger numbers can be inferred from the FAR index. However, it is considerably more labour intensive, as a minimum of 5 visits to each sett is required in order to derive the index. Therefore, the scale over which it can be applied is considerably smaller. One person working full time can realistically carry out counts at 6-10 groups in a day.

3. Unique genotypes derived from faecal samples were used to generate estimates of abundance at Woodchester that were close to the baseline estimate from live-trapping. This has potential to be a powerful, non-invasive method of assessing badger populations. However, this method is expensive in laboratory and field time. Also, when applied in an RBCT area, there was a very high sample failure rate. Until the reasons for this are clarified, application of this method in the field carried a significant risk of failure.

4. Spotlighting surveys of open land using distance sampling was used to estimate abundance at Woodchester Park. A correction factor was required to correct for the amount of time that badger spend in surveyable habitat. Radio-tracking data was used to provide this correction factor. The method produced an estimate of badger abundance close to the known population size from live-trapping. This method can form the basis of a reliable method to estimate and monitor badger populations.

Future research

The spotlighting methodology has been adopted in a large Defra-funded project to monitor the impact of any future culling policy on badger populations in the southwest. Further research on the patterns of habitat use by badgers would by extremely valuable in maximising the information that surveys such as these provide. Further research into optimising methods for studying badgers using DNA collected from the field would also be valuable. This includes both developing innovative methods of remote sample collection, and maximising the laboratory methodologies.

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Review comment

This project has fulfilled its aims well and was an essential piece of research that was needed to determine how badger population sizes could be evaluated. This work was undertaken in a rigorous and sound manner. However, there were some inconsistencies in the statistical analyses of the different methods used to estimate badger density. The methods on the use of remotely collected DNA and distance sampling are worthy of further investigation. The methodological choice of applying different methods in different circumstances to different areas is open to question. It would have been desirable to approach a comparison of this type on the performance of different methods relative to one another, and ideally relative to some 'gold standard'. Therefore, a design based more on using well known and unknown sites may have been more productive. Consequently, the performance of methods relative to one another is still uncertain. Disappointingly there was a lack of success in developing a standard cost-effective method capable of widespread application.

Scores

Conclusions based on sound evidence: 2.8
Quality of science: 2.8
Overall rating: 2.8

Table 1.1 Mean number of entrance holes per sett associated with different types of activity, and mean number of latrines and droppings.

	Spring (N=49)			Summer (N=55)			Autumn (N=33)			Winter (n=33)		
Variable	Mean	SD	Max	Mean	SD	Max	Mean	SD	Max	Mean	SD	max
Compact EH	3.5	3.2	12	2.4	2.5	10	2.6	2.5	8	3.2	2.9	12
Loose EHs	3.8	3.3	13	4.1	3.2	14	4.5	3.6	18	2.7	2.0	7
Obstruct EHs	2.1	2.7	13	1.7	2.1	10	3.1	3.3	13	2.2	2.8	9
Digging*	1.2	1.6	5	0.7	1.6	9	0.6	0.9	4	1.1	1.3	4
Bedding*	1.4	1.9	8	0.3	0.6	3	0.5	0.8	3	0.9	1.3	5
Footprint*	1.9	2.2	8	0.8	1.0	3	2.1	2.5	9	2.4	2.7	10
Polish	3.0	3.5	17	2.2	2.9	14	2.7	2.5	9	1.9	1.7	8
Dropping	0.4	0.9	3	0.6	1.4	6	1.2	2.9	16	1.1	2.6	14
Latrines	0.6	0.9	4	0.5	1.1	7	0.6	1.4	7	0.8	1.1	4

* significant variation by season (Kruskall-Wallis ANOVA, $p < 0.05$).

Table 1.2 Activity types and summary indices that had significant, positive associations with badgers trapped in spring and summer.

Spring (n=49)					Summer (n=55)				
Activity type	Slope	s.e.	r^2	p	Activity type	Slope	s.e.	r^2	p
compact EHs	0.12	0.04	0.15	0.006	compact EHs	0.21	0.05	0.26	<0.001
droppings	0.41	0.15	0.12	0.008	droppings	0.34	0.12	0.13	0.008
fresh digging	0.24	0.08	0.13	0.006	fresh digging	0.18	0.09	0.08	0.040
footprints	0.17	0.06	0.13	0.006	footprints	0.49	0.12	0.23	<0.001
-	-	-	-	-	polished EHs	0.13	0.04	0.14	0.005
activity index	0.07	0.03	0.13	0.007	activity index	0.14	0.03	0.14	<0.001
weighted activity index	0.02	0.01	0.15	0.004	weighted activity index	0.04	0.01	0.34	<0.001
badger-specific index	0.10	0.03	0.16	0.002	badger-specific index	0.23	0.06	0.20	<0.001

Table 1.3 Mean sett activity scores from random sett surveys in proactive and control areas.

TRIAL AREA	Treatment	Year 2002	2003	2004	Overall change (%)
E2	control	21.1	12.9	18.2	-13.9
E3	**proactive**	**15.2**	**6.2**	**6.6**	**-56.6**
G2	**proactive**	**10.2**	**4.8**	**6.8**	**-33.6**
G3	control	7.0	12.0	20.2	188.7
H2	**proactive**	**7.8**	**10.0**	**5.3**	**-32.3**
H3	control	9.9	8.5	5.1	-48.7
I2	**proactive**	**14.8**	**12.1**	**12.6**	**-14.7**
I3	control	21.3	17.9	13.6	-36.1

Table 1.4 Mean sett activity scores at main setts surveyed one month post-cull.

Triplet	Year 2000	*2001*	2002	2003	2004
A	-	-	17.4	9.1	13.2
B	22.9	-	19.7	8.5	7.3
C	-	-	0.0	-	9.7
D		-	40.0	13.7	9.1
E	4.7	-	63.3	21.3	12.3
F	8.9	-	11.4	-	12.8
G	13.6	-	14.2	4.4	9.0
H	4.0	*6.5*	18.0	13.3	2.0
I	-	-	14.6	5.1	4.0
J	-	-	13.5	8.6	8.0
Overall	**13.4**	***6.5***	**19.0**	**12.1**	**8.7**

Table 2.1 Pearson correlation coefficients and p-values for the relationship between and social group size and FAR in Year 1.

	Autumn	winter	spring	summer
Social group size (MNA)	0.80 (p = 0.003)	0.78 (p = 0.005)	0.72 (p = 0.010)	N.S.

Table 2.2 The estimated number of badgers present (MNA) across all groups studied, and the mean number of droppings deposited per day across all groups combined (FAR).

	MNA, all groups combined	FAR (droppings/day)
autumn 1	87	28.4
winter 1	72	33.1
spring 1	82	29.1
summer 1	82	22
winter 2	54	43
spring 2	58	17.8
summer 2	39	17
autumn 2	48	8.5

Table 2.3 Common FAR values, and the badger group size they predict from the model, and confidence intervals.

Typical FAR's	Predicted group size	lower 80% CI	upper 80% CI
1.0	4	2	7
3.0	7	4	11
5.0	10	6	14
7.0	12	7	17
9.0	14	9	19

Table 2.4 Faecal accumulation rates at main setts in D3 (2002), prior to the initial proactive cull, and after two culling operations (2003). Predicted badger group sizes, based on the preliminary work at Woodchester are given, as are the number of badgers removed.

Sett	FAR 2002	Predicted group size	lower 80% CI	upper 80% CI	Badgers taken, culling	FAR 2003	Predicted group size	lower 80% CI	upper 80% CI
1	2.6	7	4	10	1	1.1	4	2	7
2	3.9	8	5	12	2	0	0	0	0
3	5.0	9	6	14	9	1.0	4	4	2
4	1.2	5	2	8	2	2.0	6	3	9
5	2.2	6	3	9	0	0.2	3	1	5
6	1.9	6	3	9	4	0	0	0	0
			3	9		no	-	-	-
7	1.7	5			0	access			
8	5.6	10	6	15	3	0.4	3	1	6
9	0.2	3	1	5	no trap	0	0	0	0
10	1.6	5	2	8	no trap	0	0	0	0
Total		64	35	99	21		20	11	29

Table 3.1 The number of badgers live-trapped in 2001 and/or identified as having used the monitored latrines by analysis of faecal DNA. Estimates of group size from faecal samples are presented showing the median asymptote value from 1000 rarefaction curves (where the sample order was randomised each time) and the inter-quartile range.

Group	trapped and visited latrine (a)	trapped but did not visit latrine (b)	visited latrine but not trapped (c)	Totals			
				faecal genotypes a+c	trapped badgers a+b	faecal genotypes and trapped badgers (baseline estimate) a+b+c	rarefaction curve estimate (inter-quartile range)
Parkmill	7	6	3	10	13	16	**15 (13-18)**
Nettle	4	5	1	5	9	10	**9 (8-10)**
Kennel	5	3	0	5	8	8	**-**
All	16	*14	4	20	30	34	**36 (32-42)**

Fig 3.1 A rarefaction (non-linear regression) curve showing the median asymptote value for the three badger social groups combined, from a thousand iterations of the regression, with the sample order randomized each time. Extrapolation to the asymptote indicates an estimated population size of 36 individuals.

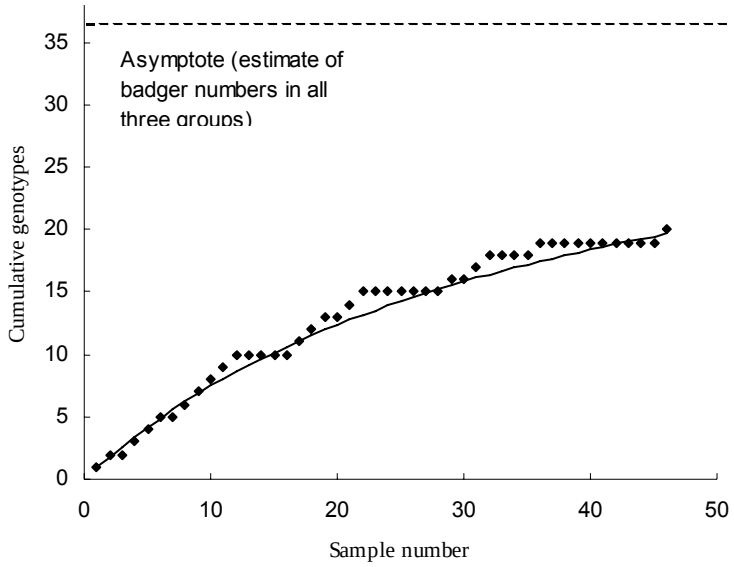

Table 4.1 Comparison of mark-recapture and three DISTANCE derived abundance estimates of badgers (*Meles meles*) in the survey area. DISTANCE estimates are given for analyses with and without the use of a correction factor (multiplier) for habitat use.

	Badger abundance (n)	% Coefficient of variation (%C.V.)
Mark-recapture estimate of badger abundance in survey area.	68	7.4
Distance sampling estimate of badger abundance without multiplier	37	21.9
Distance sampling estimate of badger abundance with multiplier (autumn mean)	76	42.2
Distance sampling estimate of badger abundance with multiplier (annual mean)	77	25.9

SE3035 Estimating badger density in Randomised Badger Control Trial proactive and control areas

Organisation Central Science Laboratory
York

Start date 01/05/05
End date 31/3/07

Total cost £306,692

Abstract
It is important to understand the effect that badger culling operations has on the population size of badgers in proactive Randomised Badger Culling Trial (RBCT) areas in order to fully interpret the results of the trial. This has not been possible to date. This project aims to estimate the difference in relative densities between the control and proactive treatments of five RBCT areas.

Aims
- Estimate relative badger densities in proactive and control treatment areas of selected triplet areas of the RBCT, immediately post-culling.
- Compare relative densities between matched proactive and control areas to provide a quantitative assessment of the effect of culling operations on badger numbers.
- Re-survey the same areas after 1 year to estimate the rates of badger recolonisation.

Relevance to Defra
Understanding the effect of the culling treatment on badger populations in trial areas is important when interpreting and generalising the results of the RBCT. Before the start of this project, no information existed on the size of the badger populations in the different trial areas. In proactive areas, data from trapping operations provides some information on the size of those badger populations. However, factors such as non-compliance land and seasonal variation in badger trappability means that an unknown proportion of the population remains un-trapped after culling. This is an unfortunate lack of information, as ideally the interpretation of the overall trial results on cattle TB should be made in the context of the effectiveness of the treatment in each area. In control areas, there is no information available on badger numbers, other than sett survey data. This project will provide Defra with independent estimates of the impact of culling on the badger populations in proactive RBCT areas.

Methods
Five control and proactive RBCT pairings were selected on the basis of suitability for spotlighting from roads, and which could be carried out around the RBCT timetable. Survey transects were set up in each area. This involved considerable reconnaissance effort to select suitable road stretches for spotlighting, measuring transects, and carrying out a PR exercise to inform local residents. Immediately

prior to each survey, leaflets describing the work and detailing the survey dates were distributed to all houses close to the spotlighting transects.

The surveys employed the technique of Distance sampling (Buckland *et al* 1993), where the perpendicular distances to observations from transects are used to generate density estimates. This method has been used to estimate densities of medium sized mammals such as badgers and foxes in a number of previous studies (Hounsome *et al* 2005; Heydon *et al* 2000; Ruette *et al* 2003) The surveys were carried out concurrently in the control and proactive areas of each of five RBCT Triplets, immediately post-culling. Surveying was carried out on as many nights as possible, before moving on to the next area culled by Defra. Around 20 transects sections with an average total distance of 30km were set up in each area. It was usually possible to survey around half the transects in any one night. Therefore each transect was repeated around 7 times in the course of the surveys in each area. Surveying was carried out by spotlight by a surveyor seated on the roof of Land Rovers modified to have a seat/roll cage assemblage on the roof. Each time a badger was encountered, the vehicle was stopped and the surveyor measured the angle and distance to the observation. These data were used to calculate the perpendicular distance from the animal to the transect.

The distance data were used to estimate relative badger densities in each area using DISTANCE 5.0 (Thomas *et al* 2005). The statistical significance of the differences between the proactive and control areas of each pair was estimated using bootstrapping. This involves iteratively calculating 1000 density estimates for each of the two areas, and subtracting the proactive value from the control value to give 1000 estimates of the difference between them. These are then ordered by magnitude, and the 25^{th} and 975^{th} percentiles identified to give 95% confidence intervals around the estimate of difference. Where the upper and lower CI's are both positive, ie do not overlap with zero, this is statistical evidence to suggest that the difference in the population estimates is real, and not merely a result of noise in the data.

It is only possible to survey open land, therefore the resulting estimates of density are for open land only. As a result, our estimates of density are underestimates, by a factor determined by the proportion of time spent by badgers in open land. Hence if badgers spend only 50% of their time in this habitat, then the density estimates are 50% of the absolute density. So the absolute density of badgers in a trial areas equals:

$$\frac{\textbf{Our survey density estimate}}{\textbf{Proportion of time badgers spend in open land}}$$

However, if we can assume that this proportion does not differ markedly between control and proactive areas of any given triplet, then the difference in relative density between the control and proactive areas of a triplet will be proportionally the same as the difference in absolute abundance. This should be a safe assumption since the triplets were originally selected as matched experimental and control areas. Badger main sett densities from the original Defra surveys were also calculated for each study area in an attempt to assess whether the pre-RBCT badger densities were

markedly different between treatments in any area. Main sett density has been used in the past as a surrogate of badger social group. (Wilson *et al* 1997).
The surveys were carried out from June to November.

Results
On average, 160km of transect were surveyed in each study area, over a period of 3 weeks each. A total of 300 observations of badgers were made, 227 in proactive areas and 73 in proactive areas.

Relative density estimates and 95% confidence intervals around the differences between proactive and control areas are given in Table 1. In all five study Triplets, estimated density was lower in the proactive culling treatment areas. The largest difference in was in Triplet E, where the density in the proactive area was 93% lower than in the control area. The next greatest difference was in Triplet D, where the estimated density in the proactive area was 86% lower than the proactive area. In Triplets I and G, the difference was much less marked, being 28% and 26% respectively. In Triplet B only 8 observations of badgers were made in the control area, and only 1 in the proactive area. These values were too low to produce density estimates. This was the last Triplet surveyed, in November. Badger activity is known to decline markedly in the winter months (Neal & Cheeseman, 1996) and it is likely that this is the main reason that so few badgers were seen. This indicates that this technique is unsuitable when applied this late in the year.

The 95% confidence intervals around the estimated difference in densities between proactive and control areas are given in Table 1. The intervals around the estimate of difference in Triplets E and D do not overlap with zero, and are therefore can be considered statistically significantly different. In areas I and G, intervals include zero, therefore we can have less confidence that these differences are real, and not artefacts of noisy data.

Sett densities in these areas prior to the start of the RBCT are given in Table 2. In Triplet I, the main sett density in the control area was half of that in the proactive area. This may indicate that prior to the RBCT, there was a larger population of badgers in the proactive area. This may partly explain why the estimated badger density in the proactive area was only 28% less than in the control area immediately post-culling, which was not statistically significant. In other words, if the badger density in the proactive area was higher at the outset, then the true population reduction would be 28% plus the initial difference between the two areas. However, in Triplet D, the density of main setts was again considerably higher in the proactive area pre-RBCT. But despite this, in contrast with Triplet I, our estimated density post-culling was much lower (86%) in the proactive area.

Conclusions
The estimates of relative badger density in proactive treatments were lower than those in control treatments in all five study pairs. This would be expected given that the surveys were carried out immediately after culling in proactive areas. However, the magnitude of the difference in density between the control and proactive areas varied between pairs. The mean difference in density between the proactive and control areas of the four study pairs where there sufficient observations to estimate density was 58%, but this ranged from 26% to 93%. These values definitively

represent the relative differences in badger density between the control and proactive areas, immediately after the final cull. However, they could only be said to reflect culling effectiveness if badger population sizes were roughly equal in the control and proactive areas at the start. Unfortunately we do not have the pre-RBCT density estimates that would be required to draw such a conclusion. To illustrate this, if the badger population was larger in the proactive areas than in the control areas before the RBCT, then the differences estimated from this survey would be underestimates of the extent of badger population reduction. The opposite would be true if the density in proactive areas were lower on average. In theory, badger populations in control and proactive areas should be similar.

The surveys will be repeated after an interval of exactly one year, which will allow an assessment of the rates of recolonisation between culls.

Future research
The surveys will be repeated in 2006, to assess recolonisation rates in the proactive areas.

A wide-scale study on the patterns of habitat use by badgers using radiotracking would enhance the value these surveys. This would provide information on the proportion of time spent by badgers in open habitats in different landscape types. This information in turn would provide correction factors that would translate these relative density estimates into estimates of true abundance.

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Review comment
The project is generating data that is essential in the interpretation of results from the RBCT. It is clearly important to know to what extent proactive culling has succeeded in reducing the density of the target badger populations, and it is important to understand the rate of re-colonisation of culled areas once culling has ceased.

However, the type of distance sampling used may not yield data that are sufficiently precise to be of much value. The accuracy is low when badger density is low and between year comparisons can be misleading since weather conditions may vary markedly between years and affect badger behaviour. This 'noise' may significantly mask pre and post cull comparisons of population, and speed of re-colonisation. The incomparability of the five triplets was acknowledged in the present study. However, pairs are compared. It is taken on 'trust' that the pairs are comparable in landscape terms, which is questionable. The value of this project would be considerably enhanced by more long-term work aimed at quantifying re-colonisation rates in relation to cull efficiency and site characteristics. Absence of pre-RBCT density data significantly affected the ability of the researchers to interpret and explain differences in population data between the sites.

Scores
Conclusions based on sound evidence: 2.6
Quality of science: 2.7
Overall rating: 2.5

Table 1 Estimated relative density of badgers in control and proactive areas of Triplets D,G,I & E. Percent difference between proactive and control areas are given in italics. The 95% confidence intervals around the estimated difference in density between proactive and control areas are given.

