Science and Research Projects

Return to Science Search homepage Return to Project List

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

Description

Attempts to control bovine tuberculosis in GB are hampered by lack of scientific knowledge in several key areas: (1). A true picture of the pathology, immunology and clinical pathology of reactor cattle; (2). The extent to which ongoing infection in herds is due to cattle to cattle spread being maintained by animals falsely testing negative to the skin test.
By undertaking detailed pathological, clinico-pathological, bacteriological and immunological examination of 200 reactor and 200 in contact non-reactor cattle, it will be possible to define many of the parameters critical for experimental and modelling studies. These include, lesion localisation, the extent of nasal shedding in the early stagers of disease and the role of concurrent infection as a covariate in pathogenesis. The detailed immunological profiling will assist in validating markers of early disease and pathology, which should help to detect animals in the early stages of infection. All results arising from the study will be closely integrated by using advanced systems of data capture, transfer and storage, and the experience gained should assist MAFF in other complex field studies, such as the current Krebs' trial.

Objective

01 31/03/2001 Develop intranet based method for data capture and transfer

02 31/03/2004 Undertake 200 detailed post-mortem examinations of reactor cattle

03 31/03/2004 Undertake relevant histological, microbiological and clinical pathological studies of samples collected from the autopsied animals

04 31/03/2004 Undertake immunological testing on 200 in contact non-reactor cattle followed by a detailed PME and relevant histological, microbiological and clinical pathological studies. The purpose of this study is to:
a) determine how commonly in contact SICCT-negative animals in multi-reactor herds are in fact infected or show an immune response to TB identified by other tests.
b) Investigate factors that may be involved in the failure of some animals to respond to the SICCT

05 31/05/2004 Undertake epidemiological and statistical analyses of the total study

Project Documents

• **Final Report :** Pathogenesis and diagnosis of tuberculosis in cattle - complementary field studies (2516k)

Time-Scale and Cost

From: 2000

To: 2005

Cost: £2,850,729

Contractor / Funded Organisations

Veterinary Laboratories Agency

Keywords

Animal Health
Biotechnology
Bovine Tuberculosis
Pathogenesis
Plants and Animals
Zoonoses

General enquiries on this form should be made to:
Defra, Science Directorate, Management Support and Finance Team,
Telephone No. 020 7238 1612
E-mail: research.competitions@defra.gsi.gov.uk

SID 5 Research Project Final Report

- **Note**
  In line with the Freedom of Information Act 2000, Defra aims to place the results of its completed research projects in the public domain wherever possible. The SID 5 (Research Project Final Report) is designed to capture the information on the results and outputs of Defra-funded research in a format that is easily publishable through the Defra website. A SID 5 must be completed for all projects.

  A SID 5A form must be completed where a project is paid on a monthly basis or against quarterly invoices. No SID 5A is required where payments are made at milestone points. When a SID 5A is required, no SID 5 form will be accepted without the accompanying SID 5A.

- This form is in Word format and the boxes may be expanded or reduced, as appropriate.

- **ACCESS TO INFORMATION**
  The information collected on this form will be stored electronically and may be sent to any part of Defra, or to individual researchers or organisations outside Defra for the purposes of reviewing the project. Defra may also disclose the information to any outside organisation acting as an agent authorised by Defra to process final research reports on its behalf. Defra intends to publish this form on its website, unless there are strong reasons not to, which fully comply with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.

  Defra may be required to release information, including personal data and commercial information, on request under the Environmental Information Regulations or the Freedom of Information Act 2000. However, Defra will not permit any unwarranted breach of confidentiality or act in contravention of its obligations under the Data Protection Act 1998. Defra or its appointed agents may use the name, address or other details on your form to contact you in connection with occasional customer research aimed at improving the processes through which Defra works with its contractors.

Project identification

1. Defra Project code: SE3013

2. Project title:
   Pathogenesis and diagnosis of tuberculosis in cattle - complementary field studies

3. Contractor organisation(s):
   Veterinary Laboratories Agency
   Woodham Lane
   New Haw
   Surrey
   KT15 3NB

54. Total Defra project costs: £ 2,850,729

5. Project: start date..... 01 October 2000

   end date..... 31 September 2005

6. It is Defra's intention to publish this form.
Please confirm your agreement to do so.....**YES** ☒ **NO** ☐

(a) When preparing SID 5s contractors should bear in mind that Defra intends that they be made public. They should be written in a clear and concise manner and represent a full account of the research project which someone not closely associated with the project can follow.
Defra recognises that in a small minority of cases there may be information, such as intellectual property or commercially confidential data, used in or generated by the research project, which should not be disclosed. In these cases, such information should be detailed in a separate annex (not to be published) so that the SID 5 can be placed in the public domain. Where it is impossible to complete the Final Report without including references to any sensitive or confidential data, the information should be included and section (b) completed. NB: only in exceptional circumstances will Defra expect contractors to give a "No" answer.
In all cases, reasons for withholding information must be fully in line with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.

(b) If you have answered NO, please explain why the Final report should not be released into public domain

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

The aim of the study was to advance the understanding of cattle-to cattle transmission of bovine tuberculosis (bTB) in Great Britain through detailed pathological and immunological investigation of cattle naturally infected or exposed to *Mycobacterium bovis*. The population sample comprised 200 cattle classified as reactors according to standard interpretation of the single intradermal comparative tuberculin test (SICTT) and 200 non-reactors ("in-contacts") which had been exposed to cattle with confirmed bovine tuberculosis, and would be classified as dangerous contacts by the State Veterinary Service (SVS). The cattle were selected from herds in England and Wales between 2002 and 2005. The reactors were randomly selected from herds with new bTB breakdowns whereas the in-contacts were selected from herds with a history of persistent recurring confirmed bTB. Selection of reactors and in-contacts occurred concurrently according to a rolling schedule over the three year data collection period.

The study design was cross-sectional with a nested cohort. Following selection and purchase, blood samples were drawn and a questionnaire administered on the farm by the SVS. Reactor cattle were then dispatched to a VLA Laboratory where an additional blood sample was drawn, nasal mucus collected and the animals humanely killed. A detailed post mortem examination was conducted. All suspect and typical bTB lesions were sent for bacterial culture and histopathological examination. The blood samples were subject to immunological assays for humoral and cell-mediated immunity, serological assays to measure past exposure to diseases and assays for levels of the trace nutrients selenium, copper and vitamin B12. A sample of livers were also sent for trace nutrient analysis. The in-contacts were subject to identical procedures as reactors except before euthanasia the animals were held and prospectively monitored for seven weeks in isolation at an ADAS Ltd research farm in Boxworth. During this period four blood samples were drawn and up to seven nasal samples collected at intervals with the aim to detect early stage disease and *M. bovis* excretion. At the end of the holding period another SICTT was performed and the in-contacts were transported to a VLA laboratory for detailed PM, culture and histopathological examination. All procedures were conducted in accordance with the Animal (Scientific Procedures) Act 1986 and standard codes of practice.

Results

At the detailed post mortem, macroscopic visible lesions characteristic of bTB were observed in 28/200(14%) of in-contacts and 111/200 (55.5%) of reactors. Overall, bTB was confirmed by culture and/or histology in 23/200 (11.5%) of in-contacts and 110/200 (55%) of reactors. The mean number of lymph nodes with TB-like lesions within each animal with confirmed bTB was 1.5 in in-contacts and 1.7 in reactors. Not all cattle infected with *M.bovis* had gross lesions typical of bTB. Bovine TB was confirmed in seven in-contacts (3.5%) without visible lesions and 12 reactors (6%) without visible lesions. No lesions characteristic of bTB were observed in tissues not specified in the Fresh Meat (Hygiene and Inspection) Regulations 1995.

Macroscopic lesions typical of bTB were most commonly found in the thorax of both in-contacts (43.5%) and reactors (55.5%) with bTB confirmed by culture or histology. In-contacts were more likely to have lesions in the abdomen (21.7%) than reactors (10.0%) and less likely to have lesions in the head (21.7 versus 40.9%). In reactors, lesions in the abdomen were rarely found without the existence of other lesions in the head or thorax.
Within the thoracic region, lesions were most commonly found in the caudal mediastinal lymph node and the left tracheobronchial lymph nodes. Within the head, lesions were most commonly observed in the medial retropharyngeal lymph nodes.

The distribution of lesions and bTB confirmation varied between production classes in both in-contacts ($p<0.05$) and reactors ($p<0.001$). Dairy cows were less likely to have visible lesions and confirmed bTB than other production classes, even after adjustment for the immunological response measured by SICTT or Interferon-γ.

M. bovis was not detected by bacterial culture in any of 1006 nasal mucus samples collected. None of the animals had more than one spoligotype isolated from any of the samples culture positive for *M.bovis*.

Immunological responses measured in blood drawn at recruitment were examined by categories of bTB severity. Interferon-γ, IL-10, and MPB83 serological responses were higher in in-contacts and reactors with confirmed bTB compared to other animals. Blood based immunological tests conducted on the in-contacts were better able to identify animals subsequently shown to have culture or histology confirmed bTB than the additional skin test conducted at the end of the holding period, even when applying the severe interpretation of the skin test. The most powerful predictor for confirmed bTB was a positive response to both PPD (Bovine-Avian) and ESAT-6/CFP-10 derived synthetic peptides with an odds ratio of 76.0 (95%CI 12.8 – 750.8).

Having antibodies to Johne's disease was associated with having confirmed bTB in both dairy (adjusted OR 3.6, 95%CI 1.1-12.4, p=0.038) and non-dairy reactors (aOR 5.4, 95%CI 1.7 -17.6, p=0.005) and the association in non-dairy in-contacts was in the same direction. There was a consistent negative association between having antibodies to liver fluke and having confirmed bTB in both in-contacts and reactors and most significantly in dairy reactors (aOR 0.2, 95%CI 0.1 – 0.7, p=0.016).

Levels of the enzyme GSPHx, a surrogate for plasma selenium, were lower in in-contacts than in reactors and lower in animals with confirmed bTB than in animals without confirmed bTB. In a logistic regression model adjusting for confounders, animals in the lowest two quintiles for selenium exposure (levels below 65 u/mL RBC) had an almost three fold higher risk of having confirmed bTB aOR 2.85 (95%CI 1.21-6.71). There was also an association between low in liver selenium and increased risk of bTB confirmation although it was not statistically significant (0.95 per μmol/kg DM (95%CI 0.9-1.0)).

Implications

The study provides a detailed description of the pathology typical of bTB in naturally infected cattle in England and Wales. Pathology observed in experimental studies can be compared to these data. The concentration of pathology in the thorax suggests that experimental protocols targeting the lower respiratory tract are likely to be most representative of natural infections. Increased pathology in the abdomen of in-contact animals could indicate a particular pathology less likely to be detected by the SICTT. The absence of *M. bovis* in any of the nasal mucus samples suggests that shedding via this route is infrequent in naturally infected cattle in England and Wales, and other shedding routes should be investigated.

A detailed PM will not identify all animals infected with *M .bovis*. Additional animals are detected by culture and immunological tests. A significant number of infected cattle not detected by the SICTT may be detected by blood-based tests and the combination of the avian/bovine PPD and ESAT-6/CFP-10 derived peptides in the BOVIGAM assay is highly predictive of infection. A heightened immunological response can be measured in the blood based tests within three weeks of a negative SICTT. The findings suggest that use of blood-based immunological tests in reducing transmission from infected skin test negative animals should be investigated. The lower risk of bTB confirmation in dairy animals with the same level of immunological response to other production classes requires investigation.

Consistent negative associations between low levels of GSPHx in blood and selenium in liver and a higher risk of an animal being infected with *M. bovis* require further investigation; particularly to establish whether the effect is causal or a consequence of disease. The lower risk of confirmed bTB related to previous exposure to liver fluke also requires further investigation.

A web-based project support system was developed and implemented successfully and demonstrated the utility of this technology for multi-centre epidemiological studies.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:
   - the scientific objectives as set out in the contract;
   - the extent to which the objectives set out in the contract have been met;
   - details of methods used and the results obtained, including statistical analysis (if appropriate);
   - a discussion of the results and their reliability;
   - the main implications of the findings;
   - possible future work; and
   - **any action resulting from the research (e.g. IP, Knowledge Transfer).**

Contents **page**

1.0 Introduction

Cattle-to-cattle transmission of *Mycobacterium bovis* (*M. bovis*) is a recognised means of introduction of bovine tuberculosis (bTB) into disease-free herds (Stamp 1944; Wilesmith 1983). The importance of the role of cattle-to-cattle transmission in the spread and persistence of bTB in contemporary British herds has gained more credence in recent years (Cox and others 2005; Goodchild and Clifton-Hadley 2001) Cox, 2005 #63}. At the time of the original project proposal the Independent Scientific Group on cattle TB initiated a program of pathogenesis research, with the assessment of "the relative importance of cattle-to-cattle transmission" as a high priority, emphasising possible deficiencies of the tuberculin

skin test and the possibility that infected animals were passing into herds undetected (Bourne and others 2001 ; Morrison and others 2000; Vordermeier and others 2004). It was also recognised that detection of bTB during abattoir inspection was not a sufficiently sensitive means of sentinel surveillance (Whipple and others 1996). Recognition of the deficiencies in contemporary routine methods of surveillance also led to a call for new tests to improve detection of infected animals (Bourne and others 2001; Vordermeier and others 2004).

The accuracy of bTB diagnosis is influenced by the stage to which the disease has progressed at the time the test is performed. For example, the accuracy of the tuberculin test has been reported to vary with stage of disease (Monaghan and others 1994), and abattoir examination relies on the observation of gross bTB-like lesions in lymph nodes or lung traditionally associated with the development of the disease . After *M.bovis* was identified as the causative organism of bTB extensive research into the pathogenesis of the disease continued to the middle of the 20th Century (Stamp 1944). However, studies of bTB pathogenesis became rarer with the introduction of the test-and-slaughter strategy based upon the tuberculin skin test in the 1950's. The introduction of the tuberculin test also meant that infected animals were more likely to be identified at an early stage of disease (Krebs and others 1997), and the relevance of historical observations from post-mortems of animals with overt clinical disease, may be limited when applied to disease observations today.