Triplet	Control Density	Proactive Density *(% less than control density)*	95% confidence intervals around difference	
			lower	upper
D	1.64	0.23 (***86***)	0.20	3.20
G	1.00	0.74 (***26***)	-0.54	1.04
I	0.90	0.65 (***28***)	-0.56	1.04
E	5.00	0.35 (***93***)	2.42	7.29

In **Triplet B**, only 8 badgers were seen in the control area and 1 in the proactive area, too few to make relative density estimates.

Table 2 Main sett densities in the study areas, from the Defra survey database.

Triplet	Main Sett Density, setts.km^{-2}	
	Control	Proactive
D	0.61	0.93
G	0.71	0.78
I	0.39	0.81
E	0.55	0.59
B	*0.30*	*0.45*

SE3108 A field study to reveal the effects of perturbation, and to model the epidemiology of TB in disturbed badger populations

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Abstract
Within the UK, attempts to control bovine tuberculosis (bTB) in cattle by culling a wildlife host, the Eurasian badger (*Meles meles*), have proved largely ineffective. Culling-induced social perturbation of badger populations and its consequent epidemiological effects may have contributed to this failure.

Here, we present a study examining the impact of culling on badger population demography, social organization, dispersal and bTB epidemiology. Two comparative study areas were established in a reactive treatment and a survey-only area within the UK government's randomised badger culling trial (RBCT).

Approximately 35-44% of badgers in targeted social groups were culled. Amongst the survivors , a greater proportion of females showed signs of breeding following culling, though cub cohort sizes were unchanged. Overlap between social groups and aggression increased in targeted groups and their neighbours. Following culling bTB prevalence increased in groups neighbouring removals, particularly among cubs.

Animals from removed groups travelled greater distances following culling. Dispersal between social groups was rare in these populations, but increased significantly following culling, in removed groups and their neighbours. Increases in bTB prevalence in groups were associated with movements both into and out of these groups. The social disruption caused by culling may increase stress within the surviving population, causing immunosuppression and thereby enhancing the expression of disease.

Social group overlap was high in a region of the survey-only area, associated with increased aggression and bTB prevalence. Reasons for this remain unclear, although the possibility of illegal badger culling cannot be ruled out.

These findings adds weight to the argument that perturbation should be considered when formulating policy regarding the control of wildlife diseases and, specifically,

illustrates a mechanism whereby culling badgers may be an ineffective approach to controlling bovine bTB in cattle.

Aims

- To investigate and compare the socio-spatial behaviour, population dynamics and bTB epidemiology in comparative study areas.
- To investigate whether the spatial and temporal scale of the perturbation processes, the pattern and speed of recolonisation and the relief of density-dependent inhibitions on reproduction and survival observed at North Nibley may be generalized.
- To investigate how characteristics associated with the badger culling operations (eg number of social groups removed, partial or complete removal of social groups) and the local badger population (eg population density, history of previous BROs in the area) may influence the nature, severity and populational and epidemiological consequences of perturbation caused by these culling operations.
- To evaluate the practicalities and logistical difficulties of badger culling operations in the field and to help identify crucial characteristics of a successful (reactive) badger culling strategy.
- To develop a new spatial and stochastic model which integrates new findings on the effect of perturbation on the outcome of different control strategies.
- To integrate the results of objectives 1 to 5, to interpret the outcome and validity of the culling trial and to advise Defra about future policy.

Relevance to Defra

Localised culling of badgers for the control of bTB has been unsuccessful (Macdonald *et al* in press). Culling has a profound impact on badger ecology and behaviour. This in turn affects the way in which bTB may spread and the risk to cattle. The contributions made to policy by this project arise by addressing stated Defra scientific objectives (ROAME A 2003-2005):

A.3.2 Control strategies to reduce the incidence of TB in cattle

E. Assessment of the effects of various TB control strategies on badger populations and subsequent disease spread to cattle;

A.3.4 Assessing wildlife populations and the prevalence of M. bovis

B. Develop innovative methods to relate badger abundance and effects of disturbance on badger populations; and

A.3.5 The pathogenesis, transmission and modelling of tuberculosis in cattle and wildlife species

A. Integrate modelling approaches to better understand disease transmission over wide spatial scales and to assess the costs and benefits of different control strategies.

Methods

Study areas were located on the borders of Wiltshire, Somerset and South Gloucestershire in South-West England. The treatment badger population was identified within the reactive culling area of the RBCT triplet E (51° 27'N 2° 25'W), occupying an area of 37.3 km^2. The comparative control population, which was not subject to culling by Defra, occupied an 18.9 km^2 area within the survey only area of triplet E (51° 28'N 2° 03'W). Both areas contained mixed farming, with arable and livestock production, the latter being principally dairy cattle. The agricultural landscapes of both areas created mosaics of woodland, pasture and arable fields.

Both treatment and control study areas were initially surveyed for badger setts in autumn 2000. Landowners also provided further knowledge of setts. Setts were categorised as either active or inactive based on the freshness of badger sign (mainly footprints, faeces and spoil). In total, 71 and 38 active setts were identified in the treatment and control study areas respectively, giving a density of approximately two setts km^{-2} in both areas.

Culling Operations

Within the treatment study area, Defra performed three badger culling operations in November 2002 (removing 38 badgers from 14 social groups), January 2003 (removing six badgers from four social groups) and August 2003 (removing 33 badgers from 13 social groups).

Badger Live Capture and Collection of Clinical Samples

Badgers were captured in cage traps, placed at or near to setts and baited with peanuts. Trapping was carried out between October 2000 and August 2003. In 2002 and 2003 both areas were trapped in winter (January), late spring (May/June), summer (August/September) and autumn (October /November). Between February and September 2001 trapping was mandatorily suspended following the outbreak of foot and mouth disease (FMD) within the UK.

Captured badgers were anaesthetised and each individual was given a unique identifying tattoo in the inguinal region on first capture. Samples of sputum, urine, faeces and pus from wounds or open abscesses were cultured to detect the presence of *Mycobacterium bovis*.

Social Group Ranges

The delineation of badger social group ranges was determined using bait-marking. Marked bait was produced by mixing small, inedible plastic chips with golden syrup and peanuts. Bait containing different coloured plastic chips was fed to each social group in both the treatment and control study areas for a 10-day period during the spring. This was followed by a simultaneous survey of badger latrines in both study areas. The locations of latrines containing markers were recorded and used to define group ranges as minimum convex polygons (MCP) connecting the outer-most latrines for each social group.

Ranging Behaviour of Individuals

The home ranges of individual badgers were determined by nighttime radio-tracking of adult badgers. Daytime positions of badgers in setts were determined by detecting underground radio signals.

Data Analysis

Badger social groups were classified according to their proximity to setts at which culling was undertaken. Within the treatment study area social groups were classified as Removed (R) if they were the target of culling, Neighbouring (N), if they were immediately adjacent to Removed groups, or Other (O), if they were adjacent to Neighbouring groups or beyond. All groups within the control area were classified as Control (C).

Badger abundance was estimated using mark-recapture analysis of trapping records using the Jolly-Seber method. Badger densities were calculated based on the areas of social group territories defined from bait marking surveys during the year in which each trapping event occurred. The productivity of social groups was calculated as the estimated number of cubs born per reproductively active female within each group. Females were recorded as reproductively active if, when examined, they showed signs of current or recent lactation.

Individual home ranges were defined as 95% minimum convex polygons (MCP) and core areas within badger home ranges were identified using 50% kernels. Radio-tracking data were partitioned into spring (Mar, Apr, May), summer (Jun, Jul, Aug), autumn (Sep, Oct, Nov), and winter (Dec, Jan, Feb) seasons. Radio-tracking data were collected from Nov 2001 to Dec 2003, but suspended during culling.

We used a definition of dispersal, using both trapping and radio-tracking data, termed 'inter-group movements' (IGM). An IGM was defined as occurring when a badger was either captured at a sett belonging to a different group than that at which it was captured previously, or when it was located at a different sett by night-time radio-tracking or daytime positioning.

The prevalence of bTB within the badger populations was calculated as the proportion of captured animals having positive culture tests from clinical samples. The prevalence of infection in each study population was estimated from all captured individuals at each trapping period. Social group prevalence was the proportion of bTB positive badgers captured from each social group at each trapping event.

Given the timing of culling with respect to data collection in this study, the culls in November 2002 and January 2003 were considered as a single winter 02/03 culling event, since we could collect no data between these culls. Given the seasonality of data collection, social group ranges, female productivity, home range, dispersal and bTB prevalence were calculated for the periods before and after this culling event, and differences compared. Changes in bite wounding frequency recorded at trapping events could potentially occur rapidly following culling and were thus analysed with respect to the two main culling events (winter 02/03 and Aug 03). Trapping events were defined as Pre-Cull (before the winter 02/03 cull), Inter-Cull (between the winter 02/03 and Aug 03 culls) and Post-Cull (after the Aug 03 cull).

Modelling

The model will operate anywhere in the UK for which habitat data are available. The region modelled can be of practically any size, although simulations presented here were conducted on a 8 km x 8 km square. The easting and northing of any location

for which GIS information if available can be input by the user. These co-ordinates represent the centre of a square in which the simulation will take place; and the width/height of the square must also be provided. The model then extracts all the habitat in this square which is usable by badgers into a separate map for simulation purposes. Parameters relating to badger demography and life-history, TB prevalence and epidemiology and simulated BRO characteristics can be manipulated to allow comparisons between BRO strategies (reactive/proactive) in an area.

The model is individual-based with the age, TB status and sex of each badger in each social group as the state variables. The model interrogates each badger at six-month time steps to determine stochastically the life history of the individual. Life history 'decisions' are made using a probabilistic approach, each individual having a particular chance that it will become infected or pass into the next stage of the disease (depending on TB status), change social groups and die (both based on age and sex), and, if female, breed.

The first 6-month season each year is the spring/summer season, and includes subroutines governing reproduction, TB transmission, TB-induced mortality, natural mortality and movement (Smith *et al* 1995, Smith *et al* 1997). The second season, representing autumn / winter, includes all these subroutines except for reproduction, which in badgers only occurs once a year. The life history and disease transmission parameters operated on a six month time-step.

Results

Over the course of the study, a total of 663 badgers were trapped in the treatment and control areas. Estimated total badger density ranged between 1.2 and 6.9 badgers per km^2 in the treatment population and between 3.9 and 7.1 badgers per km^2 in the control population (Figure 1). The earlier culls in November 2002 and January 2003, which removed 38 and six badgers respectively, did not appear substantially to alter badger density within the treatment population due to the birth of cubs in the following spring. Prior to the August 2003 cull, badger densities in treatment and control populations were similar, following the same seasonal trend. After the August 2003 cull, which removed 33 animals, the population density in the treatment area fell to its lowest level of 1.2 badgers per km^2, compared with approximately 5 badgers per km^2 for the same season in the previous year and approximately 6 badgers per km^2 in the control.

Given the estimated social group sizes, cull trapping efficiency in the treatment area, measured as the proportion of animals within each targeted group that were culled, was 35.1% in winter 02/03 and 44.4% in August 2003. This was unrelated to the size of social groups before trapping.

Overall social group productivity was lower following culling, with the greatest changes being a decline in Removed groups and an increase in Neighbouring groups. However, in Removed groups, fewer females were reproductively active in the breeding season before culling (3/14), compared with after (11/19) ($z = 2.09$; $p = 0.036$). This suggests that incomplete social group removal through culling has altered the breeding dynamics of females within these groups, possibly affecting female dominance and the potential for breeding suppression (Woodroffe & Macdonald 1995, Tuyttens *et al* 2000c).

Social Group Ranges

Range boundaries of badger social groups before culling were delineated from spring bait marking for 53 groups in the treatment area and 27 groups in the control area. After culling ranges were delineated for 55 groups in the treatment and 24 in the control areas (Figure 2). Following culling there was no significant change in range sizes in any group type with the treatment or control area. Social group range size did however vary significantly between group types, with Removed and Neighbouring groups having larger ranges than Other and Control groups. The estimated number of animals within social groups did not influence group range size either before or after culling.

Proportional overlap between social group territories of each group type changed following culling, with both Removed and Neighbouring groups increasing their overlap, whilst there was a decrease for Other and Control groups (Figure 3a). The number of surrounding groups with which each social group overlapped also changed following culling, with increases for both Removed and Neighbouring groups and decreases for Other and Control groups (Figure 3b), with the greatest relative increase being for Removed groups. Following culling, increases in proportional and numerical overlap occurred principally between Neighbour-Neighbour, Neighbour-Removed, and Removed-Removed groups, with only slight increases in Neighbour-Other overlap. Within the Control study area, a region of high range overlap was identified in spring 2002, associated with one particular dairy farm that had suffered bTB breakdowns. In the following year we were denied access to this farm and so could not conduct bait-marking, although we were able to trap at some of the setts. The reasons for this apparent social disruption within the badger population are unclear, and we cannot rule out illegal culling. It has been suggested (Godfray *et al* 2004) that illegal culling may have also taken place in other RBCT control areas, though this has not been supported in recent analyses (Woodroffe *et al* 2005).

Aggression

The proportion of badgers receiving fresh bite wounds in the treatment and control populations changed following culling, with significant increases in Neighbouring and Removed social groups following the Winter 02/03 cull and an increase in the control population in the post-cull period (Figure 4). The high level of wounding in the Control population was associated with the area of extensive social group overlap identified from bait marking. Within the treatment population, frequency of wounding remained at an elevated level in Neighbouring and Removed groups during the inter-cull and post-cull periods and decreased in Other groups, though remaining at similar levels to N and R groups. Bite wounding was significantly more likely in males, particularly cubs irrespective of timing with respect to culling, season and group type. We did not find evidence for an association between bite wounding and bTB infection.

TB Prevalence and Distribution

There was a significant increase in infection among cubs in the treatment population following culling, with one infected individual identified out of 101 cubs captured before culling, and eight out of 76 cubs (10.5%) testing positive for bTB after culling ($z = 2.86$; $p = 0.004$). Of these eight infected cubs, four tested positive for *M. bovis* from urine samples, three from sputum and one from a faecal sample. No infected

cubs were found among 47 tested in the control population. There was no overall difference between the detected prevalence of bTB in badgers in treatment and control sites, although prevalence was found to have increased significantly in both sites following culling.

Following the 2002 culling operations, a cluster of infection arose in the west of the study area (Figure 5). The majority of infected cubs were found in this cluster; however the cases in the north and east also included cubs. Infection within the control population became more widespread following culling in the treatment area, with numbers of infected individuals increasing from two to nine. This rise occurred within the area of social disruption identified from bait marking in spring 2002, being associated with a farm that had suffered a bTB breakdown in 2002.

Average prevalence rates of bTB within social groups increased significantly in Neighbouring groups following culling (Figure 6), and varied depending on social group type. Prevalence in Removed and Other groups declined following culling, whilst average prevalence remained unchanged in the control groups.

Individual Movements

Totals of 50 (32 male and 18 female) and 20 (9 male and 11 female) adult badgers were fitted with radio-collars in the treatment and control study areas, respectively. Six instrumented individuals were found to be infected with bTB by the culture of clinical samples; four males and one female in the treatment and one female in the control population. A total of 8,311 fixes were collected from tracked individuals over 239 nights.

The areas of home ranges (95% MCP) used by individual badgers in both sites showed seasonal differences between 2002 and 2003, with the largest home ranges occurring in summer in the treatment area and in autumn in the control area. No overall differences in home range size were found between the sexes, with average home ranges being 14.65 and 14.42 ha for females and males respectively. Seasonal home range areas were relatively consistent between Removed, Neighbouring and Other groups in 2002, with greater variation being apparent in 2003 (Figure 7). No difference was identified in the home range areas of infected and uninfected animals.

Nightly travel distances of badgers per unit time of observation varied seasonally in the treatment population and also between social group types. Travel distances peaked in the summer, and badgers from Removed and Other groups moved greater distances compared with Neighbouring groups. Differences between social group types were consistent across seasons, with badgers in Removed and Other groups increasing their movements relative to the same season prior to culling. No such changes in behaviour were observed amongst Control badgers.

Proportional overlap between summer home ranges of badgers from different social groups varied significantly with respect to culling for each group type, with significant increases in inter-range overlap for Neighbouring and Other groups following culling (Figure 8). Proportional overlap between home ranges in control groups did not change significantly. The number of home ranges from different groups with which

each badger overlapped in summer also increased in Neighbouring and Other groups following culling, from an average of 0.8 to 2.7 (Figure 9).

Daytime Positioning
Daytime positioning revealed inter-group movements (IGM) by radio-collared badgers on two occasions, out of 891 animals positioned underground between 2001 and 2003. Both of these were in the treatment population and were made by males to groups immediately neighbouring their original groups. One of these moves was made during the culling operation in November 2002 and the other six months after culling in July 2003.

Inter-group Movement (IGM)
Out of a total of 663 trapping events in the treatment and control study areas, only 12 instances of badgers moving between social groups (IGMs) were detected (11 individuals). Of these, nine (75%) occurred in the treatment population following the culls in winter 02/03, and one in the control study area. The *per capita* rate of IGM from the trapping data rose following the winter 02/03 culls, from 0.56% (2/358 events) to 3.21% (6/187 events), and remained elevated following the August 2003 cull at 3.39% (4/118 events).

A total of 14 IGM events were identified by combining those detected from trapping and daytime positioning data. Badgers were more likely to move to Removed groups, with either from Neighbouring or different Removed groups. IGM probability was influenced by the size of the donor social group with animals being more likely to move from large groups, but was not affected by an individual's sex or age, although nine of the movers were adults and only four were cubs.

Overall there was a greater increase in the prevalence of bTB within Neighbouring social groups, associated with both IGM donors (Figure 10a) and recipients (Figure 10b), although neither relationship was statistically significant at the 5% α-level ($F_{5,44}$ = 2.30, p = 0.061 and $F_{6,43}$ = 1.97, p = 0.092 respectively). Bovine TB infection was not detected in any of the individuals identified as having made IGMs, however the intermittent nature of bTB detectibility by culture means we cannot rule out the possibility that IGM individuals were infectious at a later stage within their receiver group. Bovine TB incidence has also found to be affected by movements within the Woodchester Park badger population (Rogers *et al* 1998).

<u>Modelling</u>
The major outputs of the model are the demography of badger populations, the epidemiology of TB and the spatial incidence of TB-infected groups over time. The following results apply to a reactive BRO following a herd-breakdown in year 11 of a 20-year simulation. The cull was assumed to be very efficient in this example scenario, with 90% of badgers whose group territories overlapped with the reactor land being killed.

The effect that this BRO had on the population dynamics of the modelled badger population is shown below (Figure 11). The population is relatively stable in numbers, with births balancing deaths. The perturbation of the population caused by the BRO can be seen in year 11, whereupon the population stabilises around a new carrying capacity.

Following culling in year 11, there is a clear decrease in the number of infected groups as a result of a 90% chance of killing each badger in each of the 5 groups affected in this example BRO (Figure 12).

The total number of newly infected badgers in a year dramatically increases immediately following the BRO (Figure 13), and it is clear that these are mainly between-group infections. The removal of badgers from a social group increases movement of individuals in nearby groups and thereby increases the overall number of new infections per year. It should be noted that this is s process-based model and does not define "perturbation" explicitly. The results obtained arise from a simple set of parameter values describing badger demography and movement (Shirley *et al* 2003).

Evaluation of the model using sensitivity analysis revealed that the simulated badger population size after 20 years was most dependent on five parameters affecting female recruitment (probability of breeding, mortality of adult females in the first half of the year, mortality of juvenile females in the second half of the year and mortality of female cubs in the both halves of the year). The simulated prevalence of TB was most affected by the population size, the rate at which infectious badgers transmit the disease to other members of their social group, and the rate at which the disease is spread outside of the social group.

Conclusions

Reactive culling gave rise to social perturbation within the treatment study area, with increased overlap between social groups and individuals, higher dispersal rates and changes in female breeding. Bovine TB prevalence increased in relation to this social disruption. Within the treatment population the emergence of bTB within Neighbouring social groups following culling and the lack of clear evidence relating bTB incidence to IGMs, suggests that other factors may be important, such as immunosuppression as a result of social stress. Increased bTB prevalence and aggression within the control population was also associated with an area of social disruption in the badger population. The reasons for this disruption are unclear, and illegal interference cannot be ruled out.

The recently published results of treatment differences in the RBCT (Woodroffe *et al* 2005) are compatible with the perturbation hypothesis, and our findings, demonstrate these effects at the levels of social groups and individuals. We anticipate that the additional evidence provided here of the impact of badger culling will aid disease control policy development, as well as adding to our knowledge about badger behaviour and ecology, and the epidemiology of bTB.

Future research

The intensity of culling within the treatment population observed in the present study was lower than reported from other populations (eg Cheeseman *et al* 1993; Tuyttens *et al* 2000a). Although generalisations can be drawn, more detail is required about the impact of different culling intensities and efficiencies. Furthermore, the effects of the scale over which culling takes place are poorly understood.

The apparent independence of post-culling bTB infection to bite wounding, and the emergence of bTB infection in cubs suggest that the disturbance itself may give rise to new infections. For example, post-culling disturbance could potentially cause social stress and induce immunosuppression (Barnard *et al* 1993; Mian *et al* 2005), resulting in the reactivation of latent disease (Gallagher & Clifton-Hadley 2000), or susceptibility to new infection from bacilli surviving within badger setts (Moore & Roper 2003). In such circumstances, cubs may provide a useful barometer for the potential role of social stress in bTB infection, since their immune systems are immature and they consequently have lower resistance to disease (Gallagher & Clifton-Hadley 2000). Currently available techniques for monitoring stress in wild animals (Montes *et al* 2004) would allow us to measure more precisely the impact of social perturbation at different scales of culling, and to evaluate the associated welfare costs.

The functioning of social dominance within undisturbed badger society (far less how it is affected by perturbation) remains a puzzle (Macdonald *et al* 2002), but the importance of female demography for the sensitivity of bTB models has been highlighted previously (Shirley *et al* 2003). Furthermore, the emergence of bTB in cubs highlights the potential need to understand pseudovertical mother-cub transmission in the presence of perturbation and more generally.

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Publications generated from the project

Riordan, P., Macdonald, D.W., Delahay, R.J., Cheeseman, C.L., Service, K., Fordham, E., & Harmsen, B.J. (submitted) The impact of culling on Eurasian badger (*Meles meles*) populations and the epidemiology of bovine tuberculosis: 1. demography and social organisation. *J. Appl. Ecol*.

Riordan, P., Macdonald, D.W., Delahay, R.J., Cheeseman, C.L., Service, K., Fordham, E., & Harmsen, B.J. (submitted). The impact of culling on Eurasian badger (*Meles meles*) populations and the epidemiology of bovine tuberculosis: 2. Individual movement and dispersal. *J. Appl. Ecol.*

Review comment

This is an important project, which utilised the RBCT experiment and north Nibley areas for research. The analysis and effort are excellent. However, the overall running of the project seemed not just exploratory but chaotic and a better-managed study might have got far more out of the situation. The lead contractor had to be changed to CSL for the final part. The project has proved important in influencing

future TB policy concerning badger culling. The results from north Nibley are fascinating, and provide sound evidence that in the reactive culling area, social perturbation led to increased overlap of social groups, increased incidence of TB, increased biting, increased number of females breeding within a group and higher incidence of disease within female cubs. The project highlighted the fact that the intensity of culling also played a role. The conclusions are limited due to the lack of replication of treatment and controls, therefore, all comparisons are confounded by area affects. Low culling rate of the RBCT area (35-40%) suggests a different situation may have arisen regarding perturbation here. Additional studies are required to provide results that are directly applicable in other habitats.

Scores
Conclusions based on sound evidence: 2.8
Quality of science: 2.7
Overall rating: 2.7

Fig 1 Badger population density in treatment and control areas between October 2001 and August 2003. Error bars show 95% confidence intervals. Arrows indicate the timing of Defra badger culling operations within the treatment area.

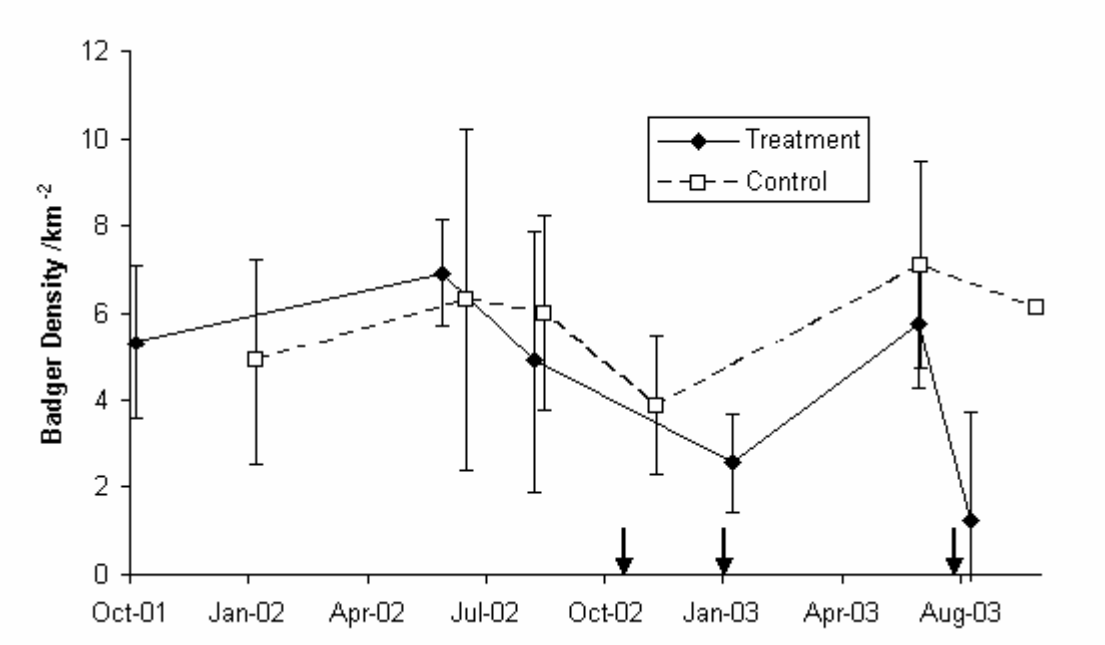
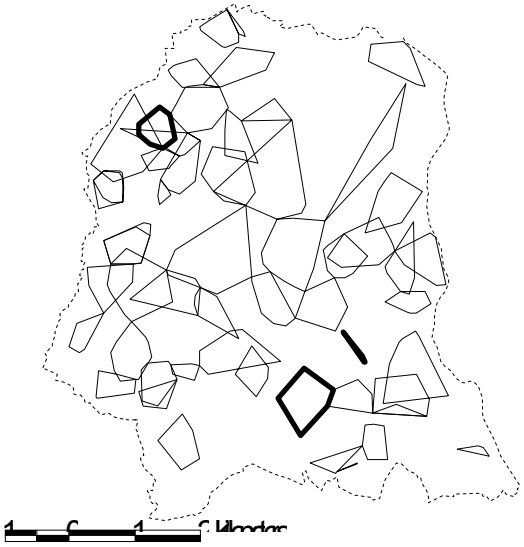
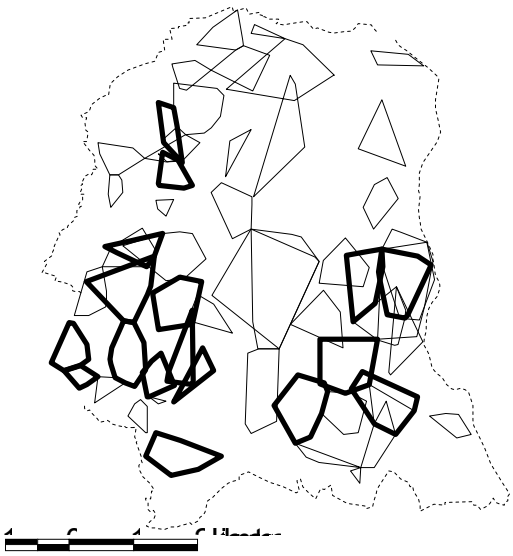


Fig 2 Social group territories expressed as 95% minimum convex polygons (from spring baitmarking), for treatment and control badger populations before and after culling. Social group territories containing badgers infected with bTB (from trapping during the year) are shown as bold.

(a) Treatment Pre-Cull



(b) Treatment Post-Cull



(c) Control Pre-Cull

(d) Control Post-Cull

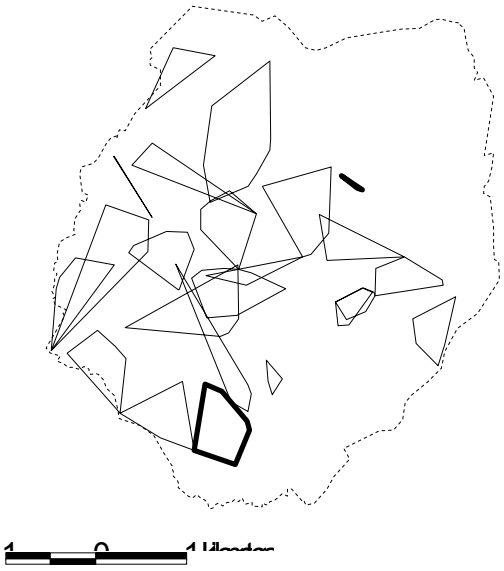


Fig 3 Mean (a) proportional overlap (+ 95% CI) and (b) number of overlaps (+ 1 sd) between social group territories for Removed (R), Neighbouring (N), Other (O) and Control (C) social groups before and after culling.

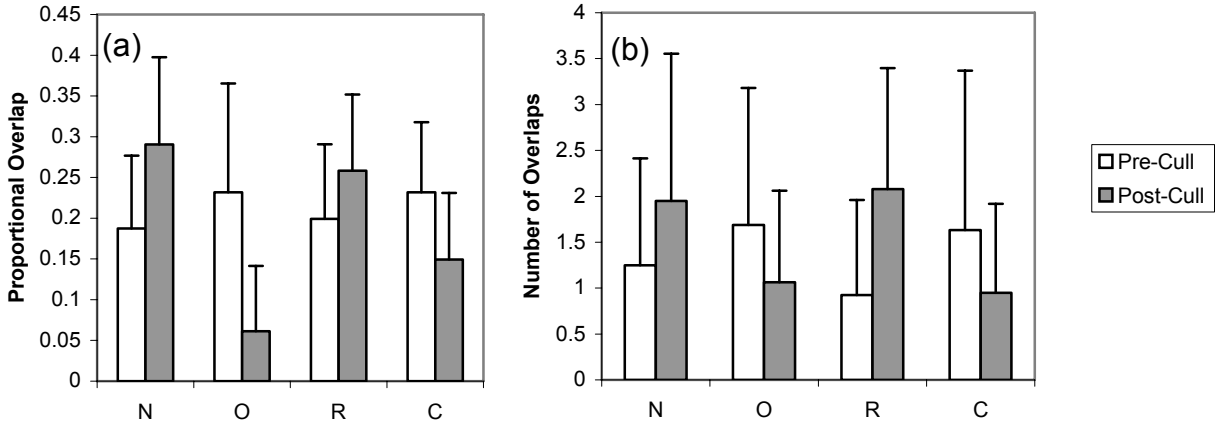


Fig 4 Proportion of badgers recorded with fresh bite wounds in Removed (R), Neighbouring (N), Other (O) and Control (C) social groups before (Pre-), between (Inter-) and after (Post-) culling events. Error bars show 95% binomial confidence intervals.

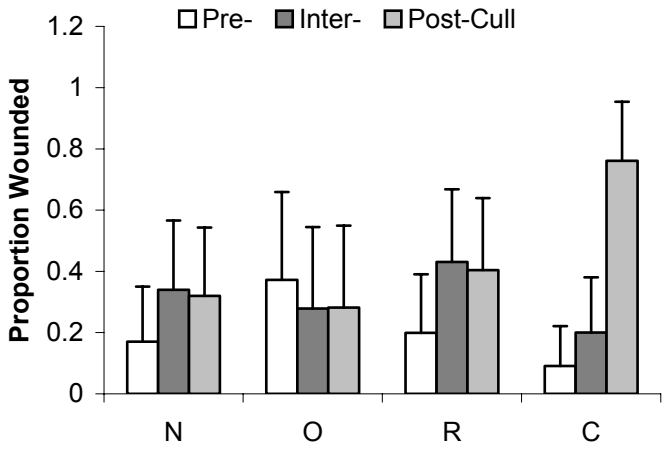


Fig 5 Distribution of bTB infection in badger populations in treatment and control areas between 2001 and 2003. Shading indicates the detected source of infection from clinical samples. The areas in which badger removal operations were carried out in each year are shown as bold polygons.

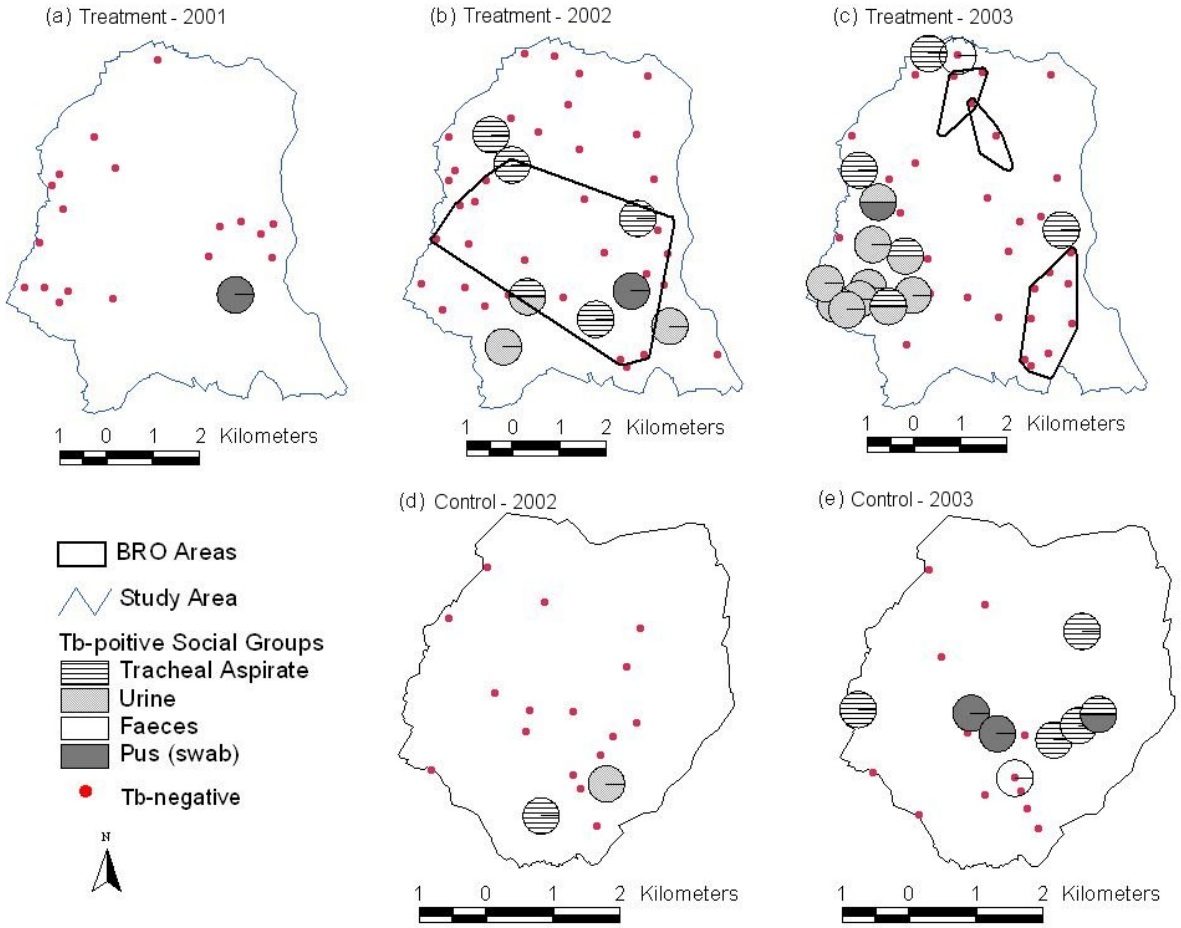


Fig 6 Mean bTB prevalence (± 95% binomial CI) in Removed (R), Neighbouring (N), Other (O) and Control (C) badger social groups before and after culling.

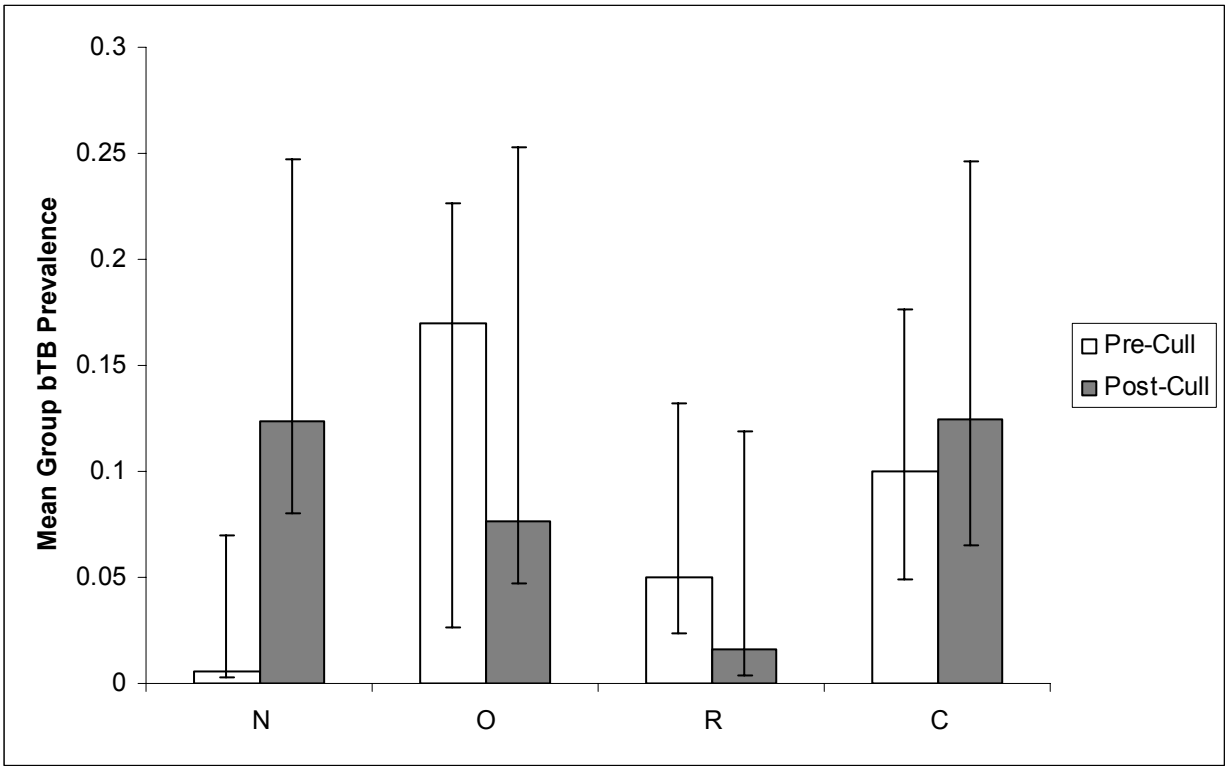


Fig 7 Mean (+ 1 s.d.) range areas (95% minimum convex polygon) of individual badgers from Removed (R), Neighbouring (N), and Other (O) social groups in the treatment (culled) population, and Control (C) social groups (survey-only area). Arrows indicate the timing of badger culling operations.