In the 1980s, a team in Northern Ireland successfully used both experimental and field-based studies to develop an enhanced understanding of routes of infection and the immunological reactions in animals infected in the UK (Neill and others 2001; Neill and others 1994). Since then, a number of experimental studies have been conducted in Northern Ireland and GB comparing different routes of infection by *M. bovis* and describing the pathological and immunological characteristics of the disease. The present study was explicitly designed to complement these studies and to provide a detailed description of the pathological and immunological responses in naturally infected animals in England and Wales.

The study began formally in October 2000 with a six-month lead-in to develop the protocols and the web-enabled database. Selection of the animals was planned to begin in April 2001 but the outbreak of Foot and Mouth Disease (FMD) in February 2001 resulted in suspension of work, and the selection of the first animals and processing of samples did not start until May 2002. By April 2005 all 400 animals had been selected into the study, and data collection was completed by July 2005.

Successful execution of the project's wide ranging and extensive protocols was only achieved through the co-operation of over 100 individuals representing four organisations:

- The Veterinary Laboratories Agency (VLA) – Weybridge and the regional laboratories of Bury St Edmunds, Luddington, Truro, Shrewsbury, Winchester and Starcross;
- The Institute of Animal Health (IAH);
- The State Veterinary Service (SVS) from Animal Health Offices in Cardiff, Carmarthen, Exeter, Gloucester, Stafford, Taunton, Truro and Worcester; and
- ADAS Ltd.

1.1 Scientific Objectives

As stated in the original research proposal, the project had five objectives:

1. Develop a web-based method for data capture and transfer
2. Undertake 200 detailed post-mortem examinations of reactor cattle
3. Undertake relevant histological, microbiological and clinical pathological studies of samples collected from the autopsied animals
4. Undertake immunological testing on 200 in-contact non-reactor cattle followed by a detailed PME and relevant histological, microbiological and clinical pathological studies
5. 15/03/2007Undertake epidemiological and statistical analyses of the total study

Scientific aims included the following:

1. Provide a detailed description of the lesion distribution in naturally infected animals

2. Assess the likely efficacy of the current abattoir post-mortem inspection
3. Describe the immunological profile of disease
4. Identify alternative immunological tests that might identify animals missed by the skin test
5. Assess whether skin test negative animals were likely to be a cause of prolonged breakdowns
6. Assess whether "intercurrent disease" is associated with reactor and/or TB status
7. Assess whether trace nutrient deficiencies are associated with reactor and/or bTB status
8. Identify other novel factors that could be relevant to bTB pathogenesis
9. Assess whether internet protocols are an effective way to conduct multi-centre epidemiological studies

2.0 Materials and methods

2.1 Animal selection

Two groups of animals were selected: a "reactor" sample comprising 200 single intradermal comparative tuberculin test (SICTT) positive animals and an "in-contact" sample comprising 200 SICTT negative animals which had been in direct contact with animals with confirmed bTB. The SICTTs were performed by official Veterinary Officers (VOs) and Veterinary Inspectors (LVIs and TVIs) from the State Veterinary Service (SVS) (specified in the European Economic Community Directive 80/219EEC, amending directive 64/432/EEC, annex B) as part of the routine herd testing program in GB.

Selection of cattle for the project was undertaken by Veterinary Officers and Inspectors according to a prescribed protocol. The protocol was designed to ensure selection of the required number of cattle meeting selection criteria over a three year period allowing sufficient time for processing in available facilities. Targets for Animal Health Offices (AHOs) were calculated from bTB surveillance data for the year (2000), which was the year before planned initiation of animal recruitment. At project start-up, AHOs were given monthly rolling targets for reactor and/or in-contact animals and based on these targets they selected animals meeting selection criteria from the breakdown notifications that came in each week. While the study aimed to ensure the reactor animals were a random sample of newly exposed and infected cattle in the bTB endemic areas, the selection of the in-contact animals was designed to maximise the chance of selecting animals exposed to *M. bovis*, but at an early stage of infection.

Inclusion criteria for animals in the reactor sample were:

ι) Animal was from a new breakdown herd

ιι) Animal was a reactor according to standard interpretation of the SICTT

The animals were selected so that equal proportions came from three breakdown groupings: herds with one reactor; herds with 2-5 reactors; and herds with 6 or more reactors. In herds with more than one reactor, the reactor selected was the animal with an eartag whose last digits most closely matched a random number generated by the project management system "Pathman".

Inclusion criteria for animals in the in-contact sample were:

i) Animal would meet the criteria for "dangerous contact" by the normal test and epidemiological criteria (VIPER Chapter 23, Sections H)

ii) Animal was from a herd with a history of repeated breakdowns and/or successive short interval tests with multiple reactors

iii) Animal had had a negative SICTT at standard interpretation

iv) Animal could be removed to the holding farm at Boxworth in Cambridgeshire within 14 days of skin test reading

In addition, because of welfare and health and safety requirements for transport and holding of the animals at Boxworth, various exclusions were imposed: animals less than three months old, bulls (entire males>12 months old), cows close to calving or in early lactation.

The locations of the animals at time of selection are shown in figure 1.

Figure 1. Source of animals by county

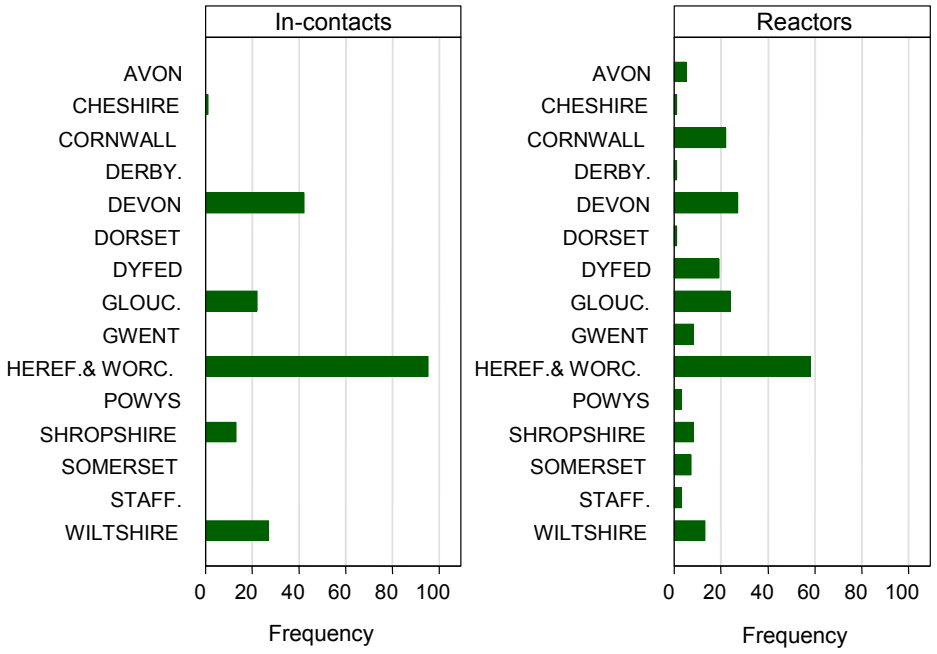


Table 1 Characteristics of animals at selection

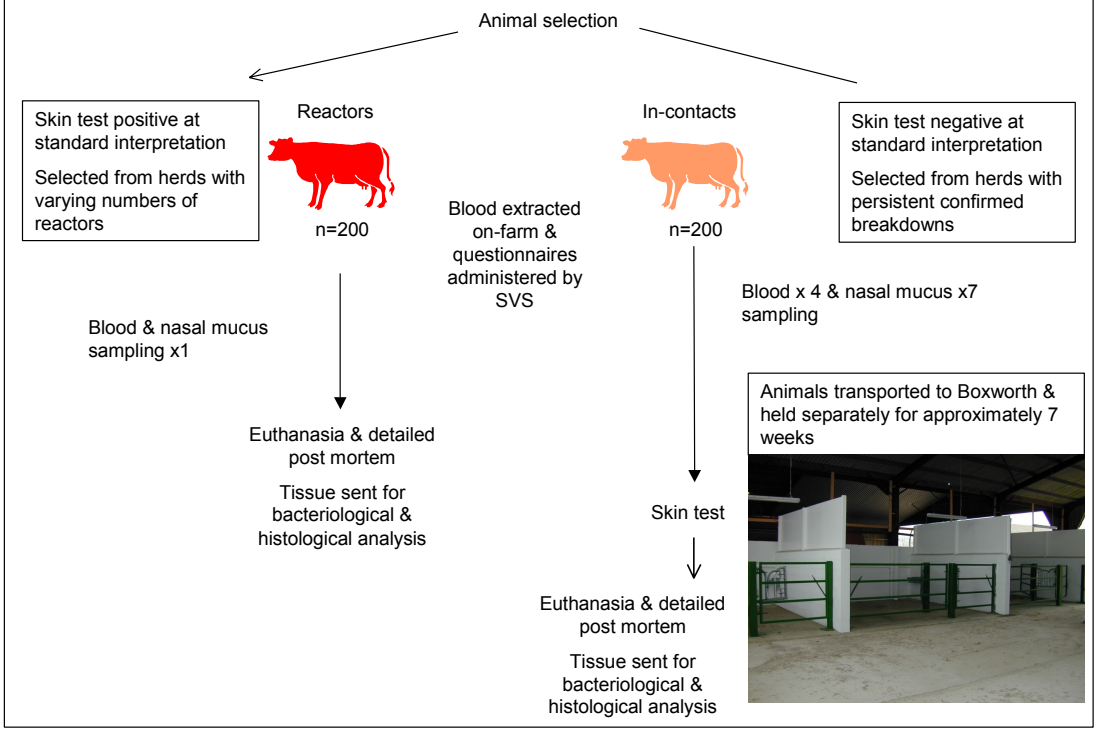
		In-contact n=200		Reactor n=200		p value*
Age [Yrs]	Mean (SD)	4.4	(3.1)	4.6	(3.1)	0.527
Sex [%]						0.653
	Female	83		85.5		
	Entire male	2		1		
	Castrated	15		13.5		
Parish bTB testing frequency of holding [%]						0.283
	Annual	73.0		78.0		
	Bi-annual	18.0		15.0		
	Every third year	0.0		1.0		
	Every fourth year	9.0		6.0		
SICTT surveillance type						
	12 month test	0.0		12.0		
	6 month test	0.0		19.0		
	Contiguous test	2.5		19.0		
	Check test	5.5		7.0		
	Inconclusive retest	1.0		5.5		
	Whole herd test	0.0		34.0		
	Short interval test	91.0		3.5		p<0.001
Bovine-avian difference in SICTT, Median (IQR)		0	(-1 to 0)	10	(6.5 to 16)	
Reactors in breakdown†, Median (IQR)		25	(11 to 46)	4	(1 to 10)	
Confirmed breakdown [%]		98		65		p<0.001
Herd size Median (IQR)		258	(189 to 346)	135	(72 to 226)	
Production class [%]						0.125
	Calf	3.5		0.5		
	Dairy cow	42.5		41		
	Finisher	16.5		14.5		
	Heifer	13		10		
	Suckler cow	18		25		
	Yearling	6.5		9		
Dairy breed‡ [%]		25.5		19.5		0.188

*Fisher's Exact test †Up to time of selection of study animal ‡Ayrshire, British Friesian, British Friesian X, British Holstein, Dairy Shorthorn, Friesian, Friesian Holstein, Friesian Holstein X, Guernsey, Holstein, Jersey X

2.2 Study design

The study design was cross-sectional with a nested cohort component whereby in-contact animals were held for approximately seven weeks for additional blood and nasal mucus sampling (see Figure 2).

Figure 2. Study design



2.3 Animal handling

Reactor cattle were transported from each farm holding to one of four VLA Laboratories (Luddington, Truro, Winchester and Weybridge) with appropriate handing facilities by hauliers who transport animals to abattoirs for the SVS. In-contact animals were transported to Boxworth in a transporter adapted to keep each animal separate and minimise the risk of between animal *M. bovis* transmission. The transport, care and sampling of the in-contact animals was sub-contracted and undertaken by ADAS who constructed special pens at the Boxworth farm during the project lead-in period. The pens enabled animals to have sight of other animals but avoided nose-to-nose contact and reduced the risk of spread of infection by coughing, bellowing or snorting (Figure 2).

The care of in-contact animals whilst at Boxworth was prescribed in detailed protocols. On arrival each animal was allocated to a pen and, after a settling period, was examined by a veterinarian to ascertain whether it was fit under the terms of the Animal (Scientific Procedures) Act 1986. Nasal mucus and blood sampling was conducted weekly and fortnightly respectively, over a period of approximately seven weeks. A skin test was conducted at the end of the sampling period and the animals were then dispatched to either VLA Bury St Edmunds or VLA Weybridge for euthanasia and post mortem examination.

2.4 Post-mortem examination

On arrival at the VLA Laboratories, the reactor cattle were unloaded into a crush and had blood and nasal mucus samples collected. The animals were then humanely euthanized, and subjected to a detailed post-mortem examination (PM) according to a standard protocol, the inspection of each animal taking two hours or more to perform. Those persons in charge of PM were either pathologists or veterinary investigation officers competent in bovine necropsy techniques. Each PM involved a general examination of all parts of the body (except the brain and spinal cord) to detect lesions of any kind, and a specific detailed examination (following dissection) of the tonsils, the head, chest and mesenteric lymph nodes and the lungs. Each of the dissected lymph nodes and tonsils was thinly sliced to detect granulomas; scalpel blades and forceps were changed between each tissue to avoid cross contamination. The detection of lesions and granulomas in the lungs was by a combination of palpation and thin slicing. The protocol required specific examination of 25 lymph node sites as well as the lungs where the lobe

location of granulomas typical of bTB was recorded. Pathologists were also instructed to collect samples from other tissues that appeared suspect.