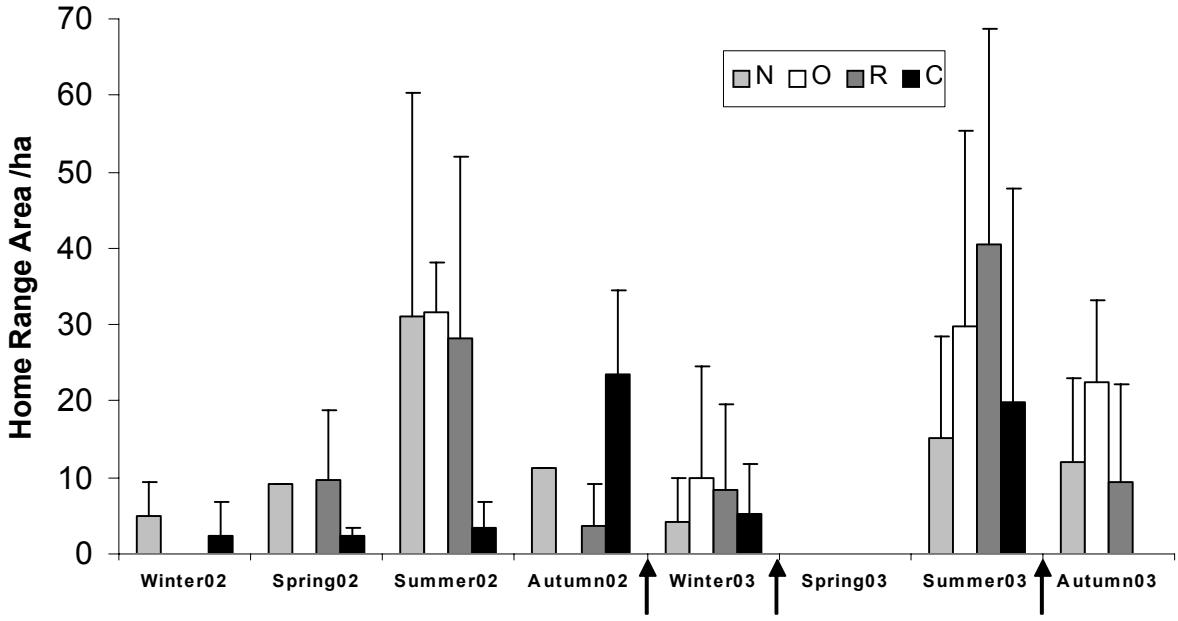


Fig 8 Proportional overlap between 95% MCP home ranges of badgers from different social groups for Removed (R), Neighbouring (N), and Other (O) social groups in the treatment (culled) population, and Control (C) social groups (survey-only area). Arrows indicate the timings of badger culling operations. Error bar indicate 95% confidence intervals.

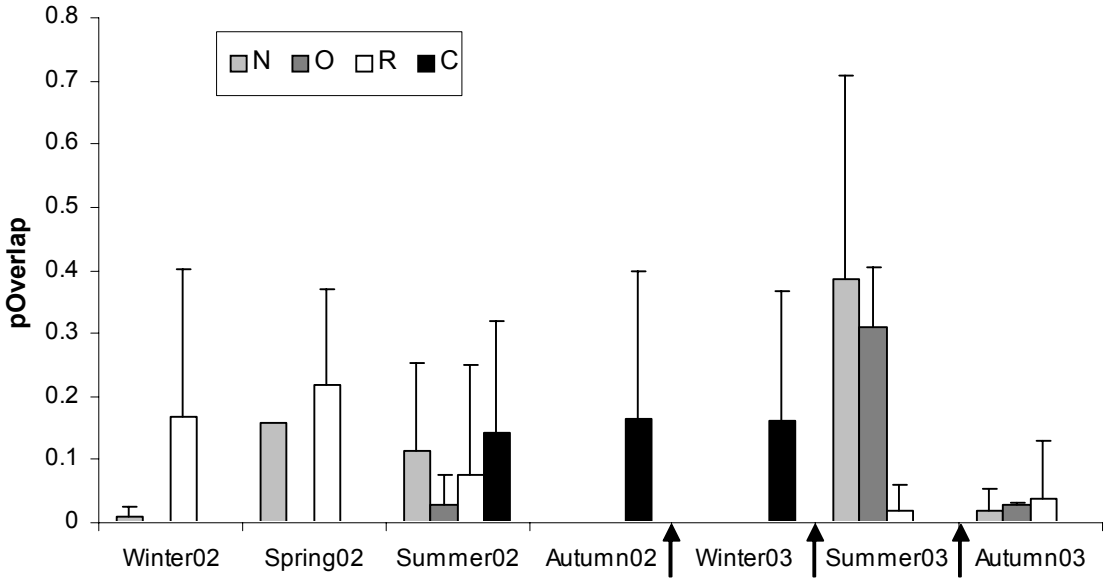


Fig 9 Mean numerical overlap (+ 1 s.d.) between the 95% MCP home ranges of badgers from different social groups for Removed (R), Neighbouring (N), and Other (O) social groups in the treatment (culled) population, and Control (C) social groups (survey-only area). Arrows indicate the timings of badger culling operations.

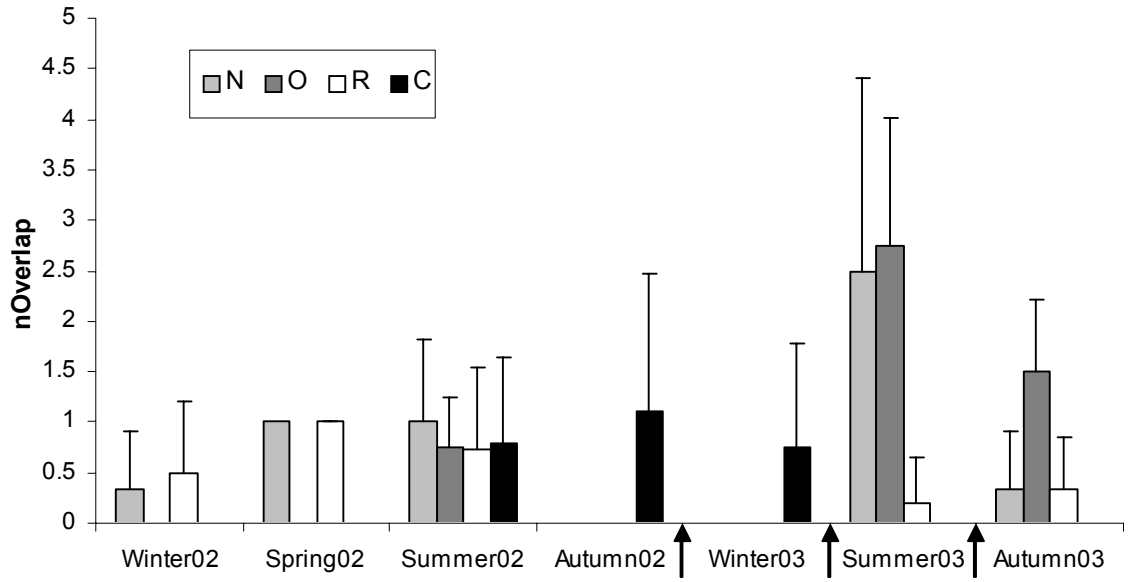


Fig 10 Mean change in bTB prevalence (+ 95% CI) following culling by social group type (RNOC) for (a) Inter-group movement (IGM) donor groups and (b) IGM receiver groups.

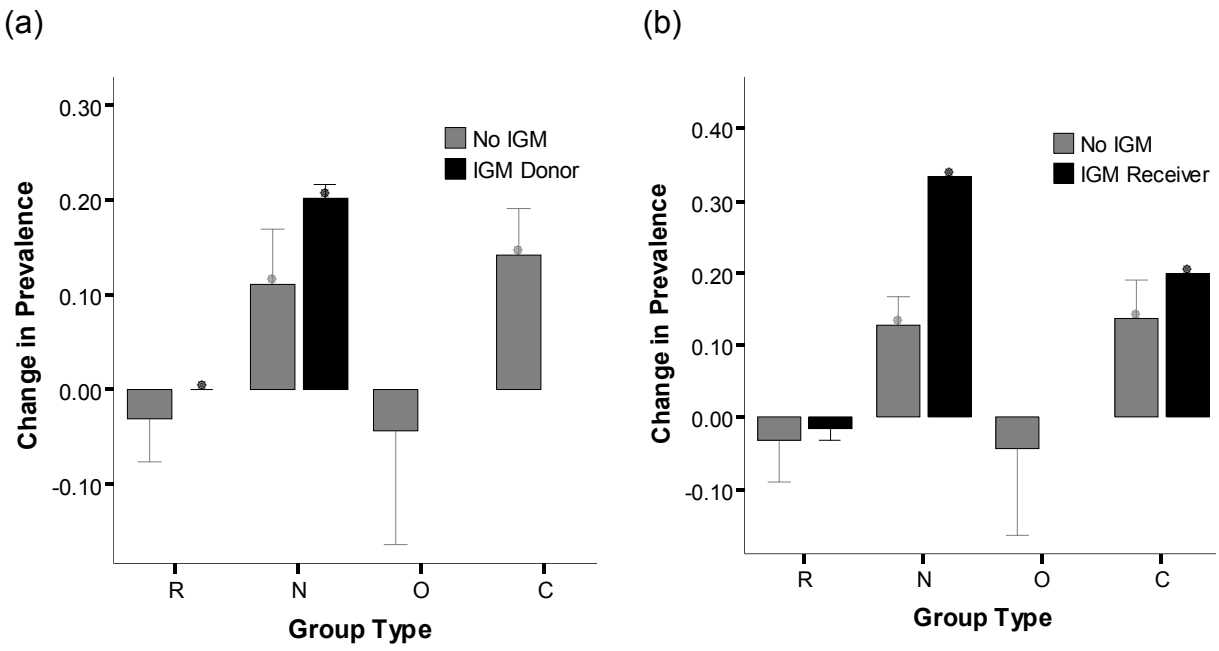


Fig 11 Modelled badger population dynamics (± 95% confidence interval) over simulated 20 year period, with a reactive BRO in year 11.

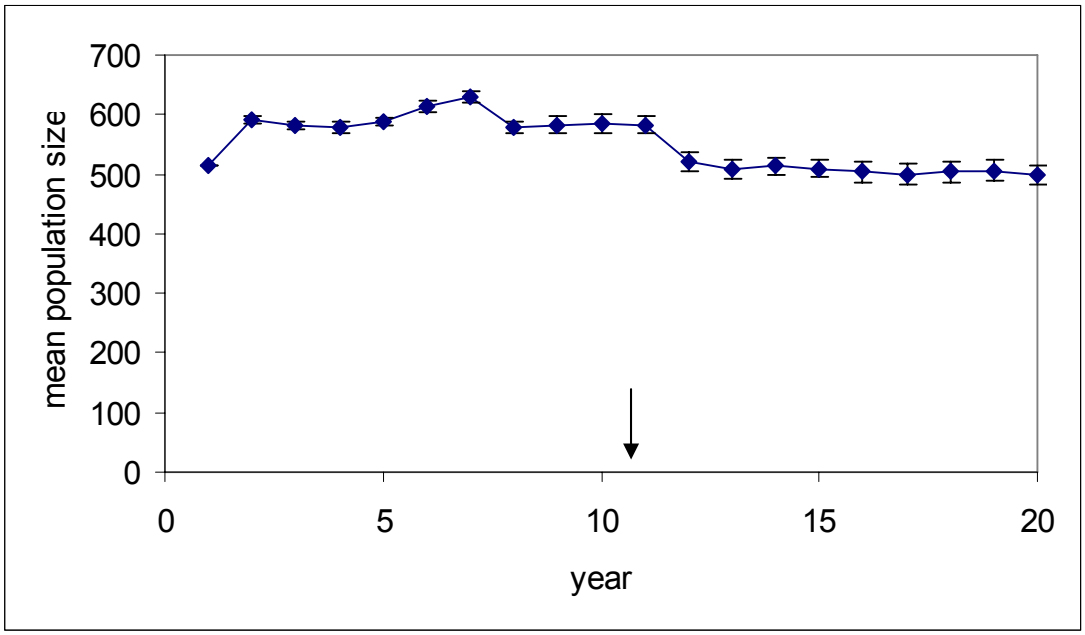


Fig 12 Modelled TB infection dynamics in a simulated badger populations over a 20 year period, with a reactive BRO in year 11. The mean number of infected social groups is represented by filled diamonds and the mean number of infected badgers is represented by open squares, both ± 95% confidence intervals.

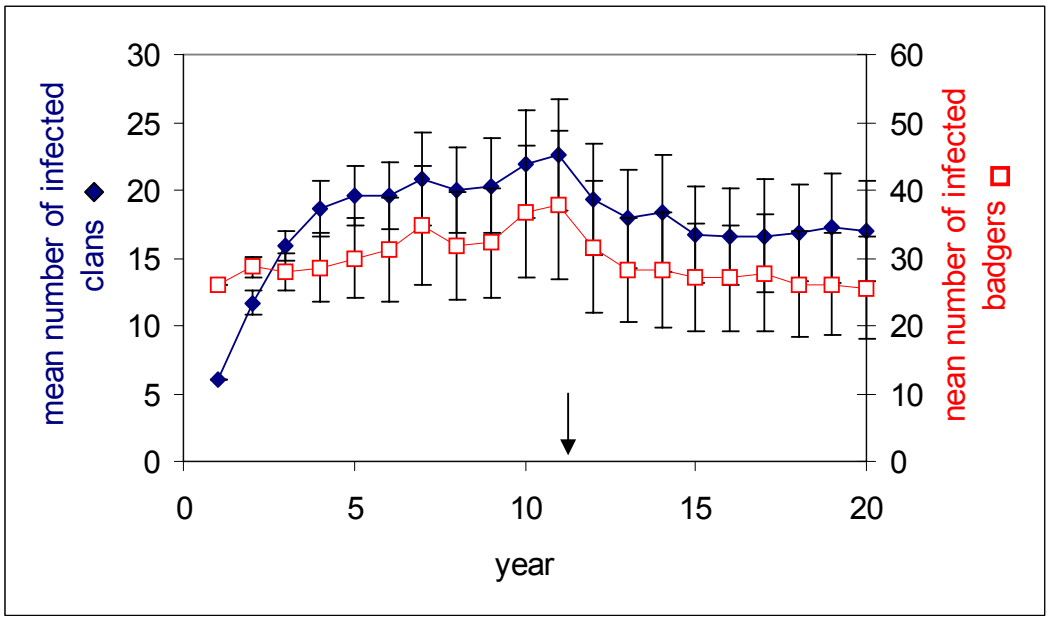
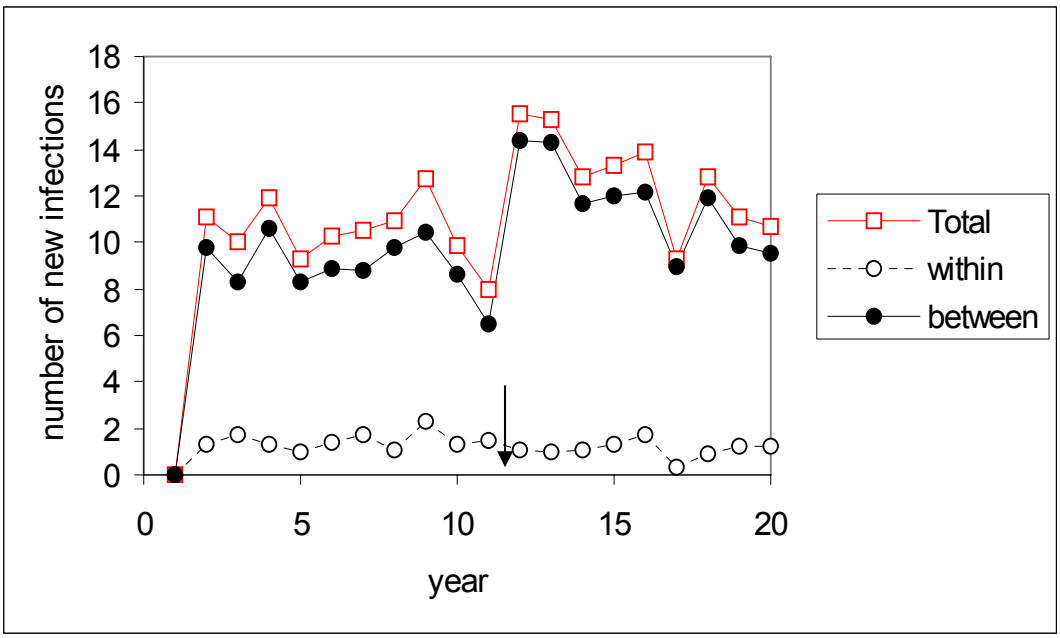


Fig 13 Number of new infections per year (± 95% confidence intervals) within a simulated badger population over a 20 year period, with a reactive BRO in year 11.



SE3110 Molecular genetic analysis of badger social structure and bovine TB

Organisation Central Science Laboratory
York

Start date 01/11/99
End date 31/12/06

Total cost £1,094,055

Abstract
The dispersal of badgers is difficult to estimate, yet even a small change in dispersal patterns can have a large impact on disease dynamics. By comparing the genetic structure of badger populations from initial culls to follow-up culls we can gain insight into the impact of culling on badger dispersal. The population genetic structure among trial sites was pronounced, suggesting that long-distance dispersal is limited. Comparison of the change in isolation-by-distance (IBD) slope between initial and first follow-up cull showed conflicting results. A significant decrease in IBD slope would indicate a significant increase in dispersal distance; however, no site showed a significant change, with only half showing a decrease in IBD slope (B2, F1, G2, E3). Site H2 differed from all other sites in that a barrier to gene flow was detected across the middle of the site, effectively dividing it into two separate populations. Differences among trials may be the result of factors such as varying culling efficiency or disturbance history. Also, our analysis of the change in IBD slope across entire sites may be too simplistic, with changes at a fine scale providing more information. Further analysis of subsequent culls will help resolve this question. All trial sites except H showed an influx of unlikely individuals (ie those with genotypes of low local probability) into the site following the initial cull, with a significant change at sites B2, E3, and G2. Further analyses are ongoing, including of additional culls, and include: estimation of dispersal distance changes through modelling, estimation of efficiency of culling through modelling, changes in fine-scale patterns of relatedness and sex differences in dispersal.

Aims
- To genotype all badger samples from proactive and reactive culling areas.
- To measure gene flow within culling areas, in order to compare between areas.
- To assess the impact of culls on the dispersal patterns of badgers.
- To assess the efficiency of the proactive culls by comparing the genetic composition of follow up culls to that of initial culls.
- To prepare the data gathered to integrate it with other research projects.

Relevance to Defra
This project aims to use genetic markers to estimate changes in the gene flow of badgers as a result of culling. It also aims to provide an estimate of the relative efficiency of culling in each trial area.

Methods

Genotyping

Ear tissue samples were taken and stored in 70% ethanol. Total DNA was extracted using a variation of a salting-out protocol (Sambrook & Russell 2001). Badgers were genotyped using a panel of 16 microsatellite loci (Mel101 – Mel117), using methods described in Carpenter *et al* (2005). The error rate was determined by blind genotyping of 4% of the previously extracted DNA samples, and positive and negative samples were included in all runs.

Statistical analyses (only proactive, first and initial follow-up culls analysed to date)

Genetic diversity within trial sites was estimated using allelic diversity corrected for sample size (Goudet 1995) and observed and unbiased expected heterozygosity (Nei 1978). An association between genetic distance and geographic distance across Britain was tested using a regression of $F_{ST}/(1-F_{ST})$ against the natural log of geographic distance (Rousset 1997).

Analyses of isolation by distance among individuals were performed through Mantel tests for association between geographic distance and genetic distance among pairs of individuals (*â*, Rousset 2000). The slope of this relationship can be used to estimate dispersal distance, with a decrease in slope reflecting an increase in dispersal.