During the detailed PM, any bTB-like lesions observed were placed into labelled Petri dishes and thence into sample bottles. In animals where at least one bTB-like lesion had been observed, a standard set of 16 samples (including individual head nodes, the left and right tonsil, individual chest nodes and a pooled sample of the mesenteric node, regardless of presence of bTB-like gross pathology) along with granulomatous lesions identified in any other tissue, was collected in duplicate for culture and histopathology. In animals where no bTB-like lesions were observed, pooled samples were collected from the tonsils, head, thoracic and mesenteric lymph nodes and sent for culture and a full set of duplicate individual samples were also collected and stored pending the culture result from the pooled samples. Samples for culture were frozen at -20°C and the samples for histopathology were fixed in buffered formalin. If *M. bovis* was isolated from any of the pooled samples, the complete standard set of samples taken from the individual lymph node sites from that animal was sent for culture and histopathology. Examination by culture was first priority, then histopathology.

The size and frequency of lesions within a lymph node or lungs were recorded. Using this information a lymph node score and lung score was created for each animal based on the modified version of the scoring system described in (Lyashchenko and others 2004). The two scores were then summed to provide a total body score. In addition the frequency of lymph nodes with lesions was also calculated for each animal.

2.5 TB50/50a

The pathologist or veterinary surgeon conducting the PM was asked to complete a TB50. This is the standard form completed by the responsible Veterinary Officer or Meat Inspector for each bovine slaughtered for food in GB. The presence or absence of lesions typical of bTB must be recorded on the form. A TB50a indicating the lesion location is additionally completed if any lesions are observed. The data are collated by the SVS and input onto VETNET, the electronic TB surveillance system in GB. The project protocol instructed the person conducting the post-mortem to complete a TB50 and TB50a if necessary as if the inspection had been conducted in an abattoir.

2.6 Bovine TB confirmation

An animal was confirmed as having bTB if at least one tissue sample from that animal was shown to be infected with *M. bovis* by:

- Bacteriological culture and/or
- Observation of characteristic bTB–like lesions during an inspection by a histopathologist of tissue sections stained by Ziehl-Neelsen (ZN) or haematoxylin and eosin (H & E) and viewed under light microscopy.

2.7 Bacteriology

The samples collected during the detailed PM were dispatched to one of three VLA laboratories with containment facilities to process mycobacteria (VLA Weybridge, VLA Truro and VLA Bury St Edmunds). This processing followed a validated standard operating procedure (SOP) BA.386 and consisted of an initial preparatory phase in which the tissue slivers were manually ground with a pestle and mortar and then decontaminated by suspension in 5% oxalic acid for 10 minutes, followed by centrifuging for a further 10 minutes at 110g to remove the acid. The resulting deposits were washed by re-suspension in 0.85% sterile saline and centrifuging for another 10 minutes, the supernatants discarded and the deposits re-suspended in 10 mL 0.85% saline. The resulting inoculum was sown onto 12 diagnostic solid-media slopes comprising two Lowenstein-Jenson base (LJ), two LJ plus pyruvate (LJP), two LJ plus glycerol (LJG), three Stonebrinks (ST) and three modified Middlebrook 7H11, each contained within an individual 30-mL thick walled glass universal bottle. These were then incubated at 37ºC, and the slopes examined after six weeks for evidence of growth of mycobacterium. *M. bovis* was discriminated from *M. avium* and other mycobacterium by a characteristic profile in the distinctive

media comprising small colonies on LJ; enhanced growth on LJ-P; little growth on LJ-G; white colonies on ST; and flat colonies with ground glass appearance on 7H11.

Culture of the nasal mucus samples was undertaken at VLA Bury St Edmunds, based on a protocol used successfully elsewhere and on experimental animals at VLA Weybridge (McCorry and others 2005). *M. bovis* was cultured on 7H11 media (Gallagher and Horwill 1977) over a six week incubation period but the protocol was modified to exclude a decontamination phase using NaOH because an earlier work at the VLA with experimentally infected cattle showed a 10-100 fold reduction in viability of *M. bovis* and associated sensitivity of the test when this decontamination step was included.

2.8 Spoligotyping

A heat-killed sub-sample from every sample from which *M. bovis* was isolated was sent for genetic typing. Spacer oligotyping (Spoligotyping) adhered closely to the methodology described by Kamerbeek (Kamerbeek and others 1997), with hybridising DNA detected by enhanced chemiluminescence and exposure to X-ray film, and the specific spoligotype identified by visual inspection of the resulting pattern (SOP CBU0245).

2.9 Histopathology

Tissue samples collected during the PM for histopathology were placed in 10% buffered formalin (BF) and dispatched to the Histopathology Unit at VLA Weybridge. Following a seven day fixation period, samples were processed according to a standard set of protocols involving trimming and placing the tissue into a plastic cassette (blocking and cassetting), processing and embedding tissue into a wax bath) and thin slicing with a microtome (sectioning). Sections were then positioned onto glass slides which, after drying, were stained with haematoxylin and eosin. A second section was also prepared and stored for subsequent staining with Ziehl-Neelsen if required.

The H & E stain slides were examined systematically under a microscope by a histopathologist for the occurrence of granulomas, and the presence or absence of any pathognomonic features indicating infection with mycobacteria, such as multi-nucleate giant cells, epithelioid cells and calcification. If granulomas were observed, the second section was sent for ZN staining and the stained slide examined for the presence or absence of acid-fast bodies.

2.10 Clinical pathology

Blood samples were collected on-farm before animals were moved to either Boxworth or a VLA laboratory. The samples were sent initially to VLA Shrewsbury, where trace nutrient levels were measured and haematology performed. The samples were then forwarded to VLA Weybridge and VLA Winchester for serology. All assays were undertaken using methods with validated SOPs and in laboratories operating under UKAS accreditation.

Red blood cell (RBC), white blood cell (WBC) and platelet counts were performed using the Sysmex F-800 Analyser system (Sysmex UK Ltd.) (SOP HA.002). The system was also used to estimate packed cell volume (PCV), although a manual estimate was also performed by the microhaematocrit method if the analyser reported values <10% or >50% (SOPs HA.003, HA.024). Differential white blood cell counts were performed by visual examination of slides of 100 white blood cells in Romanowsky stained smears (SOP HA.001). The UKAS accreditation reference for all haematology was ISO 17025.

Specific serological tests were conducted on the blood samples collected by the VOs on the farm at the time of animal selection into the study. Past or ongoing exposure to lungworm (*Dictylocaulus viviparous*) antigen (SOP PA.052), liver fluke (*Fasciola hepatica*) antigen (SOP SE.223), Johne's disease (*Mycobacterium avium paratuberculosis*) antigen (SOP BA.274 CSL "Parachek") and leptospirosis (*Leptospira hardjo)* antigen was assessed. Serological testing was also conducted to assess exposure to Infectious Bovine Rhinotracheitis (IBR) (SOP VI.035.A) and bovine viral diarrhoea (BVD) (SOP VI.027) viral antigen.

2.11 Trace nutrient analysis

Levels of vitamin B12 and copper and the enzyme Glutathione peroxidase (GSHPx) (a surrogate for selenium) were also measured in blood samples collected from study animals on the farms at selection. Deficiencies in the nutrients selected can cause diseases in ruminants (Field and others 2002; Underwood and Suttle 1999). A survey of GSHPx levels in blood drawn from British sheep reported that only 40% of flocks in England and Wales had adequate selenium levels or better (Anderson and others 1979). In addition tests were readily available and diagnostic criteria agreed for classical deficiency diseases associated with the trace elements selected. Ideally the study should have included a larger comprehensive screen of trace nutrients known to have a role in immune function. However, it was not possible to justify this level of investment in this area given the absence of supporting data relating to specifically to an association with bTB.

Selenium and copper concentrations were also measured in a sub-sample of 63 livers. The sub-sample comprised a random sample collected from animals euthanized before 2005, weighted so that equal proportions of livers from culture positive in-contacts and culture positive reactors were examined (15/31 and 17/32 respectively).

Blood selenium was estimated by determination of enzyme Glutathione peroxidase (GSHPx) activity in heparinised blood according to standard operating procedure (SOP) B1.039. Reduced glutathione was oxidised by glutathione peroxidase in the presence of cumene hydroperoxide. The reduction of oxidised glutathione by glutathione reductase causes the oxidation of NADPH, which was monitored at 340nm. A packed cell volume (PCV) was used to convert the results to units of activity per mL of red cells.

Blood vitamin B12 was measured in serum according to SOP BI.212B by the Bayer Immuno-1 analyser. The assay was performed in two steps. During the first step vitamin B12 was released from the endogeneous serum binders after sample pre-treatments. In the second step, released vitamin B12 in the sample was reacted with vitamin B12 Intrinsic Factor Reagent and incubated at 37°C. Vitamin B12 enzyme conjugate was then added and competed with sample vitamin B12 for binding sites on the intrinsic factor. A monoclonal ImmunoMagnetic Particle (mIMP) reagent was then added and following a second incubation bound to the antibody complex. After incubation, the mIMP antibody complex was washed and a para-nitrophenyl phosphate substrate added. The alkaline phosphatase in the antibody conjugate reacted to form para-nitrophenoxide and phosphate. The optical density of the assay varied with formation of para-nitrophenoxide and was read at 405nm. Increasing absorbance indicated a lower vitamin B12 concentration in the sample.

Copper was determined using heparin plasma according to SOP BI.210. The plasma samples were assayed by graphite furnace atomic absorption after a simple pre-treatment in which the sample was diluted in a surfactant and the absorbance measured against a matrix matched calibrator.

Analysis for selenium in the liver samples was sub-contracted to the Analytical Services Department of the Scottish Agricultural College, Edinburgh. Following nitric acid digestion of the sample, the digest was examined using a PS Analytical Hydride generation Atomic Fluorescence analyser.

Copper in the liver samples was analysed at VLA Shrewsbury according to SOP BI.208. A mixture of sulphuric, nitric and perchloric acids was used to completely oxidize the samples. The copper concentrations were determined using flame atomic absorption spectroscopy.

2.12 Immunology (VLA)

Multiple blood tubes were collected from the reactors at the VLA Laboratory where the PM was to be conducted. Blood samples were packaged into insulated boxes (SafTPak, http://www.saftpak.com/) to maintain a blood temperature between 16-24ºC, and transported overnight to the processing laboratories (VLA Luddington, VLA Weybridge and IAH Compton). Blood collection was similarly performed at Boxworth but was repeated four times for each in-contact animal during the holding period.

At VLA Luddington, whole blood cultures were stimulated with the *M. bovis* antigens PPD-M, PPD-A, recombinant ESAT-6 protein, a peptide cocktail containing synthetic peptides derived from ESAT-6 and CFP-10 (Vordermeier and others 2001)and the super-antigen control Staphyloccocal enterotoxin B (SEB) in accordance with SOP BAC0209. 16-24 hour plasma supernatants were harvested in duplicate plates and either tested for bovine IFN-γ using the BOVIGAM ELISA kit (Prionics, Zurich, Switzerland)(Rothel and others 1990) in accordance with SOP BAC0210, or stored frozen and sent to VLA Weybridge for testing in the IL-2 bioassay. VLA Luddington also collected serum from the clotted blood samples for detection of antigen specific (PPD-A, PPD-B, MPB70, MPB83 and *M. bovis* sonicate) IgG, in accordance with SOP BAC0208.

Immunological assays undertaken at the VLA Weybridge included measurement of IL-2, IL-4 and lymphocyte proliferation responses. Peripheral blood mononuclear cells (PBMCs) were purified by gradient centrifugation in accordance with SOP BAC0203. For the lymphocyte transformation assay (LTA), PBMC's were stimulated with PPD-M, PPD-A, ESAT6 recombinant protein, a peptide cocktail derived from ESAT6 and CFP-10 or SEB. Antigen specific lymphocyte proliferation was determined by incorporation of radio-labelled [^{3}H]-thymidine during the last 16-20 hours of a 6 day culture in accordance with SOP BAC0206.

For measurement of IL4 activity, PBMCs were cultured for 6 days in the presence of those antigens used in the LTA assay. IL-4 activity in these culture supernatants was measured using a bioassay in accordance with SOP BAC0205. Briefly, B-cells were purified from blood from an uninfected cow using magnetically labelled beads coated with a bovine B-cell specific antibody. Purified B-cells were then cultured for 48 hours in the presence of stored PBMC supernatants and B-cell proliferation was measured by incorporation of [^{3}H]-thymidine during the last 16-20 hours of culture.

The IL2 bioassay was undertaken at VLA Weybridge using frozen whole blood plasma supernatants collected at VLA Luddington. IL-2 activity was determined using a bioassay based on the principle that presence of IL-2 will support the proliferation of mitogen-activated T cells (SOP BAC0204) . Briefly, PBMCs isolated from an uninfected cow were stimulated for 4 days with concanavalin A (Con-A) to generate lymphoblasts. These were then cultured for 48 hours in the presence of the stored whole blood plasma supernatants and lymphocyte proliferation was measured by incorporation of [^{3}H]-thymidine during the last 16-20 hours of culture.

Initially, VLA Weybridge also measured IFN-γ responses in PBMC's using an ELISPOT assay. However, review of the data after the first year of the project concluded that this assay, whilst useful for measuring vaccine immunity, was prohibitively labour and resource intensive and not a practical routine diagnostic alternative to the measurement of INF-γ using the BOVIGAM assay. With the permission of the ISG and DEFRA, this assay was discontinued, and the resources used to duplicate the whole blood IFN-γ assay in order to resolve the quality issues encountered with this assay at VLA Luddington and to test an additional antigen, Rv3019c.

2.13 Immunology (IAH Compton)

Additional assays were conducted at the IAH for the detection of immunological parameters in the blood samples collected from reactors and in-contacts including detection of cytokines in whole blood; preparation of total RNA from 1:10 diluted blood; assessment of proliferation in 1:10 diluted blood; ELISPOT assay for detection of IFN-γ and IL-4 secreting cells and determination of CD4 and CD8 responses using dendritic cells.

Whole blood was stimulated with PPD-A, PPD-B or medium (control) for 24 hours. Following centrifugation, serum was removed and cytokine secretion was assessed by ELISA. Levels of IFN-γ, IL-10, IL-12, TNF-α and IL-4 were measured. Measurement of IL-12 was suspended with permission after year 1 and TNF-α levels were subsequently measured instead as this assay proved more reliable than the IL-12 assay.

For assessment of proliferation and extraction of total RNA, blood was diluted 1:10 in medium and stimulated for five days with PPD-A, PPD-B or medium (control). Parallel cultures were set up; RNA was extracted from one set of cultures and the second were pulsed with ^{3}H-thymidine for the last 18-20 hours of culture. Proliferation was assessed by scintillation counting of incorporated ^{3}H-thymidine.

Peripheral blood mononuclear cells (PBMC) were isolated from the remaining blood by density gradient centrifugation over histopaque. For detection of IFN-γ by ELISPOT, cells were stimulated for 24 hours with PPD-A, PPD-B or medium (control). The number of IFN-γ SFC was determined. In addition, PBMC were stimulated with mitogen (PMA/ionomycin/brefeldin A) for five hours. Expression of intracellular IFN-γ and IL-4 by subsets of T lymphocytes ($CD4^+$, $CD8^+$, $WC1^+\gamma\delta TCR^+$) was determined by multicolour flow cytometry.

Antigen specific expression of IFN-γ by T cells was assessed by culture of PBMC with BCG infected dendritic cells for five days, and assessment of IFN-γ expression by multicolour flow cytometry. These assays were performed using frozen PBMC prepared from blood on the day of arrival at IAH. Two vials of PBMC were prepared from each animal and stored in liquid nitrogen. Dendritic cells were prepared from one vial of cells by isolating $CD14^+$ monocytes which were cultured with GMCSF/IL-4 for three days. The monocyte derived dendritic cells were then incubated overnight with BCG (10cfu/cell) or medium (control) then co-cultured with PBMC for an additional five days. Cytokine expression by $CD4^+$, $CD8^+$ and $WC1^+\gamma\delta TCR^+$ T cells was determined.

2.14 Animal, farm and tuberculin test histories

During the recruitment of each animal into the study, a questionnaire was administered by the Veterinary Officer who selected the animal. The questionnaire ascertained the age, sex and production class of the animal and stage of reproduction if the animal was female. Animal disease history was measured by ascertaining whether the animal had diarrhoea, lameness, ill-thrift, infertility, respiratory problems, abortion, mastitis or milk drop in the last 12 months. Medication history was assessed by ascertaining if the animal had ever been vaccinated against Johne's disease, IBR or lung worm and whether the animal had flukicide, anthelmintics, IM antibiotics, BVD vaccine, Salmonella vaccine or Leptospirosis vaccine in the last 12 months. The tuberculin test histories of each of the study animals were extracted from the SVS farm files. Also recorded was any former diagnosis in the herd by a veterinarian of BVD, IBR, Leptospirosis or Johne's disease.

2.15 Data handling

Integrated involvement of staff from four organisations was critical to the project's success; and the development and deployment of a bespoke web-based project management, decision support and data-entry system, "PathMan", linking the four organisations was a key objective (Durr and Eastland 2004).

PathMan was developed during the first six months of the project. This system was based upon a SQL Server 7 database with HTML forms and reports made available to users via Active Server Pages. Data was entered into the system at each site as results became available. However, the system went beyond just data capture and reporting and involved a number of "process-support" systems, to guide users through the processing of study animals and samples. Thus, when the SVS AHDOs identified potential study animals and entered this onto *PathMan*, alerts were generated to the ADAS Boxworth or the VLA-Regional Laboratory automatically informing them of the intention to dispatch animals on the given date.

Figure 3a. PathMan web-based decision support and data entry

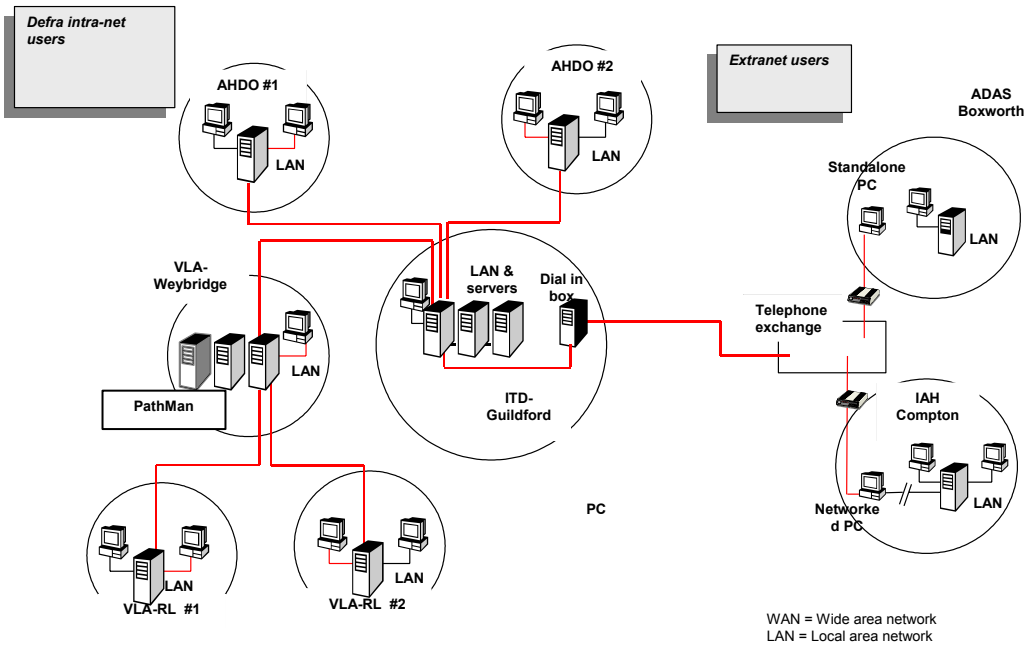
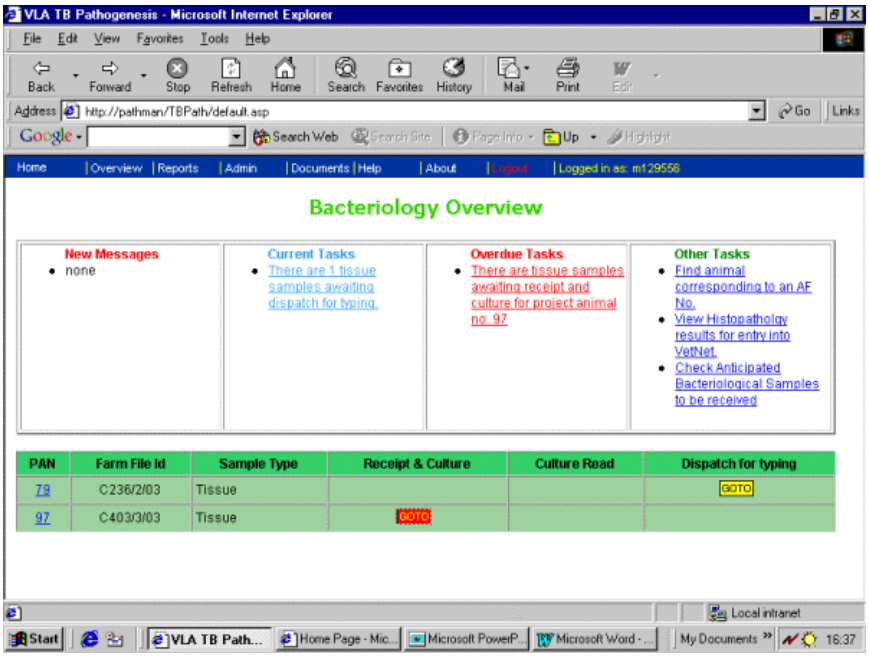


Figure 3b. Screen shot of PathMan process support matrix tracking tissue sampled cultured for *M.bovis*.



At the end of the project, the data from PathMan were extracted onto an *Access 2000* database. A fully documented finalised database was released after comprehensive data quality control measures had been carried out.

2.16 Statistical analyses

Statistical analyses were conducted using Stata 9 (StataCorp 77845, Texas, USA www.stata.com/). Both univariate descriptive and multivariate analyses were conducted. Diagnostic assays thresholds were determined using ROC curve analyses. Probability values of less than 5% were interpreted as statistically significant. Confidence intervals in all figures are 95% intervals.

2.17 Animal welfare, health & safety and biosecurity issues

On leaving the farm holdings, the animals were classified as ”experimental” and thus subject to the regulations under the Animals (Scientific Procedures) Act, 1986. The overall project required a Home Office licence, and sampling was only undertaken by staff in possession of a personal licence. Regular audits were undertaken during the course of the project by Home Office Inspectors and the conduct of the study was approved by the ethics committees of both VLA and ADAS UK Ltd.

The zoonotic potential of *M. bovis* was identified as a specific hazard to staff working on this project and required risk minimisation to be implemented. Several specific risk assessments were undertaken before the commencement of the project by both VLA and ADAS, and appropriate procedures incorporated into standard and project specific protocols; such as the requirement that all sample pots be decontaminated before leaving the PM room, and the use of safety cabinets for opening packages containing blood samples. Risk assessments were also undertaken for staff members at the Regional Laboratories and at ADAS Boxworth and procedures developed to reduce the risk of crushing injuries whilst handling the cattle for sampling and euthanasia. No accidents or injuries were reported by any staff working on the project.

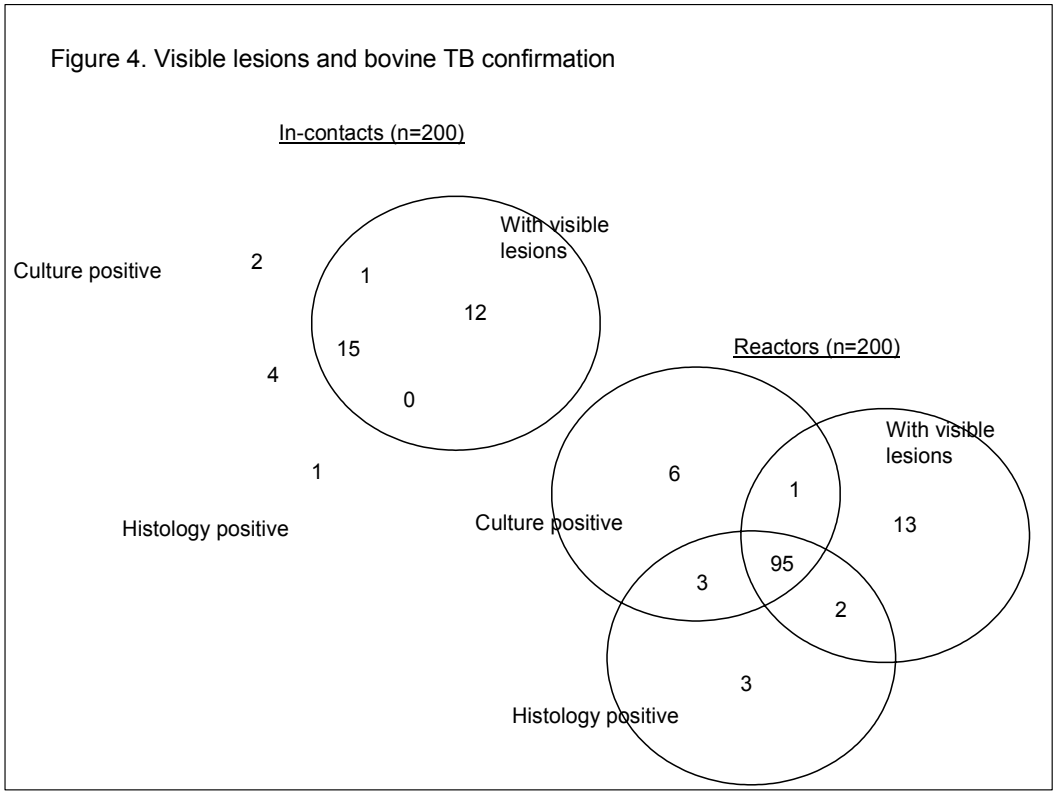
Biosecurity to prevent inadvertent *M. bovis* contamination of the environment at all the VLA laboratories and at ADAS Boxworth also followed standard operating protocols. All material, such as needles, scalpels and face-masks, that came in direct contact with the project animals or their samples, were treated as a biohazard and destroyed or decontaminated. Special attention was paid at Boxworth to prevent direct and indirect contact of the cattle with wild animals and birds. A security fence was constructed around the sheds; netting was placed to prevent entry of birds, and rodent control regularly undertaken. All waste material was either incinerated on-site or transported to an incinerator at VLA Weybridge within secured bins. Waste liquid was temporarily stored in two underground chambers, which were regularly emptied for disposal by a licensed waste disposal operator.

3.0 Results

3.1 Bovine TB confirmation in reactors and in-contacts

At the detailed post mortem, macroscopic visible lesions characteristic of bTB were observed in 28/200 (14%) of in-contacts and 111/200 (55.5%) of reactors (see figure 4).

Seventy-three percent (178/244) of tissues identified as having TB–like lesions were culture positive. Overall, bTB was confirmed by culture and/or histology in 23/200 (11.5%) of in-contacts, which comprised 16/28 (57%) of the animals with visible lesions and 7/172 (4.1%) of the animals without visible lesions. Bovine TB was confirmed in 110/200 (55%) of reactors, which was 98/111 (88.3%) of the animals with visible lesions and 12/89 (13.5%) of the animals without visible lesions. Agreement between culture and histology for bTB confirmation at the animal level was 98% for in-contacts and 94% for reactors (Kappa statistics 0.89 and 0.87 respectively where 1=perfect agreement and 0=no agreement).