Assignment tests were performed on each trial area separately to assess the influx of individuals from outside the trial sites. The initial sample was used as a known reference to which the first follow-up samples were assigned. Assignment tests use the allele frequencies of a population to estimate the likelihood of an individual occurring in that population, given its genotype. Specifically, we used the frequency based methods of Paetkau *et al* (1995, 2004). We then examined changes in the proportion of 'unlikely individuals', defined as those individuals that were unlikely to have been born in a population if their probability of assignment to that population was less than 0.05. This threshold has been demonstrated to be reasonable in an empirical study (Eldridge *et al* 2001).

Results

Results from population genetic analyses of badgers from Woodchester Park were used as a basis for the analysis of data from the culling trial. These results indicated approximately half of the paternity of cubs was through mating by adult males from outside the social group, generally from neighbouring territories (Carpenter *et al* 2005). A significant pattern of isolation by distance was observed between individuals (Figure 1). Bayesian clustering of Woodchester Park badger genotypes revealed some population substructure, but the isolation-by-distance pattern of dispersal made it difficult to define clear boundaries between groups. Assignment tests and isolation by distance or spatial autocorrelation analyses were determined to be the best approach for the analysis of trial areas.

Genotyping of the initial and first follow-up cull from all eight trial areas from which data will be available have been completed. Initial and follow-up comparisons will not be possible for trial areas A and C, as the majority of samples were not collected from these initial culls due to the closure of VLA centres. Data for the reactive and subsequent culls have not yet been analysed.

Trial area F had lower genetic diversity than the other trial areas, probably due to its relatively isolated location on the Cornish peninsula. Among trial areas there was a strong pattern of isolation-by-distance gene flow (Figure 2). This pattern has also been identified at a broader scale across Great Britain and western Europe (Pope *et al* 2006).

As for Woodchester Park, all trial areas except trial area H2 showed a significant pattern of isolation by distance among individuals. Trial area H2 differed from the other areas in that it appeared to be composed of two distinct populations, separated by a valley across the middle of the site. Dispersal across this valley appears sufficiently impeded to have impacted on population structure. Four trial areas showed a decrease in slope for the regression of *â* against ln distance (B, E, F, G), indicating an increase in dispersal. If trial area H was analysed over a 4-km window, the distance across which IBD occurred, there was also a decrease in slope. Three trial areas showed an increase in slope (D, I, J), with site D showing the only significant difference based on overlap of standard error bars. A more extensive analysis is in progress.

Based on assignment tests, in all trial areas except H there was an increase in the proportion of 'unlikely' individuals, *u*, in the follow-up cull as compared to the initial cull (Figure 3). Mean *u* from the follow-up cull was significantly less than from the initial cull for four trial areas (B, E, F, G), and followed this same trend for all trial areas except H. The variance in *u* showed a significant increase between initial and follow-up culls for three trial areas (I, E, F) and again followed the expected trend for all trial areas except H.

Conclusions

Gene flow among and within badger populations predominantly follows an isolation by distance pattern both at a broad and local scale. While all but one site showed an influx of new individuals, changes in dispersal patterns between initial and first follow-up culls were small, and varied among sites. Specific analysis tools are being developed that combine the properties of isolation-by-distance analyses and assignment tests, allowing changes in dispersal distance and direction to be estimated. Ongoing analyses will include subsequent follow-up culls and reactive culls.

Future research

- Further analysis of the changes in patterns of population structure through spatial autocorrelation analysis, rather than simply considering changes in the slope across the entire site, will provide more insight into the effects of culling on dispersal.

- Continuation of current analyses to include additional follow-up culls will determine whether the effects of proactive culling increase or reduce over time.

- We have been developing a mathematical model in collaboration with Prof. Roger Butlin that combines the properties of isolation-by-tests and assignment tests in order to extract more precise estimates of changes in dispersal distance and direction.

- Fine-scale analyses of the changes in distribution of relatives will provide information on the effects of culling on mating systems.

- Comparison of changes in male and female dispersal patterns and how there are influenced by culling.

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Carpenter P, Pope LC, Greig C*, et al* (2005) Mating system of the Eurasian badger, *Meles meles*, in a high density population. *Molecular Ecology* **14**, 273-284.

Eldridge MDB, Kinnear JE, Onus ML (2001) Source population of dispersing rock-wallabies (*Petrogale lateralis*) identified by assignment tests on multilocus genotypic data. *Molecular Ecology* **10**, 2867-2876.

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Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**, 583-590.

Paetkau D, Calvert W, Stirling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology* **4**, 347-354.

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Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics* **145**, 1219-1228.

Rousset F (2000) Genetic differentiation between individuals. *Journal of Evolutionary Biology* **13**, 58-62.

Sambrook J, Russell D (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Publications generated from the project

Carpenter P, Pope LC, Greig C*, et al* (2005) Mating system of the Eurasian badger, *Meles meles*, in a high density population. *Molecular Ecology* **14**, 273-284.

Pope L, Domingo-Roura X, Erven K, Burke T (2006) Isolation by distance and gene flow in the Eurasian badger (*Meles meles*) at both a local and broad scale. *Molecular Ecology* **online early**.

Review comment
This is potentially an important and promising project but its achievements so far are not commensurate with its cost and duration. Little has been seen so far in terms of deliverables/milestones and a great deal of analysis and interpretation of results remains to be done in order to meet the project objectives. However, the project could obtain some very interesting data on dispersal patterns. The result was that there was no evidence that culling influenced dispersal after an initial cull. It is also important to note that information provided on the movement of badgers between social groups is confined to mating movements, which may be transient, rather than contacts *per se* so does not give a direct indication of disease transmission risk. Further research is likely to be required to provide sufficient data on the relative magnitude of the effects of different parameters on *M. bovis* disease dynamics in order to inform the design of future control programmes.

Scores
Conclusions based on sound evidence: 1.8
Quality of science: 2.0
Overall rating: 2.2

Fig 1 Isolation by geographic distance between pairs of adult badgers at Woodchester Park. The genetic distance measure *a* is from Rousset (2002); female-only comparisons are represented by open circles, males by open triangles and all adults by filled diamonds.

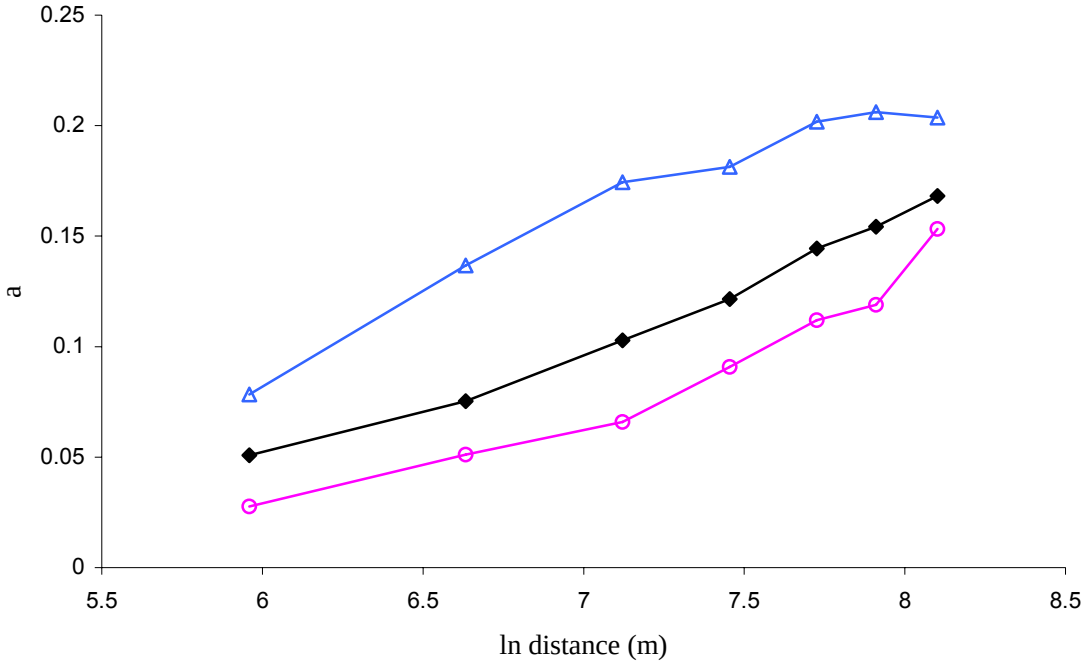


Fig 2 Isolation by distance among proactive-trial badger populations.

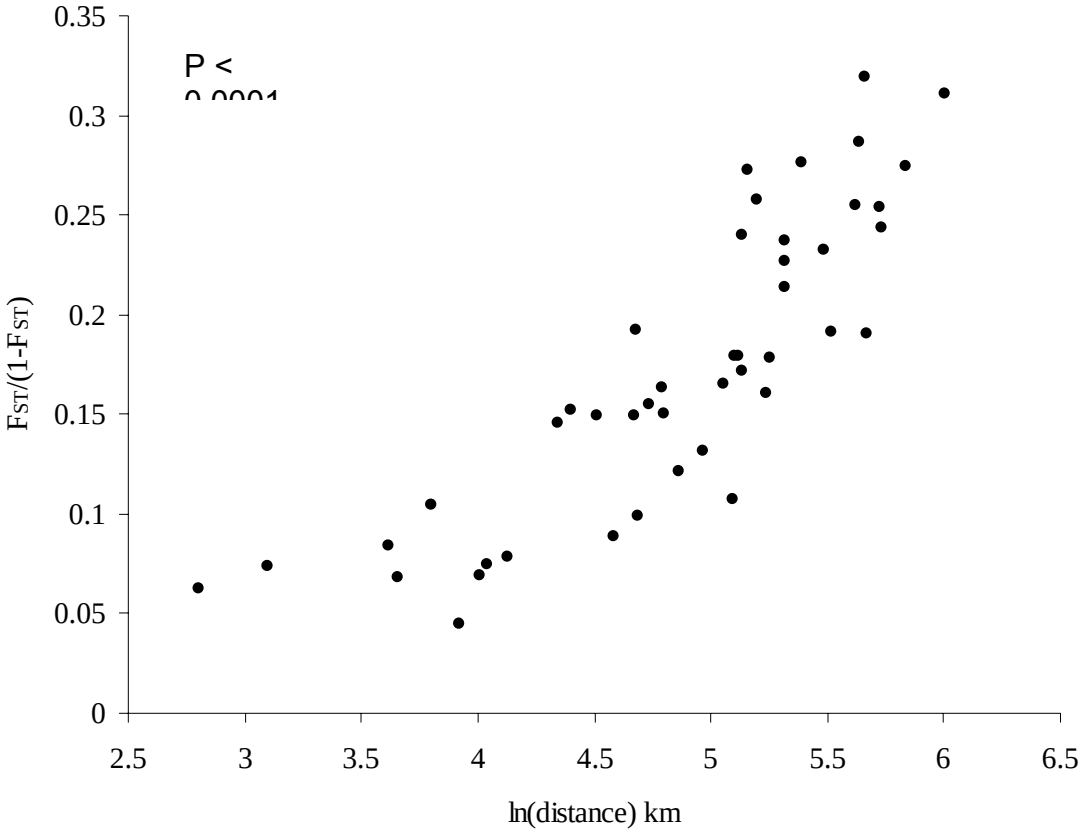
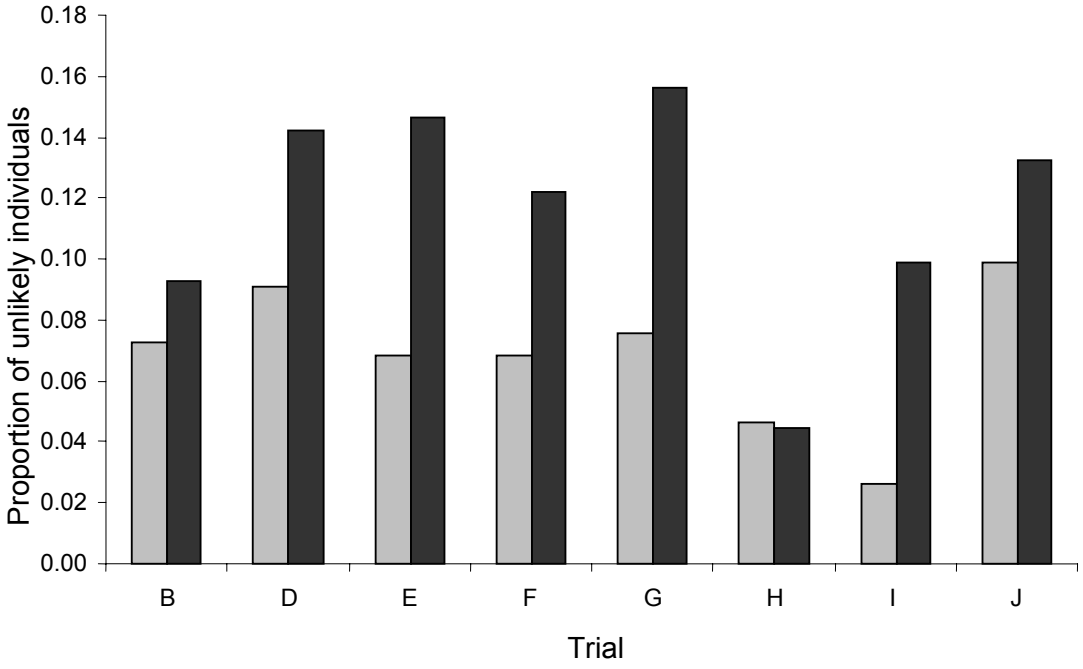


Fig 3 Change in the proportion of the number of 'unlikely' individuals sampled in a trial area, comparing the initial sample with the first follow-up sample. The proportions of unlikely individuals from the initial sample are given in pale grey and the proportions from the follow-up sample in black. Unlikely individuals were defined as those assigned to the initial trial population with probability < 0.05, using the Paetkau *et al* (2004) method.



ZF0531 The ecological consequences of removing badgers from the ecosystem

Organisation Central Science Laboratory
York

Start date 01/02/99
End date 31/03/07

Total cost £1,507,627

Abstract
Monitoring of selected species has been carried out in four of the Randomised Badger Culling Trial triplets since 2000. Surveys and other research is taking place in proactive badger cull areas, and in matched no-cull control areas. The aim of this study is to make an experimentally controlled assessment of the impact of badger removal on certain important species. From pre-cull, to the second year post cull, estimated fox densities increased in on average by 92% in proactive areas but declined by 60% in no-cull areas. Over the subsequent three years. Over the next three years while badger numbers remained depressed, fox populations in proactive areas remained fairly constant relative to control areas. The mechanism underpinning this change in fox population levels is unclear. From 2000 – 2005, hedgehogs occurred less frequently in areas with high badger sett density. Hedgehog numbers on amenity grassland sites in and around villages increased by over 100% in areas where badgers were removed, but remained stable or declined in no-cull areas. This supported previous research indicating that badgers have a role in regulating hedgehog populations. Hare numbers declined in both proactive and no-cull areas on average, but more so in proactive areas. This was not statistically significant with respect to badger removal, but would be consistent with the increase in fox numbers observed given that the fox is the hare's main predator. The results from ground nesting bird surveys with respect to badger removal were inconclusive. Skylark and meadow pipit numbers declined in the no-cull areas but remained constant in the proactive areas. This could be interpreted as a reduction in nest predation due to badger removal, in the context of a wider decline in populations. However, there are other explanations relating to the dominant habitat types in the surveyed areas that could equally well explain this pattern.

Aims
- To identify suitable sites, species and monitoring techniques to allow an assessment of the ecological consequences of removing badgers from an ecosystem.
- To conduct monitoring of predator and prey abundance, predator diet and prey fecundity over a five year period on both experimental (badger removal) and control (no badger removal) sites, concentrating on changes in abundance of other species of agricultural or conservation concern.

- To provide a replicated experimental assessment of the ecological consequences of badger removal.

- To construct a model of food web dynamics based on data from both treatment and control areas.

Relevance to Defra

Reduction in badger numbers over large regions (ten 100km^2 areas) was carried out in proactive Randomised Badger Culling Trial (RBCT) areas since the start of the Defra experiment in 1999. Badgers are Britain's largest carnivore, and present in considerable numbers in the parts of the country where the RBCT operates. It is clear that such action could have knock-on effects on local ecosystems, especially given that the reduction in badgers was sustained over several years. Potential ecological community responses to the changed food web include changes in the population size and behavioural ecology of species that are predated by, or share space and resources with badgers. These include species of economic importance and conservation value. Therefore it was considered vital to study the consequences of badger removal in order to anticipate community changes. The RBCT provides an ideal experimental framework within which to investigate these food web changes, comprised as it is of large treatment areas (proactive badger culling) matched with experimental controls (no badger culling).

Methods

The abundance of foxes, hares and rabbits was monitored pre- and post-cull in the proactive and no-cull areas of four RBCT triplets using nocturnal spotlight counts. Counts were carried out from 60 points within each trial area, from the roof of stationary Land Rovers. The distance to observations was measured using laser rangefinders and densities calculated using distance sampling theory (Buckland, 2001). Quantification of fox diet from analysis of scats collected in those same four areas was carried out to determine if fox diet changed in response to badger removal. Hedgehog abundance was estimated from nocturnal spotlight surveys of a sample of pasture fields and amenity grasslands within the trial areas. A survey of the use of badger setts by foxes for breeding dens in spring was carried out in those same triplets in 2002, 2003 & 2004. Skylark and meadow pipit abundance was monitored using surveys of singing males, in proactive and no-cull areas of 2 RBCT triplets from 2000-2004. Nest survival monitoring for those species using artificial nests was carried out concurrently with the abundance surveys. A further nest survival experiment using artificial nests at Woodchester Park in Gloucestershire was carried out in the context of an experimental manipulation of habitat and livestock grazing.

Results

1. <u>Foxes</u>

   On average, fox densities in proactive areas increased by 92% from pre-cull levels (year zero), to the second year of badger culling (no surveying was carried out in year 1 due to the FMD outbreak). At the same time in the control (no culling) areas, fox densities decreased on average by 60%. From years 2 to 3 of culling, average fox density in the proactive areas remained similar, while density on average in the control areas declined by 38%. In years 3 to 4, and years 4 to 5 the densities in the proactive and control areas remained similar relative to each other (Figure 1). The time*treatment interaction was statistically significant, implying that badger removal has an effect on fox populations. The pattern of change suggest that fox populations in cull areas responded quickly to the

reduction in badger numbers, but then reached a new equilibrium level with respect to the control areas after a period of around 3 years (Figure 1). Very few badger setts were found to be in use as fox breeding dens (0-8%) in any area. This did not change as a result of badger culling. Although there were significant variations in fox diet over time, there were no consistent differences related to the removal of badgers. Therefore, the likeliest mechanism for this population change is an increase in rates of cub production or survival following badger removal, although we have no data to support this.

2. Hedgehogs

   Hedgehogs were found to be very scarce on pasture fields in rural areas, but more abundant in amenity grassland sites in and around villages. There was a statistically significant, negative relationship between badger sett density and the density and distribution of hedgehogs ie the higher the density of badger setts in an area, the lower the probability of hedgehog presence. A full description of these results is given in Young *et al* (2005). After removal of badgers, hedgehog numbers in amenity areas increased by 100%, while there was a 55% decline in the matched no-cull areas (Figure 2). This was a statistically significant effect. In rural grassland sites, there was no apparent response of hedgehog populations to badger removal. The decline in numbers in control areas is of conservation concern, and supports recent research by other organisations indicating hedgehog population decline.

3. Ground nesting birds

   Abundance of meadow pipits and skylarks declined in the two no-cull areas over the survey period, but remained relatively constant in the proactive areas. One possible explanation for this difference is a reduction in predation pressure due to badger removal. However, both proactive study areas contained considerably more optimal habitat (upland grassland) for these species than the no-cull areas, confounding the results. So although this pattern may be due to reduction of predation due to badger removal, it could equally well be explained by range contraction into optimal areas of both species in the context of a wider decline in their numbers for other reasons. The decline in abundance of both species in the no-cull areas is of general concern. In the artificial nest survival experiment at Woodchester Park, nest failure was primarily to due to direct impact by livestock rather than predation by animals including badgers, although how these findings relate to real nests remains unknown.