Fifteen of the in-contact animals had become SICTT positive at standard interpretation when the animals were retested at the end of the seven week holding period. Of these fifteen, nine were bTB confirmed animals of which seven had visible lesions. There was one SICTT positive in-contact with visible lesions where bTB infection was not confirmed by either culture or histology. However, samples were sent to culture as a first priority and the sample available for histopathology may not have been similarly infected as the one sent for culture.

Veterinarians conducting the post mortem were asked to describe characteristics of lesions and presence of parasites such as liverfluke in notes. However, there was no requirement in the protocol to systematically record possible alternative diagnoses to bTB. Gross bTB-like lesions that were not bTB confirmed were commonly described as parasitic granulomas and bacterial abscesses.

3.2 Distribution of lesions within animals with confirmed bTB

Macroscopic lesions typical of bTB were most commonly found in the thorax in both in-contact and reactor animals (see table 2). A similar proportion of in-contacts had lesions in the head and/or abdomen. In reactors, the head was the second most common site for lymph nodes with lesions, and relatively few animals had lesions in the abdomen.

Within the thoracic region, lesions were most commonly found, in both in-contacts and reactors, in the caudal mediastinal lymph node and the left tracheobronchial lymph nodes. Within the head, the lesions were most commonly observed in the medial retropharyngeal lymph nodes. The mean number of lymph nodes with TB like lesions within each animal with confirmed bTB was 1.5 for in-contacts and 1.7 for reactors.

Table 2 Number of bTB confirmed animals with a lymph node with a lesion by body region and specific lymph node

	Incontacts bTB confirmed (n=23)		Reactors bTB confirmed (n=110)	
	n	%	n	%
Body region				
Head	5	21.7	45	40.9
Thorax	10	43.5	61	55.5
Abdomen	5	21.7	11	10.0
Other	0	0.0	8	7.3
Specific lymph node				
Head				
Left Medial Retropharyngeal	1	4.3	22	20.0
Right Medial Retropharyngeal	2	8.7	21	19.1
Left Lateral Retropharyngeal	0	0.0	2	1.8
Right Lateral Retropharyngeal	0	0.0	3	2.7
Left Parotid	0	0.0	1	0.9
Right Parotid	0	0.0	1	0.9
Left Mandibular	1	4.3	2	1.8
Right Mandibular	1	4.3	1	0.9
Left Tonsil	0	0.0	11	10.0
Right Tonsil	0	0.0	14	12.7
Thorax				
Caudal Mediastinal	7	30.4	41	37.3
Cranial Middle Mediastinal	2	8.7	20	18.2
Cranial Tracheo Bronchial	2	8.7	7	6.4
Left Tracheo Bronchial	5	21.7	20	18.2
Right Middle Tracheo Bronchial	2	8.7	5	4.5
Abdomen				
Mesenteric	5	21.7	11	10.0
Hepatic	3	13.0	0	0.0
Other				
Superficial Cervical	0	0.0	6	5.5
Popliteal	0	0.0	2	1.8

An animal may have more than one lymph node with a lesion in a body region, more than one lesion in a lymph node and more than one mesenteric lymph node with a lesion.

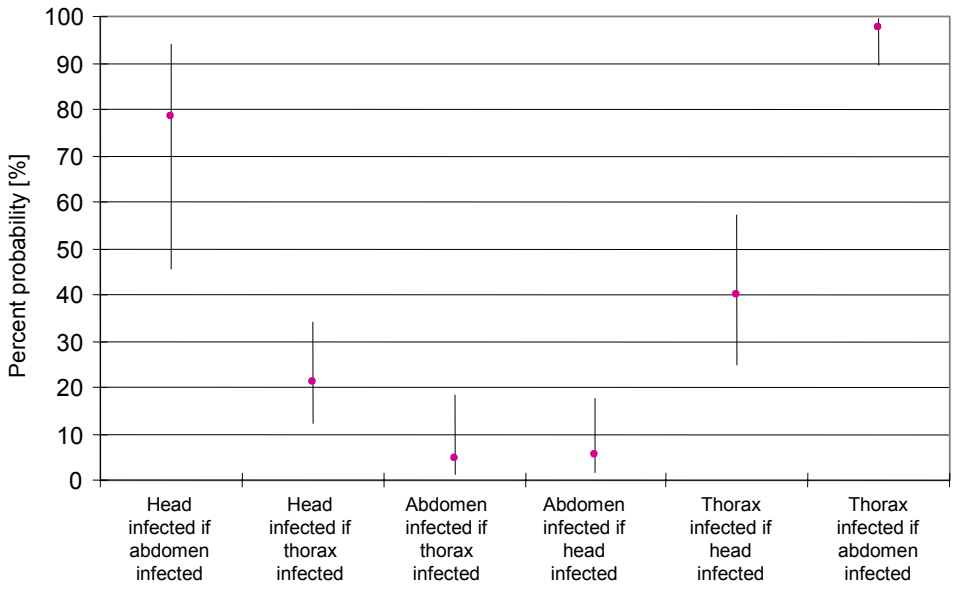
In in-contacts, lesions were frequently isolated to lymph nodes only in the head, or only in the thorax or only in the abdomen (see table 3). By comparison, in reactors lesions were commonly isolated in lymph nodes only in the head or only in the thorax but, rarely observed in the abdomen without observation of lesions in other body regions.

Table 3 Number of in-contacts and reactors with confirmed TB showing lymph nodes with lesions by body region

Region with lymph node/s with lesions	In-contact (n=23)		Reactor (n=110)	
	n	%	N	%
Head, thoracic, abdominal, other	0	0.0	1	0.9
Head, thoracic, abdominal	0	0.0	2	1.8
Head, thoracic	2	8.7	11	10.0
Head, abdominal	0	0.0	2	1.8
Head, other	0	0.0	3	2.7
Head only	3	13.0	26	23.6
Thoracic, abdominal, other	0	0.0	0	0.0
Thoracic, abdominal	2	8.7	5	4.5
Thoracic, other	0	0.0	1	0.9
Thoracic only	6	26.1	41	37.3
Abdominal, other	0	0.0	0	0.0
Abdominal only	3	13.0	1	0.9
Other only	0	0.0	3	2.7

In a logistic regression model, the association between pairs of body regions was examined in reactors whilst adjusting for lesions in other regions and for PM laboratory (see figure 5). The model showed that there was a high probability for the existence of a lymph node with a lesion in either the head or thorax if there was a lymph node with a lesion in the abdominal region. Conversely, an infected head or thorax was not a strong predictor for a lesion in the abdomen. Lesions in the head were a stronger predictor for lesions in the lymph nodes in the thorax than lesions in the thorax were for lesions in the head.

Figure 5. Adjusted probability of a lymph node with a lesion in one body region if another body region has a lymph node with a lesion (reactors only)



Adjusted for lesions in other body regions and laboratory where PM was conducted

Only two in-contacts with confirmed bTB (9%) had one or more lung lobes with lesions. Thirty reactors with confirmed bTB (27%) had at least one lung lobe with a lesion and the caudal lobes were the most commonly affected (see table 4). Both in-contact animals with a lung lobe with a lesion also had lesions

in thoracic nodes. There were three reactors with confirmed bTB that had lesions in lung tissue but none in associated thoracic lymph nodes.

Table 4. Number of lung lobes with a lesion in bTB confirmed animals

	Incontacts bTB confirmed (n=23)		Reactors bTB confirmed (n=110)	
	n	%	n	%
Lung lobe				
Left Caudal	0	0.0	12	10.9
Left Cranial	1	4.3	7	6.4
Right Accessory	0	0.0	4	3.6
Right Caudal	1	4.3	12	10.9
Right Cranial	0	0.0	6	5.5
Right Middle	1	4.3	4	3.6

3.3 Lesion and confirmation status by breakdown type, production class PM laboratory and age

In reactors, the frequency of animals with a lesion could be compared between breakdowns where bTB had been confirmed at the time of selection and breakdowns where it had not (Table 5). Macroscopic lesions typical of bTB-like lesions were more likely to be observed in animals selected from confirmed breakdowns ($p<0.001$) and bTB infection was also more likely to be confirmed by histology or culture in those animals ($p<0.001$).

Table 5. Lesion and bTB confirmation status by breakdown type, production class and PM location

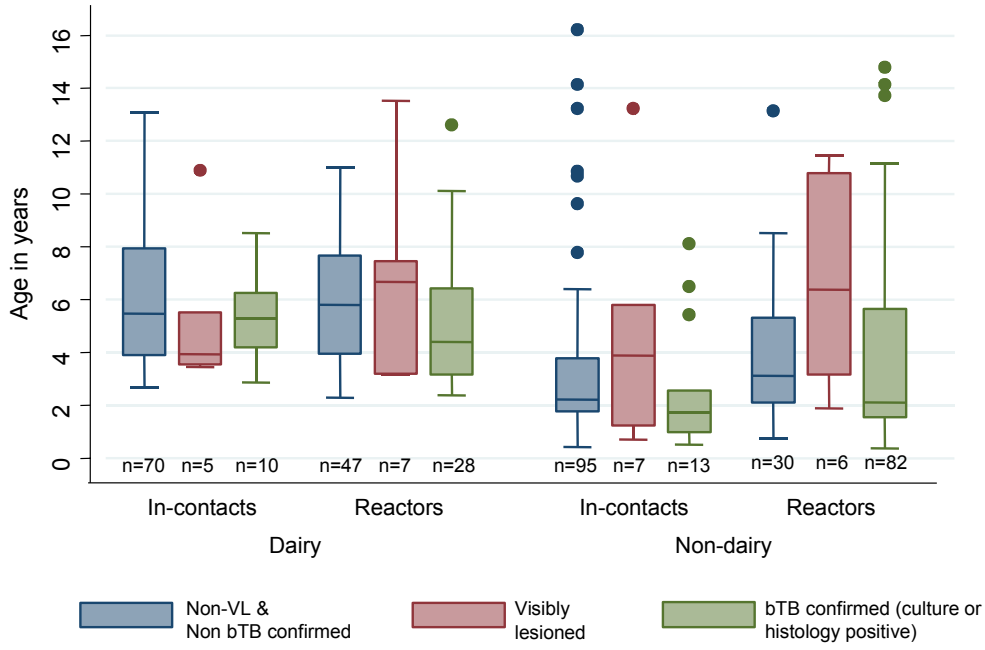
	In-contacts Visible lesions		In-contacts Confirmed TB		Reactors Visible lesions		Reactors Confirmed TB	
	n	[%]	n	[%]	n	[%]	n	[%]
All	28/200	14.0	23/200	11.5	111/200	55.5	110/200	55.0
Breakdown type								
Confirmed	28/196	14.0	23/200	11.5	99/130	76.2	107/130	82.3
Unconfirmed	0/4	0.0	0/4	0	12/70	17.1	3/70	4.3
Production class								
Dairy cow	11/85	12.9	10/85	11.8	31/82	37.8	28/82	34.2
Finisher	0/33	0.0	0/33	0.0	19/29	65.5	21/29	72.4
Heifer	6/26	23.1	5/26	19.2	12/20	60.0	13/20	65.0
Suckler cow	5/36	13.9	3/36	8.3	33/50	66.0	31/50	62.0
Yearling/calf	6/20	30.0	5/20	25.0	16/19	84.2	17/19	89.5
PM location								
Bury St Edmunds	12/69	17.4	13/69	18.8	2/2	100.0	2/2	100.0
Luddington	0		0		45/83	54.2	42/83	50.6
Truro	0		0		27/54	50.0	29/55	53.7
Weybridge	16/131	12.3	10/131	7.6	17/22	77.3	16/22	72.3
Winchester	0		0		22/41	53.7	18/41	56.1

The distribution of lesions and bTB confirmation also varied between production classes in both in-contacts ($p<0.05$) and reactors ($p<0.001$) (see Table 5). The most striking observation was that reactor dairy cows were less likely to have visible lesions and to have bTB confirmed than other production classes.

The proportion of reactors with visible lesions or with bTB confirmed was slightly higher in animals examined at Weybridge compared to other laboratories, but the differences between laboratories were not statistically significant.

Figure 6 shows the age of animals at selection. Reactors with confirmed bTB tended to be younger than other reactors. This was also true of non-dairy in-contacts with confirmed bTB. In-contact dairy animals with confirmed bTB were as old, or older, than other animals.

Figure 6. Box plot of age at selection by reactor, visible lesion (VL) and bTB confirmation status in dairy and non-dairy animals



3.4 TB50

The veterinary pathologists indicated that the number of animals identified as having a visible lesion if the PM had been conducted in an abattoir was slightly less than the number identified at the end of the detailed PM, particularly for in-contact animals (see table 6). The intra-rater agreement as measured by the Kappa statistic was 0.677 for the in-contacts and 0.870 for the reactors (0=only chance agreement and 1=perfect agreement).

Table 6 Classification of animals by TB50 PM compared to detailed PM

		Detailed PM			
		In-contacts n=200		Reactors n=200	
		NVL	VL	NVL	VL
TB50	NVL	171	12	89	13
	VL	1	16	0	98

3.5 Spoligotypes

There were 321 *M. bovis* cultures sent for spoligotyping; a median of 2 (IQR 1-3) per culture positive animal. The frequency distribution of spoligotypes in reactors was similar to that in the national herd over the period of the project (source VETNET surveillance data 2002-2005) (see figure 7). Proportionally fewer spoligotype 9s and more type 17 and 10s were isolated from in-contacts than from cattle in the national herd. Figures 8a and b show the location of infected animals by spoligotypes in-contacts and reactors and the distribution of spoligotypes in infected animals the National Herd over the study data collection period 2002-2005. Eight cattle including two in-contacts were from breakdowns

where more than one spoligotype was isolated from herd during the breakdown. Three of the eight animals were yearlings, two were dairy cows and there was one finisher, one suckler and one heifer. None of the individual animals in the study had more than one spoligotype isolated from any of the culture positive samples, including those cattle from breakdowns where more than one spoligotype had been identified.

Figure 7. Distribution of spoligotypes between reactors, in-contacts and the national herd

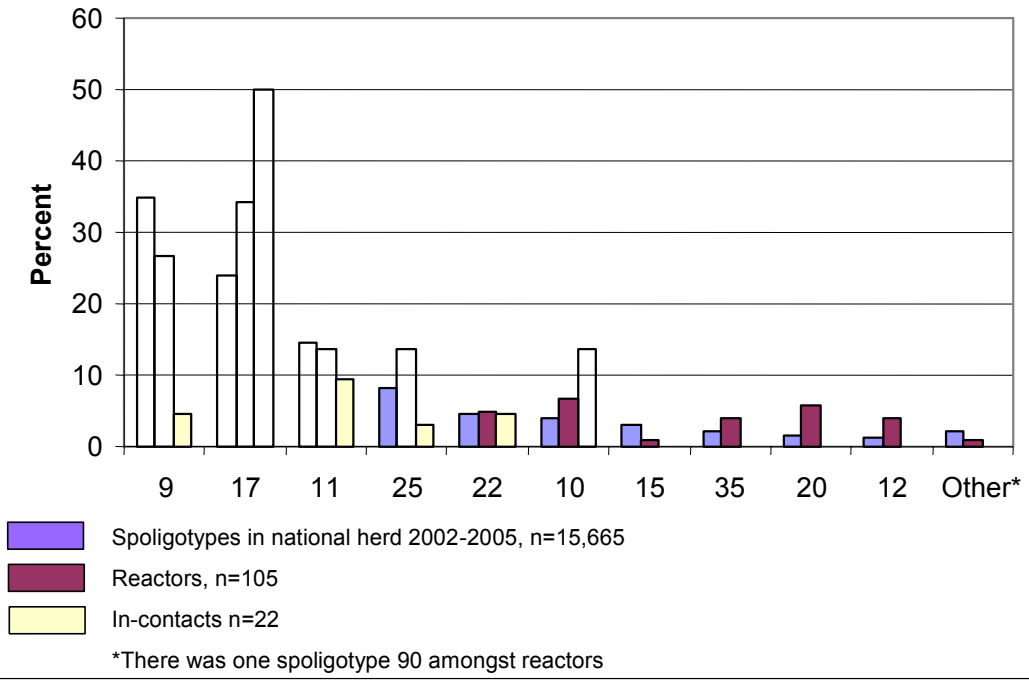


Figure 8a. Geographical distribution of spoligotypes of culture positive animals by farm holding at selection

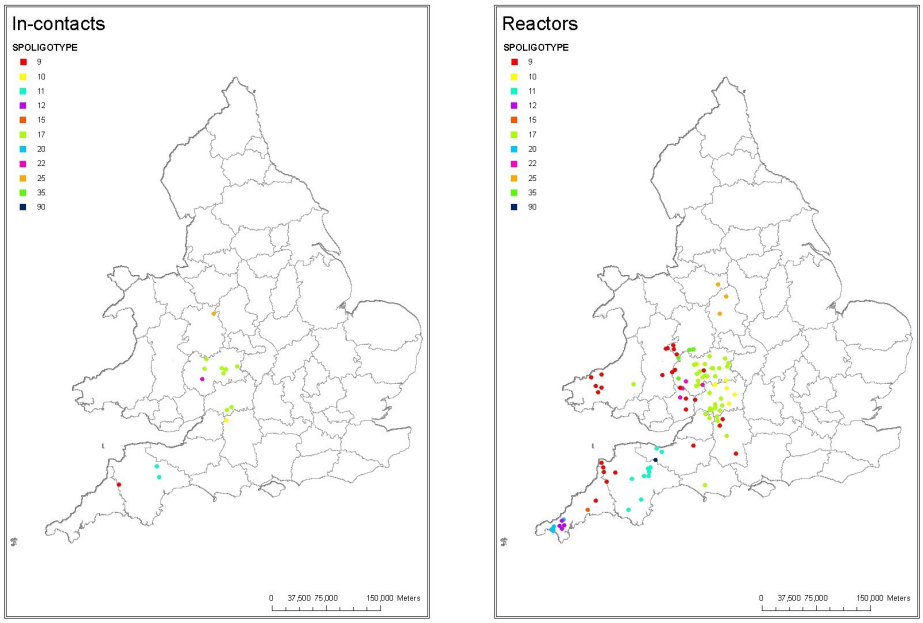
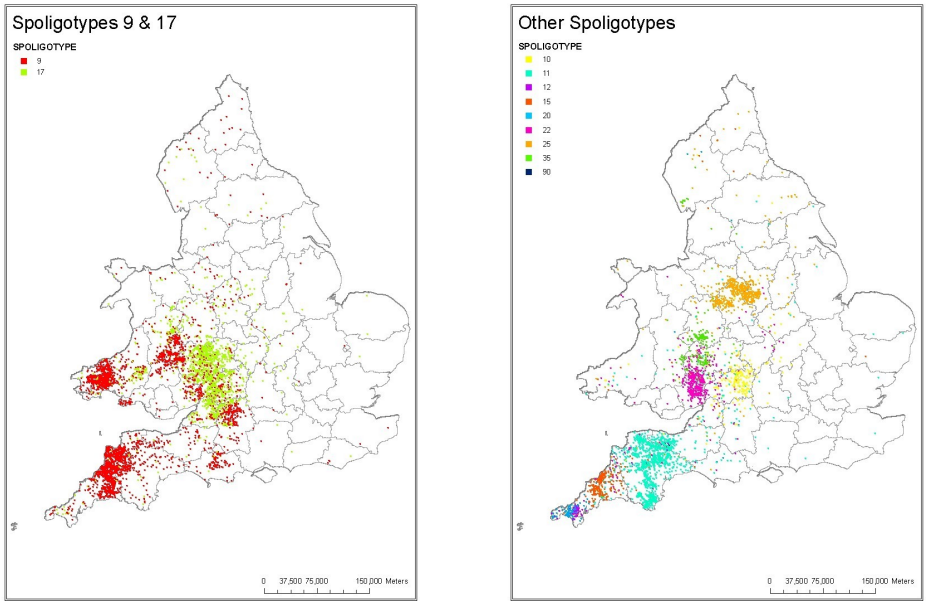
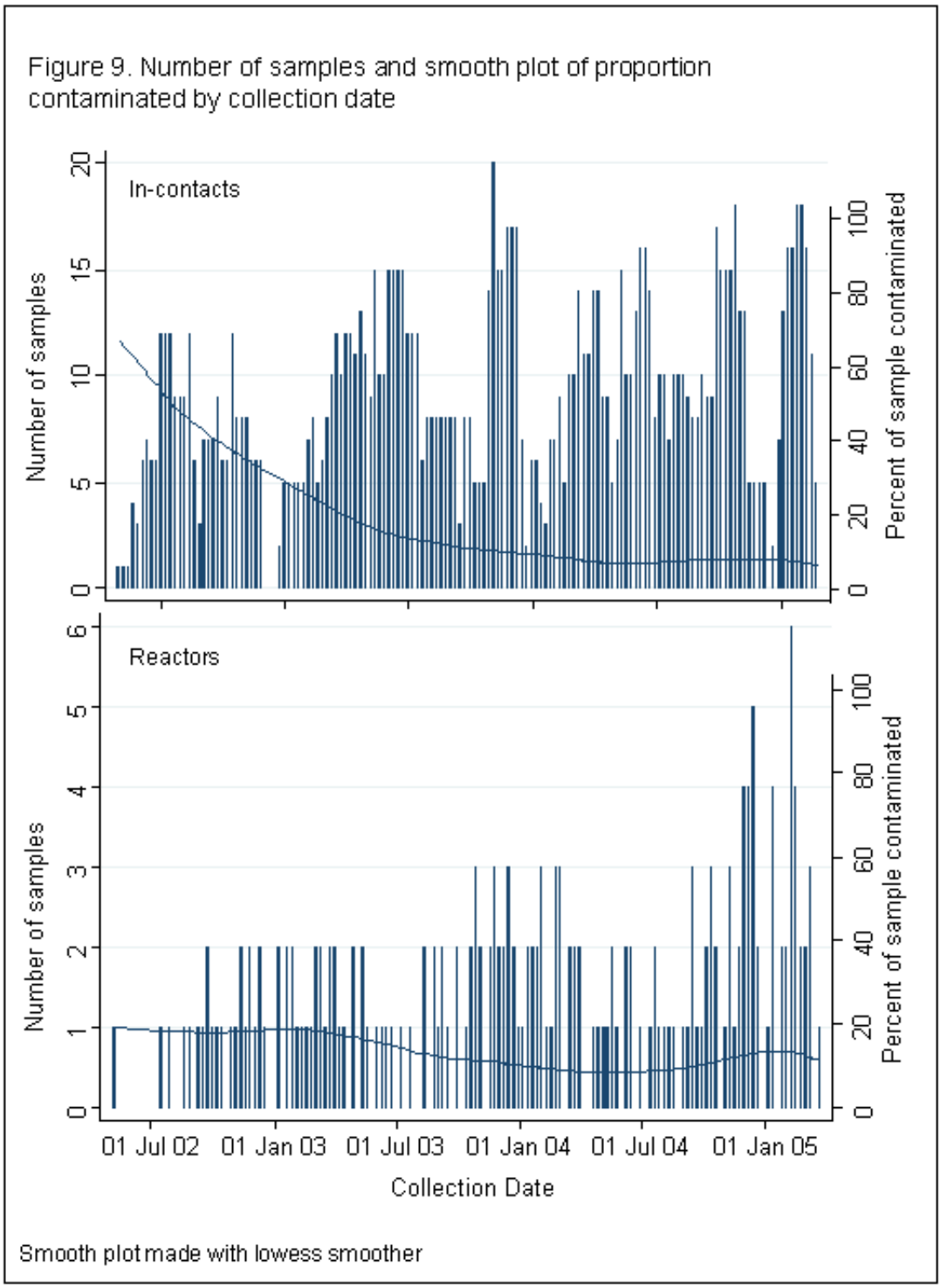


Figure 8b. Geographical distribution of spoligotypes from confirmed bTB breakdowns in England and Wales 2002-2005



3.6 Nasal shedding

In total, 1543 nasal mucus samples were collected for the detection of possible nasal shedding of *M. bovis* in infected animals. This comprised a median of 4 samples (IQR 2-6) collected per in-contact animal during the seven week holding period, and one sample collected from each of the 195 reactors just before euthanasia. Contamination by fungi and other sources occurred in some samples, particularly in samples taken early on in the project (see figure 9). There were, in total, three in-contact animals and 62 reactor animals for which there were no uncontaminated sample results. However, 868/1348 (64%) and 138/195 (71%) samples were completely uncontaminated from in-contacts and reactors respectively, and on average less than 50% (3/6 slopes) of the sample was contaminated when contamination did occur. *M. bovis* was not detected by bacterial culture in any of the nasal mucus samples.



3.7 Immunology – VLA results

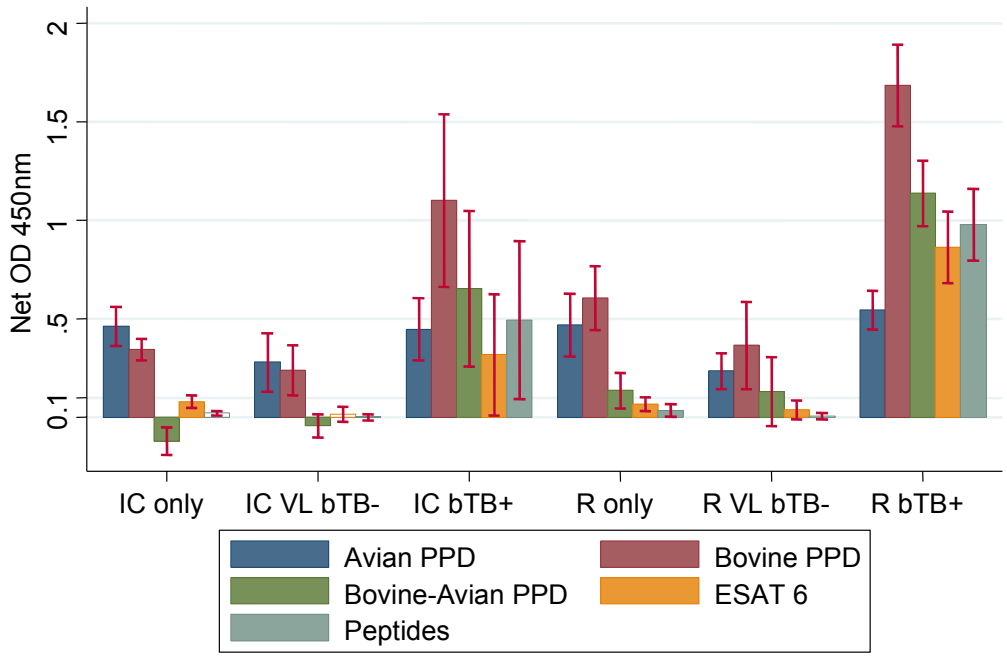
Immunological responses measured in blood drawn at recruitment were examined by categories of bTB severity. Responses were compared between animals where immunology results that met valid QC criteria existed for all tests (see table 7 & figures 10 & 11).