4. Rabbits

   There was no obvious pattern in rabbit populations in general across all study areas. There was a great deal of variation within and between areas. There was no statistical difference between proactive and no-cull areas in terms of changes in rabbit numbers ie no effect of the removal of badgers.

5. Hares

   There was a decline of 56% in average hare densities in proactive areas, while over the same period densities in control areas remained constant on average (Figure 3). There was considerable variation in estimates over time both within and between areas. Therefore, despite being apparently markedly different, this was not a statistically significant effect with respect to badger removal. However, it should be noted that this overall pattern of results is the opposite of those seen with foxes with reference to the proactive and no-cull treatments. Foxes are the most important predators of hares (Reynolds & Tapper 1989) and this may be evidence that the increase in fox numbers after badger removal is having a knock-on negative effect on hare populations. Therefore the hare population pattern should be analysed with respect to fox numbers.

Conclusions

The removal of badgers appears to have led to an increase in fox numbers. The reason for this is unclear, but we speculate that this may be due to interference competition (eg Begon *et al* 2006). Badgers and foxes both breed in underground dens, and foxes are known to use abandoned badger setts (Neal & Cheeseman, 1996). However, badgers are heavier and stronger than foxes and tend to win disputes. Therefore they are likely to claim the best breeding sites, and removal of badgers may mean less competition and hence lowered stress in foxes. Stress has been shown to be a contributory factor in reproductive failure (Hartley *et al* 1994). Therefore this numerical response may be due to an increase in productivity. Fox numbers can also be affected by levels of shooting, but it seems unlikely that these should differ systematically between proactive and control areas.

Hedgehog numbers also increased in response to badger removal. This is more easily explained, and supports earlier research indicating that badger predation acts to restrict the growth of hedgehog populations. We do not know whether this pattern is a permanent escape from regulation by badgers, or whether badger recolonisation would lead to a reduction in hedgehog numbers. Only repeat surveys beyond the end of the RBCT could answer this question.

Hare numbers declined more in proactive areas than in no-cull areas, although this was not a statistically significant difference. However, this pattern might be anticipated given the observed changes in fox numbers, and should not be ignored. Hares are also very sensitive to change in land use. Therefore any analyses of hare population growth in relation to fox numbers must be done with land use data included in any model. These analyses are planned.

Skylarks and meadow pipits declined in no-cull areas, but remained constant in the proactive areas. This might be evidence of a general decline in these species, offset by the removal of predator pressure when badgers are removed. However, this result should be treated with caution, due to the confounding effects of habitat composition in the study areas. However, the decline in numbers in the control areas should be viewed with concern in conservation terms.

Future research

Data collection on nocturnal mammal populations is continuing in 2006, following the final year of proactive culling in the RBCT in 2005. However, data collection beyond this on certain species would be extremely valuable in future years, to determine whether the population responses observed are reversed or otherwise. For example, hedgehog populations have increased markedly in proactive culling areas relative to control areas. With the recolonisation of badger populations, this trend may be reversed, indicating population regulation by badgers. Alternatively their populations may have escaped the regulatory effects of badgers and reach a new stable state. Additionally given that important species such as foxes, hedgehogs and hares have shown marked population responses, it is important that their populations are monitored during badger recolonisation. This could only be achieved by continuing the surveys beyond the end of badger culling, ie into 2007 and beyond.

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Publications generated from the project

J. Davison, R.P. Young, T.D.Hounsome, I.D.Trewby, M. Claridge, R.W.Yarnell, N. Walker, P.A.Henrys & G.J.Wilson (In Review) A comparison of distance sampling and scat counts for monitoring fox populations. *Journal of Applied Ecology.*

Hounsome, T.D., Young, R.P., Davison, J, Trewby, I.D., Yarnell, Garnett, B., Delahay, R.J. & Wilson, G.J. (2005) Estimating badger abundance by distance sampling: an evaluation study on a intensively studied, high density population. *Journal of Zoology,* **266***, 81-87*

Hounsome, T.D. & Delahay, R.D. (2005) Birds in the diet of the Eurasian badger (*Meles meles*): a review and meta-analysis. Mammal Review, **35**, 199-209.

Hounsome, T.D., Leech, D., Gorman, M., Raffaelli, D. & Delahay, R.J. (Submitted) Evidence for the direct and indirect of livestock on the nest survival of ground nesting birds: an experiment at three scales. *Journal of Applied Ecology.*

Young, R.P., Davison, J., Trewby, I.D., Wilson, G.J., Delahay, R.J. & Doncaster, C.P. (In Press) Spatial variations in hedgehog abundance in relation to badger density and distribution. *Journal of Zoology.*

Review comment
The objectives of this project are important in that they seek to answer fundamental questions about the consequences of any potential badger culling policy. The project provides reassurance that culling of badgers does not have disastrous ecological effects. Following badger removal, there was a significant increase in fox numbers, with a corresponding decrease in hare abundance, as well as a marked increase in hedgehog numbers. An important part of this project centred on ground nesting birds for which progress was disappointing, but understandable given that the study areas could not be chosen for the purpose due to FMD, and that all nest-predation studies are procedurally difficult. A criticism is that the project is extremely expensive and centred on just a few species and due to the limited number of species being examined, the ability to build a food web is questionable. Given indirect effects, perhaps further species should have been included. Further investigation in the indirect relationship between badger and hare may be required given that the hare is a Biodiversity Action Plan (BAP) species. This project suggests possible increase in predation by foxes but this has not been proven.

Scores
Conclusions based on sound evidence: 2.5
Quality of science: 2.7
Overall rating: 2.5

Fig 1 Mean fox densities in proactive badger culling areas and matched control areas over time. Cull.Year zero refers to the pre-cull densities, cull.year 1 refers to the first year post-culling etc.

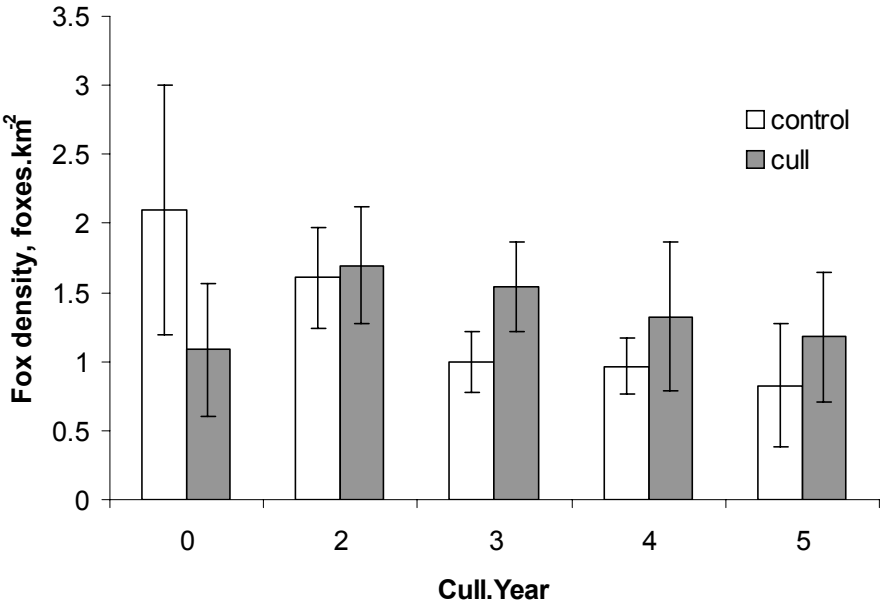


Fig 2 Mean hedgehog densities in amenity grassland areas of proactive badger culling and matched control areas over time. Cull.Year zero refers to the pre-cull densities, cull.year 1 refers to the first year post-culling etc.

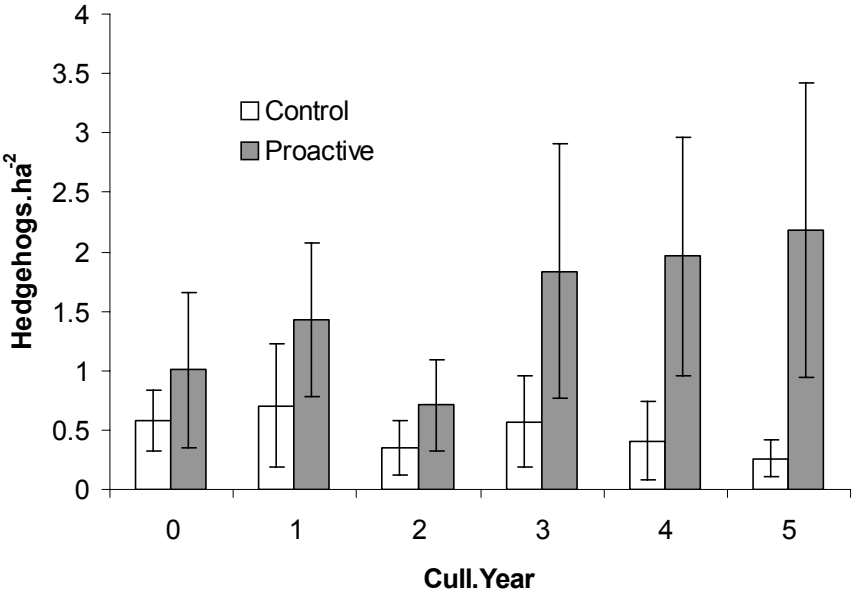
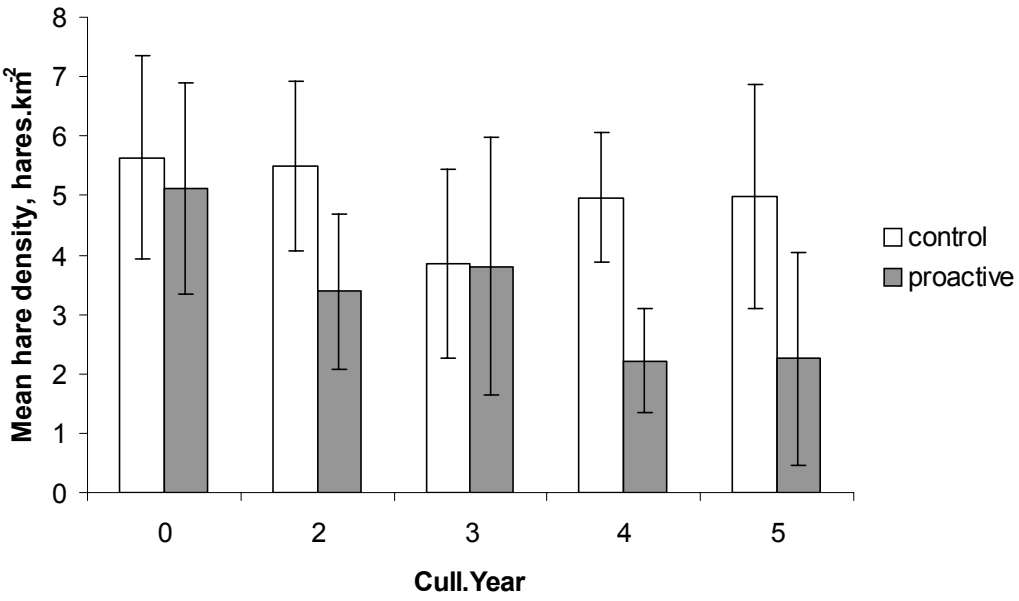


Fig 3 Mean hare densities in amenity grassland areas of proactive badger culling and matched control areas over time. Cull.Year zero refers to the pre-cull densities, cull.year 1 refers to the first year post-culling etc.



SE3029 An investigation of potential badger/cattle interactions including the extent of badger visitations to farm buildings and food stores, and how cattle husbandry methods may limit these

Organisation Central Science Laboratory
York

Start date 01/01/03
End date 31/12/05

Total cost £556,66.31

Abstract
Bovine TB, caused by *M. bovis*, is a widespread and increasing problem in cattle in the southwest of England. Badgers have been implicated as the main wildlife reservoir of this disease. Contamination of pasture by badgers has been considered the main potential transmission route to cattle. However, during extensive surveys of 36 farms in TB hotspots badgers were found to visit 39% of farmyards, leaving *M. bovis* infected contaminants on 29% of these. Intensive monitoring on six of these farms indicated that visit frequency was likely to be much higher than these extensive estimates. During these intensive studies badgers were observed frequenting feed stores, cattle housing, troughs and silage clamps, with observations on excretion or scent marking in feed stores and cattle housing. Direct, nose-to-nose contact was observed on four occasions within cattle housing. Patterns within the frequency of badger visits to farmyards appeared to be regulated by food availability, which was mediated by season and may be influenced by rainfall too.

Badger visits to farmyards and buildings pose a considerable potential TB transmission risk to cattle yet very few farmers implemented methods to reduce or prevent cattle contact with badgers either within the farmyard or at pasture. Simple barrier methods, such as electric fencing, may provide a simple, cheap option to manage this problem.

Aims
The project sought to answer the following questions about the use of farm buildings by badgers:

1. What is the extent of badger visits to farm buildings in TB hotspot areas;
2. What is the seasonal and weather related frequency of badger visits to farm buildings and troughs;
3. What food types are exploited during visits to farms;
4. Do badgers and cattle come into closer contact on pasture or in buildings;
5. Can electric fencing deter visits to buildings and where do excluded badgers go;
6. What are the main risks to cattle associated with badger excretory products/behaviour; and
7. What factors make some farms more attractive to badgers than others?

Relevance to Defra
Despite the potential disease risks to cattle associated with badgers entering farm buildings, we still know relatively little about this subject. However, in order for farmers to be able to adopt practical husbandry measures that can reduce disease risks, we must further our understanding of the interactions between badgers and cattle. This will enable Defra to make informed policy decisions on what practical advice to give farmers.

Methods
Question 1 was addressed by extensive surveying of farmyards, buildings and surrounding land for signs of badger activity on 36 farms in TB hotspots in the southwest of England. During these surveys building and field use, contents, and a measure of the level of biosecurity practised were recorded to address question 7.
Remote surveillance with video and still cameras was used to measure the frequency of badger visits to farmyards and buildings on a subset of six farms. This data and assessments of biosecurity during farmyard surveys addressed question 4. Correlating surveillance data with local meteorological data and comparing with the dates on which the data were collected allowed question 2 to be addressed. During surveillance, cameras were deployed at various facilities to assess the relative frequency of visits to feed stores containing different feeds. In addition, badger faecal samples were collected in the field each quarter and examined microscopically. These were compared with samples collected during feeding trials on captive badgers to identify components of badger diets. These two techniques provided data to address question 3.

Ten badgers were fitted with radio collars and tracked for up to two years. Obtaining spatial fixes by remote triangulation or direct observation provided data to support answers to questions 2, 3 and 4.

Remote video surveillance inside cattle housing on six farms was undertaken to estimate direct contact rates between badgers and cattle within buildings. Direct observations on badgers traversing cattle pasture on 18 farms was used to estimate direct contact rates between badgers and cattle at pasture. Surveys of farm buildings for badger excreta on 36 farms and information gleaned from the literature on cattle contact with excreta at pasture were used to assess indirect contact rates between badgers and cattle. These methods were used to address question 5.

An experiment was undertaken to address question 6. Seven badgers were fitted with radio collars and tracked for six weeks to assess their movement patterns a) before farmyard resources were enclosed within electric fencing, for two weeks b) while farmyard resources were enclosed within electric fencing for two weeks and c) when fencing was removed, for two weeks.

Using data on badger/cattle contact rates from remote and direct observation and from farm surveys and the literature the infective probability of each potential transmission event type was calculated. These estimate the likelihood of each event type explaining the prevalence of TB infection in cattle herds. Comparing the magnitude of each estimate indicated the relative risk presented by each event type, therefore addressing question 7.

Data on farm husbandry practices, building use and contents, biosecurity, field use and badger activity outside farmyards from 36 farms were used to identify factors associated with the presence or absence of signs of badger activity within farmyards, to address question 8.

Results

- Signs of badger activity were found within farmyards and buildings on 39% of farms, ranging from Gloucestershire to Cornwall. Two of 33 faecal samples and two badger carcasses found in farmyards and facilities cultured positive for *Mycobacterium bovis*.
- Badgers visited farmyards and facilities all year round, but visits were more frequent during the spring and summer months and less so during the autumn and winter (χ^2 = 30.63, *d.f.* = 6, *P* = 0.044). The frequency of badger visits was much higher in the spring of 2005 than spring of 2004. At the time of writing, visit frequency had not been correlated with meteorological data.
- Cattle cake/concentrates was present in more badger faecal samples than any other feed derived from the farmyard (15.5% of 419 samples in comparison to Barley in 8.8% and wheat in 5.3%). Cereal silage was rarely found (maize silage was identified in 1.9% of samples). The most prolific food item was grass (in 74.0% of samples), which was presumably taken whilst foraging for invertebrates, but may have been taken as grass silage. Invertebrates were highly abundant (adult arthropods in 65.9% and larvae in 53.9%. Earthworms were present in 29.4% of samples but this represents a considerable underestimation since chaetae, which were used to identify their presence, generally passed through the fraction that was examined.
- Video surveillance indicated that stored cattle cake was visited by badgers more frequently than all other food resources.
- Standards of biosecurity were generally of a very low standard on farms surveyed. Badgers generally had access to any buildings except dairies. Badgers visited all farmyard facilities surveyed, but visited feed stores more frequently than other facilities, although this difference was not statistically significant (χ^2 = 8.25, *d.f.* = 6, *P* = 0.220).
- Badgers and cattle came into direct contact (within 2m and clearly responding to each other) 50 times over 120 camera nights of observation in cattle housing and troughs but were not observed making direct contact at pasture. The closest badgers and cattle got in buildings was 0m (direct, nose-to-nose contact on 4 separate occasions) and at pasture 12m.
- The specification of electric fencing used during the experiment successfully excluded badgers from accessing protected farmyard facilities on all occasions when it was working. At the time of writing, movement patterns during each phase of the experiment had not been analysed.
- At the time of writing the infective probabilities of each potential transmission event had not been calculated.

Conclusions
Badger visits to farmyards and facilities were a widespread and frequent occurrence on farms surveyed in the southwest of England. Husbandry and biosecurity measures implemented by farmers did not aim to reduce or prevent badgers from entering their buildings, with few exceptions. Badgers were observed defecating, urinating and scent marking at pasture, in cattle housing and in feed stores and direct, nose-to-nose contact with cattle was observed in cattle housing and troughs. It is likely that all of these pose a considerable disease transmission risk to cattle. Cheap, simple barrier methods, such as electric fencing, can reduce badgers' access to farmyard facilities and consequently reduce the risk of transmission to cattle.

Future research
SE3119 "An experiment to assess the cost effectiveness of farm husbandry manipulations to reduce risks associated with farmyard contact between badgers and cattle" was designed as a direct result of this project and has received Defra funding.
Sufficient information exists within the published literature to assess the costs and benefits of preventing cattle from contacting badgers and their excreta at pasture. Pending the findings of SE3119 or extrapolating from SE3029, it would be possible to undertake an investment appraisal to assess which husbandry manipulations would probably yield the greatest net benefits.

Publications generated from the project
Pending.

Review comment
Information obtained on contact rates in buildings and the potential effect this may have in developing hotspots of infection is very important. In particular identifying which badgers visit which buildings and how this could contribute to herd breakdowns. The results identified a potential level of contact between tuberculous badgers and cattle both in cattle sheds and at troughs. This supports the case for promoting targeted biosecurity measures against exposure from this source. Limitations set by the number of herds investigated, and by the use of a less than complete means of assessing the true tuberculosis status of all the badgers recorded, restricted the extent to which the team could report on differences in the behaviour of tuberculous and uninfected badgers. However, the results obtained from the project will be extremely useful in creating precise biosecurity plans for farms at risk of TB. A wider study on the estimation of infectivity probability involving herds/holdings in known endemic areas in which prior badger removal has not occurred in the recent past is required.