Table 7. Animals with valid immunological blood results from blood drawn after recruitment (sample 1)

	Animals n
In-contacts	
Confirmed bTB*	20
Visible lesion & unconfirmed bTB	11
No visible lesion & unconfirmed bTB	143
Reactors	
Confirmed bTB*	96
Visible lesion & unconfirmed bTB	11
No visible lesion & unconfirmed bTB	61

*Confirmed by culture or histology

Figure 10. IFN-γ response at different stages of disease



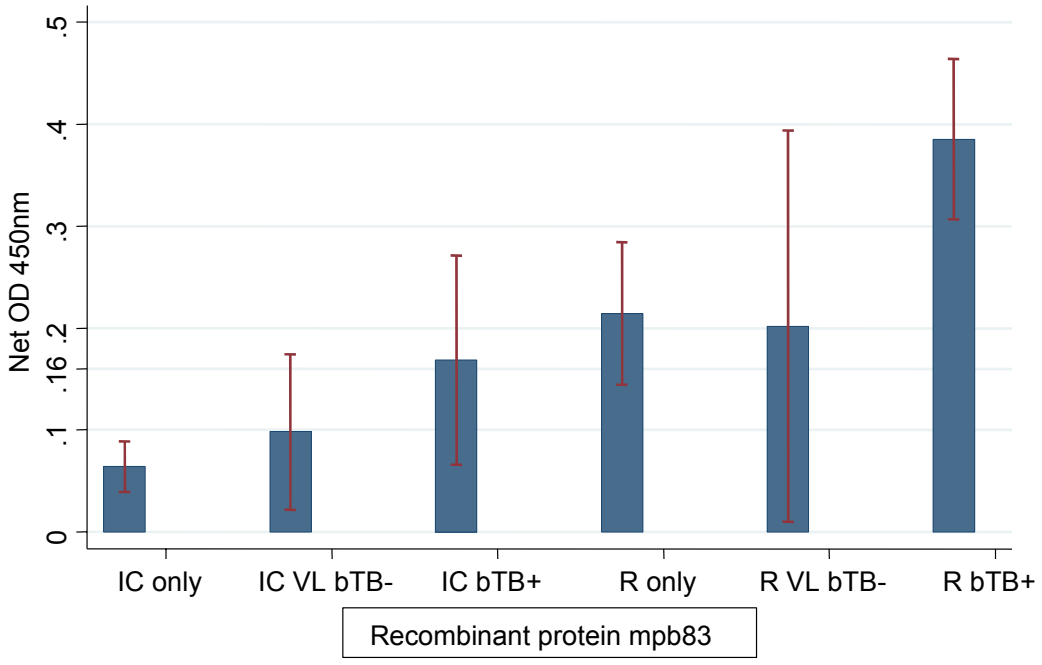
A response to Bovine-Avian PPD above 0.1 is interpreted as indicating infection with *M. bovis*. IC only = in-contact with no visible lesion and both culture and histology negative. R only = reactor with no visible lesion and both culture and histology negative. VL bTB- =visible lesion but both culture and histology negative. bTB+ =confirmed bTB (culture positive and/or histology positive).

IFN- γ responses to avian purified protein derivative (PPD) were similar across disease categories whereas the response to bovine PPD varied. Mean responses to bovine PPD were highest in in-contacts and reactors with confirmed bTB ($p<0.001$). This finding was reflected in the mean difference between the response to bovine and avian PPD which was highest in reactors with confirmed bTB. There was minimal response to ESAT6 protein except in in-contacts and reactors with confirmed bTB and the response was highest in reactors with confirmed bTB ($p<0.001$). The same pattern was observed in the IFN-γ response to peptides derived from ESAT6 and CFP10 ($p<0.001$).

Mean antibody responses to the sero-dominant antigen MPB83 are shown in figure 11. The lowest mean responses were observed in in-contacts without confirmed bTB or in-contacts with visible lesions; mean responses were below the threshold set for a response indicative of *M. bovis* infection. Mean responses were similar for in-contacts with confirmed bTB and reactors without confirmed bTB. The strongest response was observed in reactors with confirmed bTB where the mean response was significantly

higher than the response in reactors without confirmed bTB and in-contacts with confirmed bTB p=0.033).

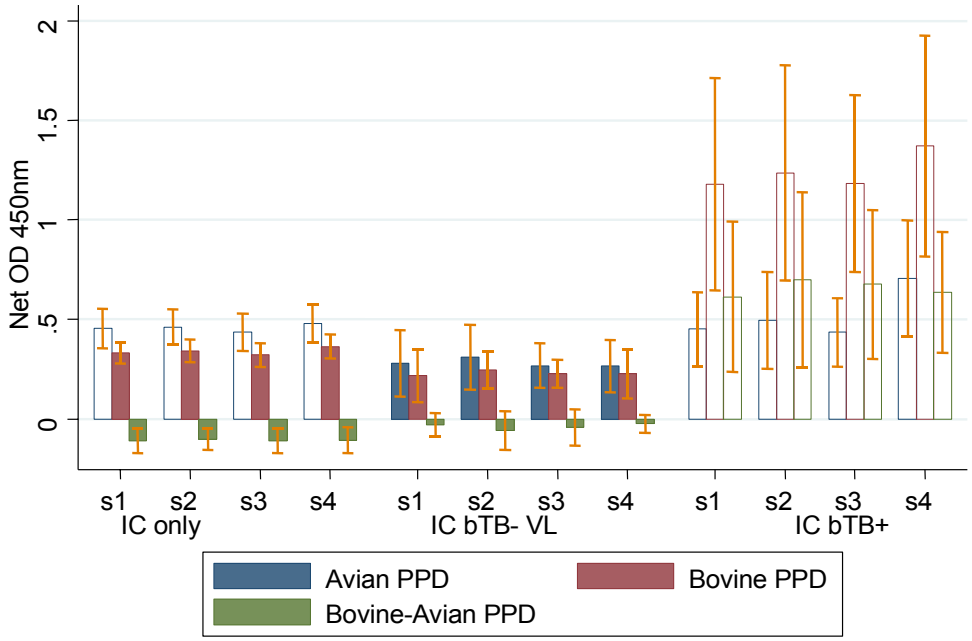
Figure 11. Serological response at different stages of disease



A response to MPB83 above 0.16 is interpreted as indicating infection with *M. bovis*. IC only = in-contact with no visible lesion and both culture and histology negative. R only = reactor with no visible lesion and both culture and histology negative. VL bTB- =visible lesion but both culture and histology negative. bTB+ =confirmed bTB (culture positive and/or histology positive).

Four blood samples were taken during a seven week holding period in in-contacts (mean days between bleeds 13.9 (SD 0.2)). Mean Bovine-Avian PPD IFN-γ responses did not vary over the holding period (see figure 12). Slightly higher responses were measured to ESAT6/CFP10 peptides and ESAT6 protein in the in-contacts with confirmed bTB in the last blood sample compared to previous samples but differences, were not statistically significant (p>0.300).

Figure 12. Mean IFN-γ response over holding period



A response to Bovine-Avian PPD above 0.1 is interpreted as indicating infection with *M. bovis*. IC = in-contact. VL bTB- =visible lesion but both culture and histology negative. bTB+=confirmed bTB (culture positive and/or histology positive).

Next, the predictive value of the immunological responses measured in the in-contact animals at selection for confirmed bTB were compared. To facilitate this, cut-off values were used to categorise immunological responses as positive or negative. The standard cut-off (Optical Density (OD) value of >0.1) for a positive PPD (PPDB-PPDA) response and positive ESAT6/CFP10 peptide response were used (SOP BAC0100) which provided respective specificities of 96.6% and 95.8% in the recent IFN-γ Specificity Trial. The specificity for that cut-off in this population (85%) was then used to identify an equivalent cut-off for the MPB83 response (OD>0.16) (the lower apparent specificity of the IFN-γ test in this study reflecting the absence of a true negative cohort). The cut-off applied for ESAT-6 protein was the same as the standard cut-off for ESAT6/CFP10 peptides.

Blood-based immunological tests conducted on skin test negative animals were better able to identify infected animals than an additional skin test conducted at the end of the holding period (see table 8). The most powerful predictor was a positive response to both Bovine-Avian PPD and ESAT6/CFP10 peptides. However, based on the blood samples taken from reactors at selection we also found that some skin test positive animals would have been missed by these tests at selection. There were 6/96 (6%) reactors with confirmed bTB and valid blood based immunological test results which tested negative to both Bovine-Avian PPD and ESAT6/CFP10 peptides at selection. Five reactors (7%) with unconfirmed bTB had positive responses to both Bovine-Avian PPD and ESAT6/CFP10. Considering the IFN-γ response to Bovine-Avian PPD alone, 9/96 (9%) of reactors with confirmed bTB tested negative, and 30/72 (42%) of reactors with unconfirmed bTB tested positive.

Table 8. Proportions of skin test negative in-contacts with confirmed bTB that would be detected by blood tests and an additional skin test

Immunological response	Confirmed bTB (n=20)		Unconfirmed bTB (n=154)			
	n	%	n	%	Odds Ratio	95%CI*
Blood tests at beginning of holding period†**						
Positive INF-γ response						
PPD (Bovine-Avian)	13	65	22	14.3	11.1	3.6 - 36.2
Peptides	11	55	9	5.8	19.7	5.6 - 68.8
ESAT 6	7	41	20	13.7	4.4	1.2 - 14.3
Peptides & PPD	10	50	2	1.3	76	12.8 - 750.8
Positive serological response						
MPB83	9	45	29	18.8	3.5	1.2 - 10.3
Positive response to SICCT at end of holding period‡						
Standard interpretation	2	10	3	2	5.6	0.4 - 51.4
Severe interpretation	3	15	6	3.9	6.6	2.2 - 20.0

*exact confidence intervals †conducted a mean of 19 days (SD16) after the last SICCT on farm
‡conducted a mean of 65 days (SD15) after the last SICCT on farm

The predictive power of the tests in the in-contacts were also compared using logistic regression where the outcome was the binary response bTB confirmed/unconfirmed. Variables for the responses to all tests (PPD, peptides, ESAT6 and MPB83), were placed in the model initially and the effect of the removal of each variable from the model was then examined using Akaike Information Criteria (AIC) and likelihood ratio tests. The model that provided the best fit to the data, regardless of whether binary or continuous data were used to indicate immunological response, was a model confined to responses to ESAT6/CFP10 peptides and PPD.

The immunological results reported above are only for those blood-based assays that could be practicably applied to routine diagnostic applications. Additional immunological assays were undertaken, for example lymphocyte transformation assay and IL-2 and IL-4 bioassays were performed at VLA Weybridge. However, review of these datasets indicated that they offered no additional diagnostic or predictive information over IFN-γ or serology, and since their methodologies would be unsuited to routine applications, this data is not reported.

3.8 *Immunology – IAH results*

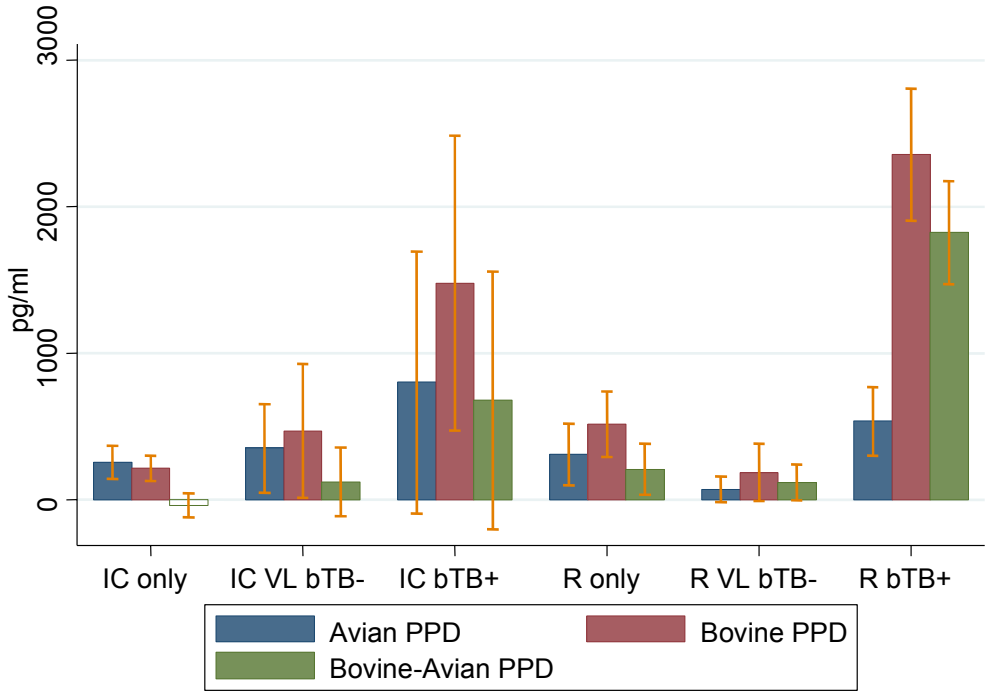
Table 9 shows number of animals for which valid immunological results existed for selected assays conducted at IAH. Measurement of T cell responses to BCG infected dendritic cells did not begin until September 2003, 18 months after the first animals were recruited.