Scores
Conclusions based on sound evidence: 2.5
Quality of science: 2.3
Overall rating: 2.3

SE3119 An experiment to assess the cost effectiveness of farm husbandry manipulations to reduce risks associated with farmyard contact between badgers and cattle

Organisation Central Science Laboratory
York

Start date 01/11/05
End date 31/10/09

Total cost £1,114,730

Abstract
Recent research at the Central Science Laboratory (CSL) has identified visits to farm buildings by badgers (*Meles meles*) as potentially important in the transmission of *Mycobacterium bovis* (the causative agent of bovine tuberculosis) to cattle. Project SE3029 has indicated that this may be a common and widespread problem throughout the southwest of England and certain farm husbandry characteristics may influence the frequency of visits. Experimental investigation of husbandry practices to reduce badger visits to farm buildings has been recommended by the Independent Husbandry Panel and the Godfray Review.

This project aims to identify and measure the benefits and costs associated with four broad husbandry practices by manipulating them on a series of farms within a factorial experiment. Each measure may achieve a different result. Therefore, an investment appraisal will be conducted to identify and estimate the potential benefits and costs of each husbandry practice. The benefits derived from each measure, both in isolation and in combination with others, will be assessed as the ability to affect a) a change in the frequency of badger visits to farm buildings and b) a detectable change in the probability of a herd breakdown and/or herd restriction status. This will allow estimation of the benefits (valued in £GB) based on market prices.

The results produced will be directly relevant to Defra's policy on controlling TB in cattle by providing a quantified estimate of the financial benefits that can be realised by farmers through improved farm husbandry methods. This will also be of direct benefit to the farming community who will be provided with information on which to make an informed, economically rational judgement on whether and how to invest in improved husbandry methods to reduce risks to herd health.

Aims
The project will answer four fundamental questions about the costs and benefits associated with husbandry manipulations:

- What husbandry measures are effective at reducing or preventing badger visits to farm buildings;
- Is a detectable change in the probability of a herd breakdown associated with each measure;
- What economic costs are associated with each measure; and
- How cost effective is each measure?

Relevance to Defra
In order for farmers to be able to rationally decide on implementing practical husbandry measures that can reduce disease risks, we must develop specific husbandry methods that can be demonstrated to reduce these risks for a quantified investment. This will enable Defra to make informed policy decisions on what practical advice to give to farmers. At present, the potential options for management of the reservoir of *M. bovis* infection in badgers (eg vaccination and culling) are limited and problematic. However, information on the cost–effectiveness of different husbandry practices obtained from this study will contribute to the development of more attractive policies based on improved farm management practices.

Methods
A sample of 64 farms throughout Gloucestershire and surrounding counties will be engaged in a factorial experiment to test the ability of four different measures to limit or prevent badger visits to farm buildings and facilities. These will include badger-proof doors on cattle sheds and feed stores, a novel trough design and electric fencing across silage clamps. Farms will be monitored for signs of badger activity using remote camera surveillance, tracking plates and hair traps each month for 3 years. During the first year the background level of badger activity will be estimated for each farm. During year 2 treatments will be implemented on 60 of the farms, with 4 farms as full controls. Badger activity and farmer compliance (in terms of the frequency with which the treatments are used) will be monitored as before. During year 3 all treatments will be removed and badger activity measured as before. During each year the tuberculin test results will be obtained for each herd and compared with the level of badger activity. Test results for all farms in the southwest of England will be used as control data for comparison with the treatment farms.

The costs (in GB£) of treatments will be compared with the benefits (valued in GB£) of; a) changing the frequency of badger visits to farm buildings and b) changing the probability of a cattle herd breakdown or being under movement restriction. This will be assessed within an investment appraisal. Wider social benefits (eg potential benefits to farming communities) will be identified from an extensive literature review and discussions with the NFU and Defra. The cost–effectiveness of the manipulations will be analysed using profitability indicators such as net present value (NPV), benefit:cost ratio and internal rate of return. Risk and uncertainty will be assessed via sensitivity analysis.

Results
At the time of writing, farmers' names and addresses and herd test histories had still to be received by CSL from a third party, preventing us from recruiting farms to the study. However, we have received an assurance that progress is being made on delivering these data.

Conclusions
Pending.

Future research
Should no measures tested during this experiment be able to effect a reduction in the frequency of badger visits to farm buildings and facilities or should there be no demonstrable benefit to implementing husbandry best-practice within the farmyard, the value of limiting or preventing cattle contact with badgers on grazing land would present a further opportunity to investigate methods of reducing interspecific transmission of disease.

Publications generated from the project
Pending.

Review comment
The project addresses important issues regarding the technical and economic feasibility of controlling *M. bovis* transmission from badgers to cattle by investments to increase farm biosecurity. The real data generated by this project will relate to the effectiveness of different measures in limiting badger visits to parts of farmyards. However, it appears that the measures will vary in their implementation depending on the idiosyncrasies of each farmyard and this is an issue. The scaling back of the project and its objectives significantly reduces its impact and prolongs the delivery of sound, well proven, husbandry techniques for reducing badger contacts and bTB on cattle farms. It is unlikely that changes in breakdown rates will be detected, however the design will detect effects on badger exposure with different treatments. The power analysis was felt to be questionable. This is due to farms being selected at random, therefore, some farms will experience very different levels of badger visitation to others and there is not sufficient replication by treatment combination to accommodate this variation. The economic aspect is an unconvincing appendage to the main experiment, which is unlikely to be generalisable. Therefore, the project needs to be reconsidered and redirected before it goes any further, so that the full potential of the project can be realised.

Scores
Conclusions based on sound evidence: 1.6
Quality of science: 1.8
Overall rating: 1.5

SE3109 Novel methods of estimating badger numbers in the wider countryside

Organisation University of Bristol

Start date 01/10/99
End date 31/12/03

Total cost £308,972

Abstract
Badgers have been implicated in the spread of bTB. By understanding the dynamics of TB in badger populations you may be able to identify the risk they pose to the spread of the disease to cattle. Consequently having quick and simple methods to estimate badger numbers in the field will help develop badger control, and hence bTB control, strategies and aid the prediction of future bTB problem areas. This project aimed to develop simple field techniques for estimating badger social group size which could subsequently be used to predict badger numbers. Using a two pronged approach – predicting badger numbers from field signs, and habitat data using GIS – results from the project suggested that apart from in arable landscapes, none of the sett or latrine variables monitored during the field survey were able to predict badger group size. Examination of models derived from both field and digital datasets suggested that this model performed better than other models that were developed with badger data alone and as such were more robust and transferable than previous models.

Aims
- To develop simple field techniques for estimating badger social group size from a variety of field signsTo relate badger numbers (in terms both of numbers of social groups and number of badgers) to habitat structure and use GIS to develop badger signatures.
- To test the accuracy of the models produced.
- To provide Defra with protocols that will enable the estimation of badger numbers.

Relevance to Defra
It is important that Defra is able to estimate badger density in a variety of different landscapes. Without such data, it will be impossible to implement effective badger control strategies, since field staff will not be able to gauge the success of any particular trapping operation. This project will provide a simple technique for Defra field staff to estimate badger-trapping efficiency and hence improve the efficiency of any badger culling.

Please note the following information is from the executive summary of the final report.

Approach

A dual approach was taken to address these objectives. Firstly, the relationship between a suite of badger signs and group size was studied using field data from a range of habitat types and badger densities in Britain. Secondly, correlates between badger density and habitat type (badger signatures) were identified based on both field and digital data using GIS. These two approaches were then merged to assess the potential for the combined use of field survey and digital habitat data to improve the predictive models.

Predicting badger numbers from field signs

Data on badger signs and group size were collected from badger social groups throughout UK. Data were collected by paid field staff and volunteers. Sett observations were used to provide estimates of the number of badgers living in a group. These data were scored for quality and data with the lowest quality scores were excluded from the subsequent analysis. Group size estimation at badger groups studied by field staff generally took the form of mark-resight estimates (Bailey's and Bowden's estimators).

Data collected on sett characteristics included location, type (main, annexe, subsidiary or outlier), number of entrances, habitat type, slope and soil type. The number of entrances and the activity status (well-used, partially-used or disused) of each were also recorded.

Volunteers were provided with guidelines as to how to carry out a baitmarking survey and were asked to record the location of all latrines found on an enlarged map of their study area. At each latrine, they were asked to record the total number of pits, faeces (or estimate when this was unclear), fresh faeces and the number of faeces containing coloured pellets. Paid field staff carried out baitmarking in exactly the same manner, except that all data were recorded using a GPS data recorder. When the data were received, a line was drawn around the outermost latrines containing coloured baitmarkers on the map, to estimate the territorial boundary. The baitmarking data were scored for quality and data with the lowest quality scores were excluded from the subsequent analysis.

The data were stratified by landscape in order to eliminate variation in the relationship between landscape types. The landscape types used were arable, pastoral, marginal upland and upland, derived from the Centre for Ecology and Hydrology's land classification system. Stepwise (backward) linear regression was used to select the variable, or combination of variables, which best predicted badger group size in each landscape type. The variables included in this analysis were number of well-used holes at main setts, number of latrines, number of dung pits and number of fresh droppings.

The model predicting badger numbers for arable landscapes was validated by surveying a new area. The validation site was located northeast of Tetbury in Gloucestershire, comprised 15.3 square km and encompassed 10 main badger setts (social groups).

Volunteers provided data on 104 badger social groups. A further 11 badger groups were studied by LMJS and eight by GNC. Hence, the total number of social groups which provided data was 123. Numerous other badger groups were studied but group size could not be successfully estimated for these groups. When the 123 records were assessed for quality, 63 scored '1'(good), 39 scored '2' (fair) and 21 scored '3' (poor). The regression analysis was run with just credit 1 and 2 data (*n* = 102).

Group size was smallest in pastoral landscapes and greatest in marginal upland, but the difference was not significant. Mean territory size was smallest for pastoral landscapes and greatest in marginal upland. The only significant model predicting badger numbers based on field signs was for arable landscapes. This model consisted of two predictor variables; the number of well-used holes at main setts and the number of fresh faeces (i.e. number of fresh faeces inside territory + number of fresh faeces on boundary/2). These two variables accounted for 84% of the variation in badger group size. The regression equation is: Number of adult badgers = 1.827 – (0.148 x number of well-used holes) + (0.342 x number of fresh droppings).

On the basis of this model for arable landscapes, 36 (95% CI 20-51) adult badgers were predicted to be living in the Tetbury validation area. The mean mark-resight estimate of population size was 33 (Bailey's estimate) or 34 (Bowden's estimate) adults.

Predicting badger numbers from habitat data using GIS

The aims of this part of the project were to develop logistic regression models that could be used to predict the distribution of badger main setts across Scotland, England and Wales, using habitat data recorded by surveyors during both the 1980s and 1990s national badger surveys. Further logistic regression models were also developed using data derived from digital sources, in order to assess the potential for predicting badger distributions in previously uncensused areas.

Data on the presence and absence of main setts in 1km squares across the UK were provided by the 1980s and 1990s national badger surveys. Environmental data were used as independent variables. These were from both field-based and digital datasets. Field-based data comprised the habitat (vegetation) data recorded at the same time as badger activity, during the surveys of each 1km square. Digital data comprised the habitat, soil, geology, topographical and road data for each 1km national badger survey square, extracted from digital datasets sourced from various environmental survey organisations in the UK.

Separate logistic regression models were developed for both field-based and digital data, and for each badger survey period. Four datasets were therefore carried forward to further analyses: '1980 field-based', '1990 field-based', '1980 digital' and '1990 digital'. The models were estimated using a backwards stepwise approach. Problems of multicollinearity were minimised by removing all variables with more than 90% of cases with zero values. Threshold-independent receiver operating characteristic (ROC) plots were used to identify the optimum probability threshold for assigning cases into dichotomous classes and therefore enhance the overall

prediction success of the models. Cohen's Kappa was selected to measure model performance.

Because robust measures of prediction success make use of independent data, i.e. data not used to develop the prediction model, the four datasets were each divided into two groups of data, one for training the model and the other for testing it.

The overall classification accuracies (training/testing) of the models were 71.9/68.6% (1980 field-based), 72.9/71.6% (1980 digital), 73.1/69.7% (1990 field-based) and 69.9/69.0% (1990 digital). For each of the four logistic regression models produced, a distribution map was generated in a GIS, showing the presences and absences in the entire datasets (testing and training combined).

The variables selected in both the habitat and digital models were very similar to those found significant in other badger studies. Positive predictors of main sett presence included the amount of pasture and broadleaved woodland in all models; hedgerows, treelines and semi-improved grassland in field-derived models; and hilliness, length of major roads, podzol and till soils in digital models. Negative predictors of main sett presence included the amount of heather moorland in field-derived models, and elevation and the amount of shrub heath in digital models.

The models generated from digital data were slightly more accurate than those generated from field-derived habitat data alone. This is probably due to the fact that the digital models were able to incorporate topographical, road, soil and geological data in addition to habitat data, which, in combination, were able to explain more of the observed variations. Obtaining the digital data is much less labour intensive than its field equivalent, although it does require specialist GIS skills and very high computing power and some datasets were expensive.

Almost 60% of all wildlife-habitat models published in refereed journals have no evaluation at all. Most of the previous badger models produced used poor validation techniques, and the majority of previous models developed to predict badger distribution and density are flawed since they focused on predicting the presence of badgers, and did not give equal emphasis to the prediction of absences. The fact that the models produced here are able to achieve classification accuracies of between 69 – 74% for test datasets across Britain, incorporating a variety of landscape types, suggests that these models are much more robust than previous models predicting badger distribution and numbers.

Predicting badger numbers using both field survey and digital habitat data

No previous model has attempted to combine field data on badger signs with digital habitat data to predict absolute badger numbers. Therefore the aim of this part of the research project was to develop predictive models using a combination of the field data on badger signs with the digital habitat data, in order to predict badger densities.

All of the data used were collected using the methods detailed above. A multiple linear regression model to predict the total number of adult badgers, based on all digital data and the number of active entrances at all main setts, was significant. Based on this model, the predicted number of badgers in the Tetbury study area was

within the 95% confidence limits of the Bailey's estimate based on the mark-resight data, but was some distance from the mean estimates produced by either Bailey's or Bowden's methods. None of the other multiple regression or Discriminant Function models performed well when tested against independent datasets.

Protocols for estimating badger numbers from field signs and digital habitat data

Field surveys should be conducted during spring as the abundance and detectability of badger field signs varies significantly throughout the year. All field boundaries should be searched on both sides all areas of scrub and woodland should be thoroughly searched. Badger tracks across open areas should be followed and searched for badger signs. All evidence of badgers should be recorded, either using a GPS data recorder or enlarged maps and data recording sheets. The formula in point 11 should be used to estimate badger numbers.

Digital habitat data should be extracted using a suitable GIS (e.g. ArcInfo / ArcView) from the following data sources: habitat (CEH Landcover Map 2000), soil and geology (Cranfield Natmap1000 for England and Wales; Macaulay Institute National Soil Map of Scotland), topography (Ordnance Survey Digital Elevation Model (Landform Panorama)), roads (Bartholomew Road Atlas). This should be done for each area for which the presence or absence of badger social groups needs to be predicted. Logistic regression models can be used to predict badger presence/absence. Prior to running the logistic regression, it is essential that variables with more than 90% zeros are removed. ROC plots should be used to maximise the efficiency of the classification coefficients.

Conclusions

This project took a new approach to developing an index of abundance. Rather than concentrate on a single or small number of study sites, we collected data from as wide a range of landscapes and badger densities throughout Britain as possible. The results of the stepwise regression based on the field survey data including badger signs indicated that apart from in arable landscapes, none of the sett or latrine variables were able to predict badger group size. The highly significant model produced for the arable landscapes accounted for a high proportion of the variation observed in badger group size. When this model was applied to data collected from a new area, it predicted the number of adult badgers with great accuracy.

For the predictive models based on badger presence/absence and habitat data from the national badger surveys, there were clear consistencies in the variables being selected using both field-derived and digital datasets. Overall, the models developed using both field-derived habitat data and digital data performed well in comparison to other badger models, and other logistic regression models predicting the presence and absence of various species. Since these models have been developed for the whole country, they are likely to be more robust and therefore more transferable than any previous models. By using digitally-derived data much larger areas of land could potentially be included in study areas, with the ultimate goal of predicting the distribution of badger setts across the whole of the UK. Building predictive models using the national badger survey data provides a good basis for extrapolation, since considerable effort was taken to collect an even spread of data from different habitat types in different parts of Britain.

The attempt to combine digital and field data on badger signs to predict the number of adult badgers was not as successful as the use of digital and field data independently. There may be some potential to use both digital and field-data to predict badger numbers, given further data, but whether such combined models would offer significant benefits over those produced solely from field or digital data is uncertain.

Review comment

The aims were important for Defra, since in planning counter-TB measures it is clearly advantageous to know the likely limits to badger density in different landscapes. It was encouraging to see a project that encompasses a large variety of landscape types and badger densities across Britain, including Wales. However, it was found that none of the sett or latrine variables was able to predict badger group size except in arable landscapes. The use of digital datasets to develop models to predict badger distribution was slightly more accurate than using purely field-derived habitat data. This shows some promise in providing badger population information at a large scale but is unlikely to give the accuracy at a local level that is needed for badger/TB studies. It was not sufficiently clear how the researchers determined the 'quality' of the data for inclusion in their analysis. In addition, the researchers' statistical expertise was not sufficient for the analyses undertaken and expert statisticians should have been collaborators. Despite the substantial efforts of this project, the overall policy aim was not achieved, namely to develop a novel, quick, easy and reliable method of estimating badger numbers in the field and survey protocols.

Scores

Conclusions based on sound evidence: 1.8
Quality of science: 2.0
Overall rating: 1.6

Annexes

Annex A

Review of Defra's Bovine TB R&D Programme
3rd – 6th July 2006
PROJECT APPRAISAL FORM

PROJECT NO.:	**ASSESSMENT COMPLETED BY:**
PROJECT LEADER:	

FOR ALL PROJECTS:

1. Given the information on the Department's objectives and the background of the Bovine TB R&D programme, do you think Defra should be funding this work?

Yes / No

If not please state your reasons:

2. Has the rate of progress in achieving scientific aims been adequate?

Yes / No

If not please state your reasons:

3. Has the rate of progress in achieving the policy aims been adequate?

Yes / No

If not please state your reasons:

3a. What value to Policy has this project delivered (**DEFRA TBD ONLY**)?

FOR PROJECTS NOT COMPLETED AT THE TIME OF THE REVIEW:

4. Do you think the project is likely to achieve all its stated aims?

Yes / No

If not please state your reasons:

FOR QUESTIONS 5 to 9 PLEASE AWARD SCORES AS FOLLOWS:

0 = Not at all 1 = Partial 2 = Satisfactory 3 = Good 4 = Extremely good

5. Soundness of methods	-Personnel	
	-Facilities	
6. Conclusions based on sound evidence		
7. Quality of science		
8. Value for money		
9. Overall rating (it is important to provide a score here)		

10. Overall opinions of the project:

11. Has the project raised further questions that need addressing with Defra funded research?

12. Are the areas within the project addressing topics of highest priority?

Yes / No

If not, which topics would you identify as high?

Signed: …… **Name:** ……

Date: ……

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Annex C ROAME A for 2003-2005

Tuberculosis R&D

A.1. Summarise the policy problems to be addressed

Defra has a statutory obligation to control tuberculosis in farm animals in Great Britain under the Animal Health Act of 1981, the Tuberculosis Orders, the Zoonoses Orders and various EC Directives (e.g. 64/432/EC - trade in cattle and pigs).

The main problem is TB in cattle and since 1937, apart from a brief interruption in 2001 because of the Foot and Mouth Disease epidemic, there has been a successful programme of regular testing of the national cattle herd using the single comparative intradermal tuberculin test. This resulted in a reduction in the incidence of bovine TB from 40% of cattle in the 1930s to less than 1% of total cattle herds with a confirmed incident in the 1990s. However, persistent pockets of disease remained in the South West of England and to a lesser extent elsewhere in Great Britain, and in the past few years the number of incidents has risen steadily with, in 2002, just over 4% of unrestricted herds suffering a confirmed new herd incident.

TB has also been found in wild mammals, particularly badgers, and from the 1970's until 1997 policy was based on the assumption that badgers can act as a reservoir for the disease and transmit it to cattle. Although the nature of this link was highly controversial, various badger control strategies were implemented during this period in an attempt to reduce the tuberculosis in the national cattle herd.

The Krebs Report commissioned in 1997 by Defra's predecessor, MAFF, made several recommendations for research and the appointment of independent experts, the Independent Scientific Group on Cattle TB (ISG), to monitor such research. These recommendations included the setting up of an experiment, the Randomised Badger Culling Trial (RBCT), to quantify the contribution badgers make to tuberculosis in cattle and assess the impact of culling badgers on the incidence of the disease in cattle.

It was also recommended that long-term research into development of a cattle vaccine and associated diagnostic tests to distinguish infected from vaccinated cattle should be put in place. Work on a badger vaccine should also be continued as an option. As a result of these and further recommendations including genome sequencing, epidemiological and ecological studies, a major programme of research was implemented. The total expenditure for Defra on bovine tuberculosis in the 2002/3 Financial Year is expected to be £75 million, comprising approximately £25 million on tuberculin testing, £32 million on compensation, £7million on the RBCT and £11 million on other research.

A.2. What are the policy objectives(s) for which you are considering commissioning research? (These objectives must be testable)

1. To gain a better understanding of the epidemiology of *Mycobacterium bovis* infection (TB) in cattle and wildlife.

2. To evaluate the benefits, costs and effectiveness of the present strategies that attempt to reduce occurrences of TB in cattle.

3. To develop better strategies to reduce occurrences of TB in cattle.

A.3. What are the scientific or technical objectives of the proposed research programme? (these objectives must be testable)

1. Improved diagnosis and detection of M. bovis.
   A. Investigate the performance of the tuberculin test in cattle under experimental and field conditions.
   B. Utilise the gamma interferon tests for *M. bovis* in cattle, incorporating existing or new diagnostic approaches and develop alternative immunological measurements.
   C. Determine the effect of freezing samples on the viability of *Mycobacterium bovis* prior to culture testing.
   D Develop and evaluate strain typing methods for *M. bovis*.

2. Additional control strategies to reduce the incidence of TB in cattle.
   A. Assess whether husbandry of cattle could be improved to reduce the risk from bovine TB.
   B. Assess the economic effects of bovine TB and its control at the farm level and to develop a framework for the assessment of the economic impacts of TB and its control on the agricultural sector and on the national economy.
   C. Study the kinetics of skin test response in bovine TB.
   D. Conduct a feasibility study into the prospects for developing successful biological control techniques to destroy *M. bovis* in the environment.
   E. Assess the effects of various TB control strategies on badger populations and subsequent disease spread to cattle.

3. Development of effective vaccines for use in cattle and badgers.
   A. Investigate vaccine candidates currently available for testing, such as BCG and crude antigenic derivatives of *M. bovis*, for the development of a vaccine for TB in cattle.
   B. Investigate the oral sensitisation of cattle with BCG and on the effect of vaccinating already infected animals particularly with respect to lesion development.
   C. Develop a live attenuated vaccine for TB in cattle, based upon a mycobacterial strain, genetically modified to ensure efficacy, stability and safety.
   D. Develop a vaccine for TB in cattle based upon antigenic elements of *M. bovis*.
   E. Investigate the immune responses of cattle to *M. bovis* with the aim of identifying antigens which may be useful in vaccination or diagnosis.
   F. Develop a diagnostic test to differentiate between infected and vaccinated cattle alongside the development of a cattle vaccine.
   G. Develop epidemiological models to evaluate the level of vaccine efficacy required to effect control of TB in cattle and badgers and to determine the level of efficacy required when additional control measures are used in addition to vaccination. Determine the cost benefit ratios of alternative control systems.
   H. Develop a vaccine for TB in badgers in collaboration with work under the cattle vaccine programme.

I. Develop oral/respiratory delivery systems that are effective in stimulating protective immune responses without capture of badgers.
J. Investigate the use of field studies on bait uptake and bait targeting in badgers and on the level of bait uptake by non-target species including cattle.

4. Assessing wildlife populations and the prevalence of *M. bovis.*
   A. Develop innovative methods to estimate badger populations by using field evidence of their activity.
   B. Develop innovative methods to relate badger abundance and effects of disturbance on badger populations.
   C. Produce an anatomical atlas of the lymphatic system of badgers.
   D. Develop tests, for use in the living badger and based on its cellular immune response, to establish *M. bovis* prevalence.
   E. Investigate the role of other wildlife species, in areas with high herd incident rates, in the transmission of TB to cattle using field studies and risk analyses.
   F. Investigate the extensive literature already available on BCG safety in wildlife species, based on laboratory experimentation.

5. Understanding the pathogenesis, transmission and modelling of tuberculosis in cattle and wildlife species.
   A. Integrate modelling approaches to better understand disease transmission over wide spatial scales and to assess the costs and benefits of different control strategies.
   B. Assess the correlates of local variation in risk associated with TB transmission between badgers and cattle, taking into account badger density, *M. bovis* prevalence, husbandry, climate and landscape variables.
   C. Develop sensitive and specific molecular typing techniques to enable a long-term study of TB transmission between wildlife, cattle and other species (including man) and to assess the variation of different genotypes of *M. bovis*.
   D. Develop novel improved research techniques to establish TB transmission routes and to help elucidate local variation in risk.
   E. Investigate the pathogenesis and transmission of tuberculosis in cattle.
   F. Investigate low dose TB infection in cattle, the dynamics of such disease and diagnostic strategies.
   G. Investigate sequence analysis of the *M. bovis* genome, to enable future work on improving diagnosis, control and advance understanding of the pathogenesis of TB.
   H. Carry out multivariate analysis of the risks associated with herd breakdowns.

A.4. Describe any alternative research means of fulfilling the Department's policy objectives. Have these alternatives been rejected and, if so, why?

There are a number of alternative research approaches which could be taken. For example, studies of climate and TB transmission, transmission via feedingstuffs, concurrent diseases, such as BVD and BIV, and trace element deficiencies. However, Defra has been advised by the ISG and others that these areas are not yet sufficiently promising or are areas not sufficiently amenable to research studies to merit inclusion, given resource pressures and competing priorities.

The current RBCT and associated research covers all the aspects required by the Krebs Report and recommended by the ISG.

A.5. Are there non-research ways of achieving these objectives? If so, why is R&D considered appropriate?

Research is the only method of achieving these objectives.

The following additional TB control measures could be considered whilst the research programme is in progress, and their effect could be measured.

1. The introduction of more stringent cattle testing procedures including a statutory requirement for pre- and post-movement testing of cattle.

2. The introduction of a statutory or farm licensing requirement rather than solely the current advice for improved farm husbandry practices in order to reduce the risks to cattle from possible wildlife contact. Until the RBCT ends in 2005/6, there is a moratorium on any badger culling outside trial areas, other than in exceptional circumstances under license as stated in the Protection of Badgers Act 1992.

A.6. It may be possible to get the research results by buying in the information from other countries or by participating in internationally funded and organised research. Have these alternatives been considered and/or rejected and, if so, why?

Although wildlife reservoirs for TB are found in other countries, the actual species differ. Thus, research into the control of the disease, whilst complimentary, is not specific enough for direct application to the GB situation. Collaboration already exists in areas of mutual benefit, particularly with the Republic of Ireland, Northern Ireland and New Zealand, which have many similar problems to GB. This extends to vaccine development in cattle and badgers, work on pathogenesis of TB in cattle and the development of new tests in cattle, such as the gamma interferon test. Contact is also maintained with research groups from Australia and the Institut Pasteur in France where genome work is being carried out.

The essential laboratory support services and the necessary multidisciplinary approach for research to control tuberculosis has centred on Defra's Veterinary Laboratories Agency and has led to an internationally recognised centre of expertise. In addition, most of the new research programme has been put out to open competition to ensure input from the best expertise throughout the UK and the international research community.

A.7. Why should Defra fund the research rather than private industry or other public bodies?

Defra has a statutory responsibility to control bovine TB in cattle for reasons of animal health and welfare, protection of human health and to support the export of animals and their products (Defra is the competent authority for a number of EU Directives and Regulations). Some fundingfor the GB bovine TB research programme does come from other funding bodies such as BBSRC and the Wellcome Trust. However, as bovine TB is a statutory disease and covered by EU legislation, it is appropriate that Defra funds the majority of such research.

Annex D Defra funded research

Project code	Title	Contractor(s)	Start date	End date	Total cost
SE3001	A spatial analysis using GIS of risk factors associated with TB Incidents in cattle herds in England and Wales	VLA	01/01/1999	31/05/2003	£188,373
SE3002	Ecological Correlates of Tuberculosis Incidence in Cattle	University of Warwick	16/08/1999	15/12/2003	£436,784
SE3006	Quantification of the risk of transmission of bovine TB from badgers to cattle within localised areas	VLA	01/04/1999	31/12/2002	£167,504
SE3007	Integrated modelling of *M. bovis* transmission in badgers and cattle	CSL	01/04/1999	31/03/2003	£902,769
SE3008	Detection and enumeration of *Mycobacterium bovis* from clinical and environmental samples	VLA	01/04/1999	31/12/2004	£548,808
SE3009	The Risk to Cattle from *Mycobacterium bovis* Infection in Wildlife Species other than Badgers	University of Oxford	01/05/1999	30/04/2004	£1,214,788
SE3010	The risk to cattle from wildlife species other than badgers in areas of high herd breakdown risk	CSL	01/01/2000	28/02/2004	£762,623
SE3013	Pathogenesis and diagnosis of tuberculosis in cattle - complementary field studies	VLA	01/10/2000	30/09/2005	£2,850,729
SE3015	Mycobacterium bovis pathogenesis	IAH, University of Belfast, VLA	01/07/2000	31/03/2004	£2,440,159
SE3017	Development and evaluation of strain typing methods for *Mycobacterium bovis*	VLA	01/09/1999	31/03/2005	£1,275,223
SE3020	An integrated approach to the application of *M. bovis* genotyping for the control of bovine tuberculosis in GB	VLA	01/04/2001	30/09/2004	£927,801

SE3024	Low dose TB infection in cattle: disease dynamics and diagnostic strategies	QUB, VLA	01/10/2002	30/09/2006	£2,560,207
SE3026	Bovine TB transmission in restocked herds: risk factors and dynamics	University of Warwick	01/06/2002	31/03/2006	£1,114,496
SE3027	Pathogenesis and immunology of *Mycobacterium bovis* infection in cattle	IAH	01/04/2002	30/09/2005	£1,506,135
SE3028	The development of improved tests for the diagnosis of *Mycobacterium bovis* infection in cattle	VLA	01/04/2002	31/03/2005	£428,428
SE3029	An investigation of potential badger/cattle interactions and how cattle husbandry methods may limit these	CSL	01/01/2003	31/12/2005	£556,851
SE3030	Application of postgenomics to reveal the basis of virulence, pathogenesis and transmissability of *M. bovis*	VLA	01/04/2001	31/03/2006	£3,318,624
SE3032	The long term intensive ecological and epidemiological investigation of a badger population naturally infected with *Mycobacterium bovis*	CSL	01/04/2003	31/03/2006	£1,761,990
SE3033	Housing of naturally infected cattle (field reactors) at VLA for immunological and bacteriological analysis	VLA	01/04/2004	30/09/2007	£775,076
SE3034	Exploratory investigation of cattle movement records in Britain to enhance animal disease surveillance and control strategies	University of Oxford	01/06/2003	31/03/2004	£84,780
SE3035	Estimating badger density in RBCT proactive and control areas	CSL	01/05/2005	30/04/2006	£153,346
SE3036	A QRA on the role of wild deer in the perpetuation of TB in cattle	CSL	04/07/2005	31/10/2006	£146,656
SE3037	A QRA on the role of wild deer in the perpetuation of TB in cattle	Risk Solutions	01/07/2005	20/01/2006	£49,718
SE3107	Develop Innovative Methods to Estimate Badger Population Density	CSL	01/04/1999	31/03/2005	£1,150,521
SE3108	An Integrated Study of Pertubation, Population Estimation, Modelling and Risk	CSL, University of Oxford	01/04/1999	31/03/2004	£1,376,056
SE3110	A molecular genetic analysis of badger social structure and bovine TB	CSL	01/01/2000	31/12/2006	£1,094,055

SE3112	Assessment of the economic impacts of TB and alternative control policies	University of Reading	01/05/2001	31/04/2004	£156,959
SE3116	The economic value in changes in badger populations	University of Reading	01/06/2003	31/11/2004	£75,330
SE3117	Cost-benefit analysis of badger control	CSL	16/08/2004	31/05/2007	£443,714
SE3118	Review and economic analysis of the use of PCR assays for *M. tb* complex	VLA	01/07/2005	31/12/2005	£46,506
SE3119	Cost effectiveness of farm husbandry manipulations	CSL	01/11/2005	31/10/2009	£1,114,730
SE3206	Genome sequence analysis of *Mycobacterium bovis*	VLA	01/01/1999	31/03/2005	£1,156,293
SE3208	Generation of Vaccine Candidates Against *Mycobacterium bovis*	VLA	01/04/1999	31/03/2005	£1,566,005
SE3209	Testing of Vaccine Candidates for Bovine Tuberculosis using a Low Dose Aerosol Challenge Guinea Pig Model	VLA	01/07/1999	30/06/2004	£1,068,045
SE3212	Testing TB Vaccines in Cattle	VLA	01/04/1999	31/03/2005	£1,609,963
SE3215	Development of immunological assays for the detection of *Mycobacterium bovis* infection in badgers	VLA	01/04/2002	31/03/2005	£525,041
SE3216	Development and testing of vaccines against badger tuberculosis	VLA	01/04/2002	31/03/2005	£477,994
SE3217	Kinetics of skin test response in bovine TB	IAH	01/04/2004	31/03/2005	£252,100
SE3220	Analysis of the PPD diagnostic reagent	VLA	01/10/2005	30/09/2007	£274,970
SE3221	Volatile organic compound analysis for the rapid diagnosis of disease	VLA	01/01/2006	31/12/2008	£457,390
SE3222	Development of improved diagnostic tests for detection of bovine TB	VLA	01/07/2005	30/06/2008	£1,907,392
SE3223	Development of an oral BCG vaccine bait formulation for badgers	VLA	01/01/2006	30/12/2008	£1,691,251
SE3224	Continuation of cattle vaccine development	VLA	01/04/2005	30/09/2008	£5,622,823
SE3225	In depth histopathology of lymph node granulomas in natural and experimental cattle TB	VLA	01/10/2005	30/09/2006	£46,590

SE3226	Development of tools to study immunopathology in badger TB	VLA	01/10/2005	30/09/2006	£44,036
SE3227	Evaluation of the protection efficacy of vaccines against bovine TB in a natural transmission setting	VLA	01/10/2005	31/03/2011	£6,781,127
SE3228	A safety study of BCG vaccine in wild badgers - prep work	VLA	01/04/2005	30/12/2005	£478,375
SE3229	Enhanced modelling and prediction of the spread of bovine TB in mainland Britain impacts of cattle movements, climate and spoligotype	VLA/ERGO	01/11/2005	31/10/2007	£588,361
ZF0531	The ecological consequences of removing badgers from an ecosystem	CSL	01/02/1999	31/03/2007	£1,846,627

Annex E Work supported by other funders

This annex contains a summary of on-going and completed bTB research funded by the Biotechnology and Biological Sciences Research Council (BBSRC), Department of Agriculture and Rural Development Northern Ireland (DARDNI), Food Standards Agency (FSA), Wellcome Trust and the Welsh Assembly Government.

Further details may be obtained directly from the funding bodies should they be required.

BBSRC

Title	Institution	Start date	End date	Cost to date
Institute				
Milkalyser: sensors to allow integrated monitoring systems for dairy cows	Silsoe Research Institute	01/07/2002	31/12/2002	£74,200
Antigen presenting cell populations in cattle	Institute for Animal Health	01/04/2005	31/03/2008	£83,968
Proteomic signatures of bovine tuberculosis	Institute for Animal Health	01/10/2004	30/09/2007	£2,035
Molecular analysis of the interaction of intracellular pathogens with bovine antigen presenting cells and host immune responses	Institute for Animal Health	01/04/2003	31/03/2006	£312,216
Characterisation of bovine natural killer (NK) cells and their role in determining immune bias	Institute for Animal Health	15/02/2003	14/02/2006	£19,224
Low dose TB infection in cattle: Disease dynamics and diagnostic strategies	Institute for Animal Health	01/10/2002	30/09/2006	£1,583
Pathogenesis and immunology of *Mycobacterium bovis* infection in cattle	Institute for Animal Health	01/04/2002	30/09/2005	£109,243
Integrated management systems using biosensors	Silsoe Research Institute	01/04/2003	30/09/2005	£704,328
Grants				

Laser microdissection microscope for Institute for Animal Health, Pirbright	Institute For Animal Health	21/06/2002	21/06/2003	£51,644
The programme underlying dormancy and autocrine resuscitation in *Mycobacterium bovis* BCG	University of Wales, Aberystwyth The University of Manchester	01/05/2001	30/06/2004	£289,436
Identification of serum biomarkers for bovine TB	Imperial College London	01/12/2003	31/03/2006	£181,388
Characterisation of bovine natural killer (NK) cells and their role in determining immune bias	Institute For Animal Health	01/04/2003	31/03/2006	£194,972
Cell sorting for functional and post-genomic analysis of rare immune and infected cells	Institute For Animal Health	01/10/2005	30/09/2006	£110,000
The biology of environmental *Mycobacterium bovis*, and its significance to the epidemiology of bovine tuberculosis	University of Warwick	01/05/2004	30/04/2007	£404,850
Proteomic signatures of bovine tuberculosis	Institute For Animal Health	01/10/2004	30/09/2007	£17,065
Proteomic signatures of bovine TB	University of Southampton	09/05/2005	08/05/2008	£210,018
Immune inductor and effector sites in the upper airways of cattle and influence of site of antigen expression on induction of mucosal immunity	Institute For Animal Health	02/01/2006	01/01/2009	£321,463
Do post-receptor binding events decide the fate of mycobacteria in bovine macrophages?	Institute For Animal Health Royal Veterinary College	03/01/2006	02/01/2009	£202,526
Studentships				
Determination of the roles of major secreted *M. bovis* proteins in bovine tuberculosis pathogenesis	University of Leicester	28/09/2004	27/09/2005	£15,050
Non-linear frameworks for epidemic and endemic diseases eg FMD and bovine TB	University of Warwick	29/09/2002	28/09/2005	£36,250
The nature of CD8 T cell memory in bovine tuberculosis	University College London	01/10/2002	30/09/2005	£36,250
Oxidative stress response and its role in bovine tuberculosis	University of Reading (work address Institute for Animal Health)	01/10/2001	30/09/2004	£35,000

DARDNI

Title	Duration	Cost to date
Mycobacterium bovis antigen studies in relation to bovine immune responses	From 1996-	£817,671
Early cell-mediated immune responses in bovine tuberculosis	From 1996-	£755,876
M. bovis strain typing applications: molecular epidemiological studies of bovine tuberculosis and biological properties of Mycobacterium bovis strains	From 2000-	£635,887
Estimation of the prevalence of tuberculosis in badgers from road traffic accident (RTA) casualties	From 1999-	£102,844

FSA

Title	Duration	Cost to date
Methods for the determination of Mycobacterium bovis within edible tissues from TB-suspect cattle following slaughter and salvage	From April 2002-June 2003	£141,224

Wellcome trust

Title	Duration	Cost to date
Bovine Tuberculosis in the developing world	5 years	£2,160,022

Welsh Assembly Government

Title	Duration	Cost to date
Found Dead Survey	Minimum of 1 year but no maximum set	Work ongoing therefore no finite figure
Wildlife Survey	Approx. 6 months but with possibility of extension	£370,000
Case-Control Study 2005	Data collection finished. Due to report mid 2006	£75,000