Table 9. Animals with valid immunological results from blood drawn after recruitment (sample 1)

	Animals	
	IFN-γ & IL-10	CD4, CD8, WC1* responses to dendritic cells
In-contacts		
Confirmed bTB†	23	5
Visible lesion & unconfirmed bTB	12	3
No visible lesion & unconfirmed bTB	162	39
Reactors		
Confirmed bTB†	107	66
Visible lesion & unconfirmed bTB	13	5
No visible lesion & unconfirmed bTB	70	36

*Confirmed by culture for *M. bovis* or histology
*WC1=workshop cluster 1, expressed on a subset of gamma delta T cells

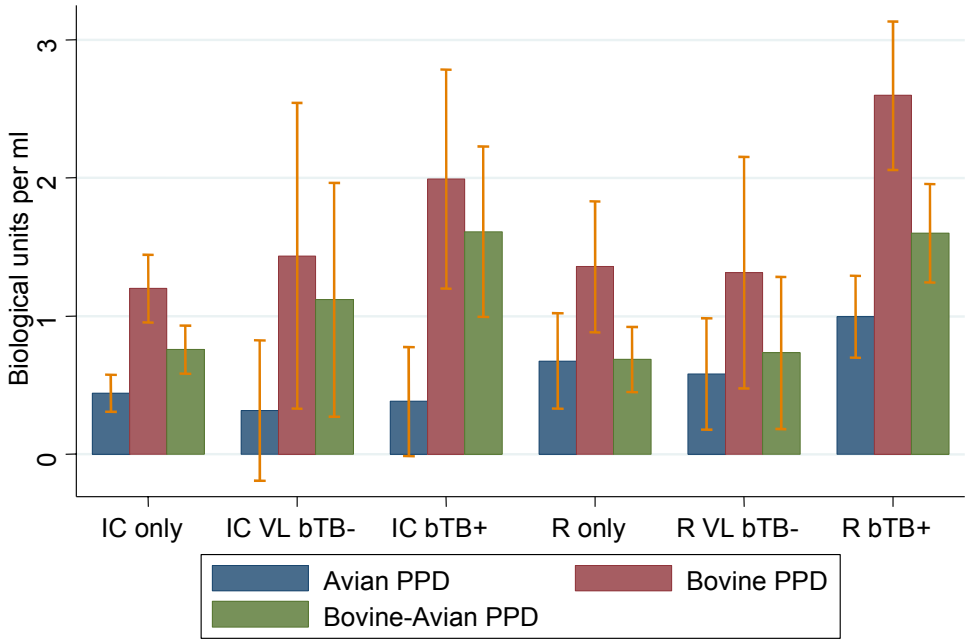
Figure 13. IFN-γ response at different disease stages



IC only = in-contact with no visible lesion and both culture and histology negative. R only = reactor with no visible lesion and both culture and histology negative. VL bTB- =visible lesion but both culture and histology negative. bTB+ =confirmed bTB (culture positive and/or histology positive).

Mean IFN-γ responses to bovine PPD were highest in in-contacts and reactors with confirmed bTB and significantly higher in reactors with confirmed bTB compared to all other groups ($p<0.0001$ comparison of geometric means) (see figure13). Mean responses to avian PPD were also higher in the animals with confirmed bTB, particularly in-contact animals but this value was based on responses from only 23 animals and the differences between groups were not significant. Responses in reactors with visible lesions but which were not culture positive were significantly ($p=0.007$) lower than those with lesions and confirmed TB.

Figure 14. IL-10 response at different disease stages

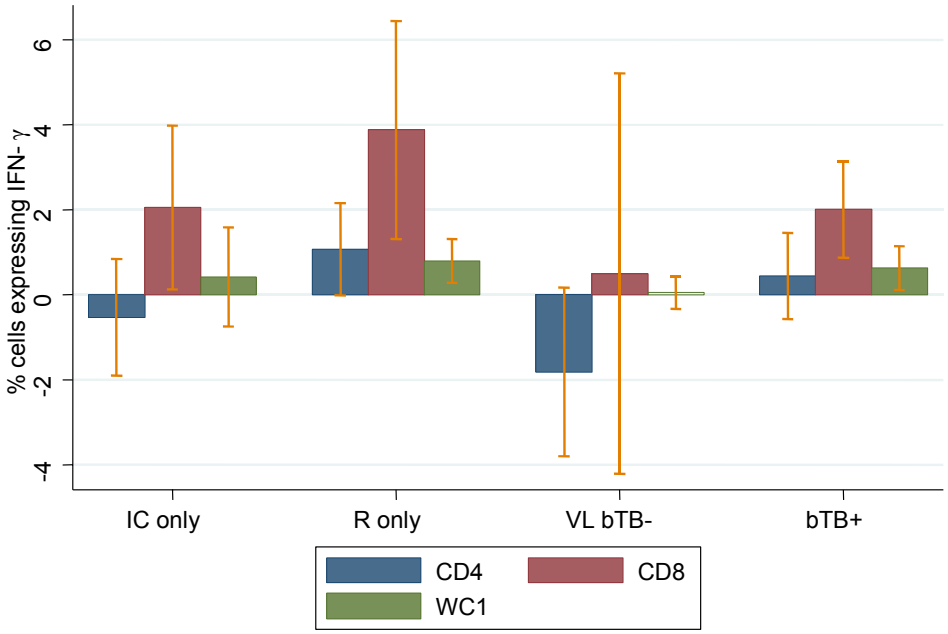


IC only = in-contact with no visible lesion and both culture and histology negative. R only = reactor with no visible lesion and both culture and histology negative. VL bTB- =visible lesion but both culture and histology negative. bTB+=confirmed bTB (culture positive and/or histology positive).

Interleukin 10 responses showed a similar pattern to that observed with IFN-γ (see figure 14). Mean responses to bovine PPD and bovine-avian PPD were highest in the reactors and in-contacts with confirmed bTB. Differences in the ratio of PPD-B specific IL-10:IFN-γ were observed between groups. In particular the ratio of IL-10:IFN-γ was lower in in-contacts with confirmed bTB compared to those without (data not shown).

Figure 15 shows the T cell response to BCG infected dendritic cells in animals classified by bTB severity group. Some differences between responses, particularly those of $CD8^+$ T cells, were observed but none were statistically significant.

Figure 15. T cell subset IFN-γ responses after stimulation infected dendritic cells

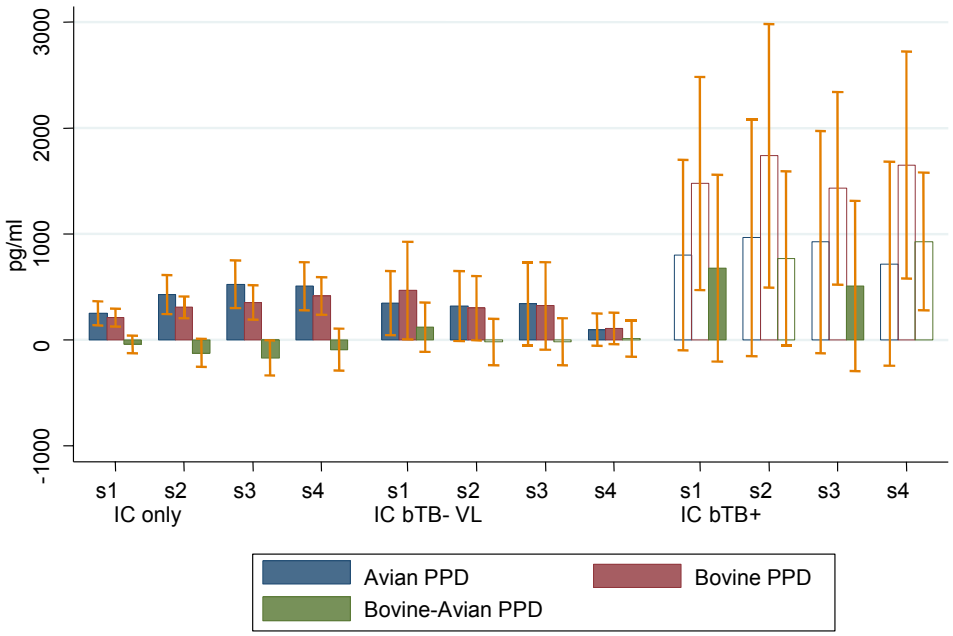


IC only = in-contact with no visible lesion and both culture and histology negative. R only = reactor with no visible lesion and both culture and histology negative. VL bTB- =visible lesion but both culture and histology negative. bTB+ =confirmed bTB (culture positive and/or histology positive). Reactors and in-contacts have been combined in visible lesion and bTB confirmed categories because of the small number of in-contacts for which there are sampling results.

Next the responses measured in the additional blood samples taken over the seven week holding period in in-contacts were compared (mean days between bleeds 13.9 (SD 0.2)). The mean responses (shown in figures 16, 17 and 18) varied somewhat but no time related trends were observed within groups. Overall mean IFNγ (figure 16) and IL-10 (figure 17) responses to bovine PPD were higher throughout the entire follow-up period in animals with confirmed bTB compared to other animals.

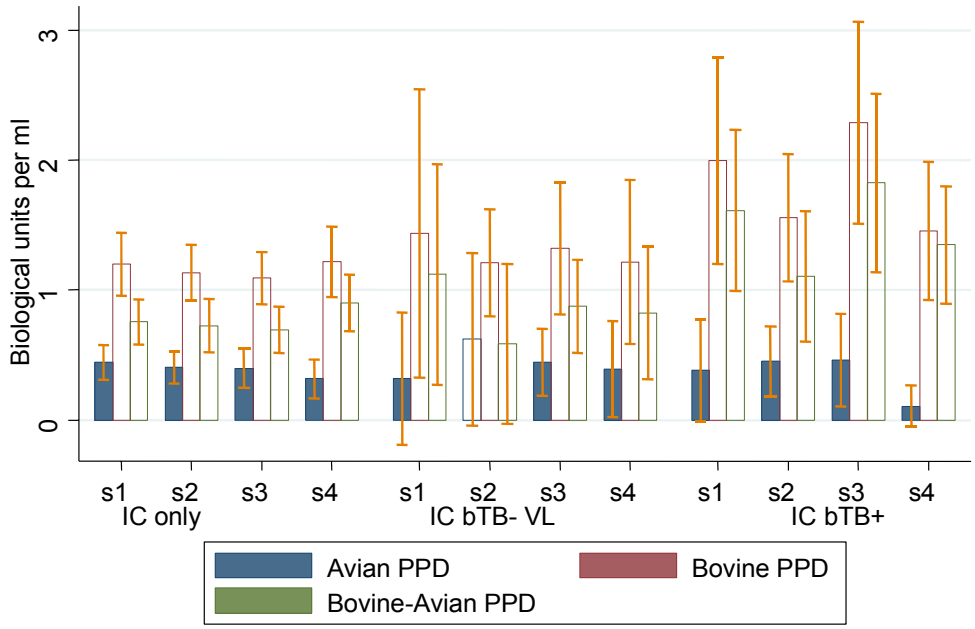
Although there appeared to be a reduction in $CD8^+$ and $WC1^+$ T cell responses to BCG infected dendritic cells (figure 18) over the holding period in bTB+ in contact animals, the sample numbers were too small to draw firm conclusions.

Figure 16. IFN-γ response over holding period



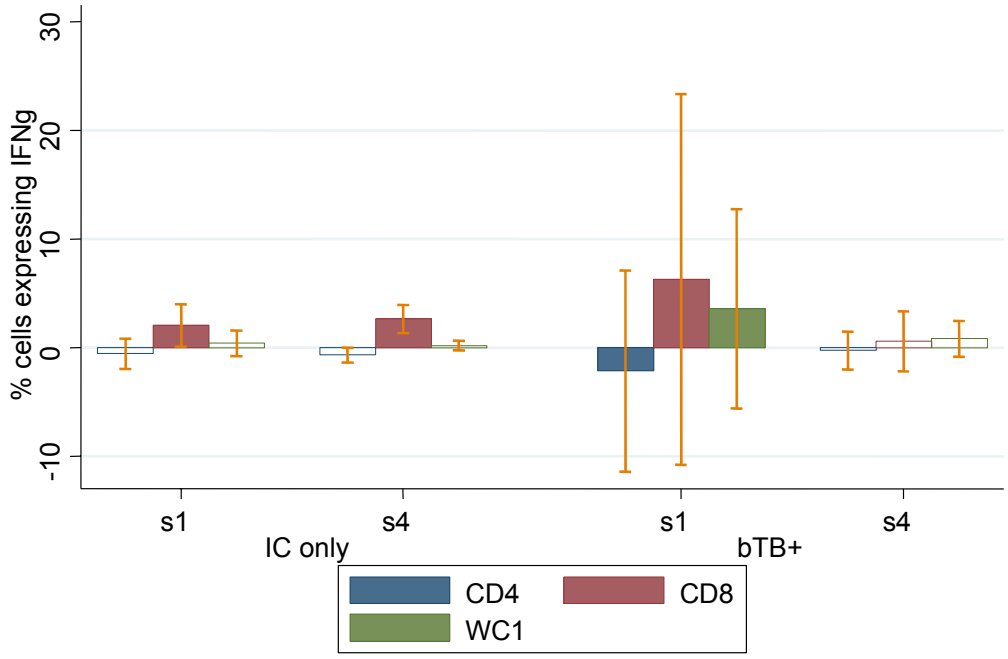
IC only = in-contact with no visible lesion and both culture and histology negative. VL bTB- =visible lesion and both culture and histology negative. bTB+=confirmed bTB - culture positive and/or histology positive.

Figure 17. IL-10 response over holding period



IC only = in-contact with no visible lesion and both culture and histology negative. VL bTB- =visible lesion but both culture and histology negative. bTB+=confirmed bTB (culture positive and/or histology positive).

Figure 18. T cell subset IFN-γ responses after stimulation with infected dendritic cells over holding period.



IC only = in-contact with no visible lesion and both culture and histology negative. bTB+=confirmed bTB (culture positive and/or histology positive).

Additional immunological parameters that were measured at IAH Compton included TNFα, IFN-γ by ELISPOT, IFN-γ by flow cytometry. Measurement of IFN-γ by either of these two assays did not provide any novel information when compared to IFN-γ ELISA measurement. TNFα levels were low in all cases and these data were not subjected to detailed analyses.

3.9 Inter-current disease

Clinical disease and medication history of dairy and non-dairy production classes were examined separately because of known differences in the management of dairy cattle compared to other cattle. The proportions of animals in each class with reported disease, disease exposure measured by antibody status, reported medication and vaccination are shown in table 10. In dairy animals, intra-mammary injections and Leptospira vaccinations were more common in in-contacts than in reactors. Antibodies to Leptospira were also more common in in-contacts compared to reactors. Antibodies to Johne's disease were more common in reactors than in-contact animals.

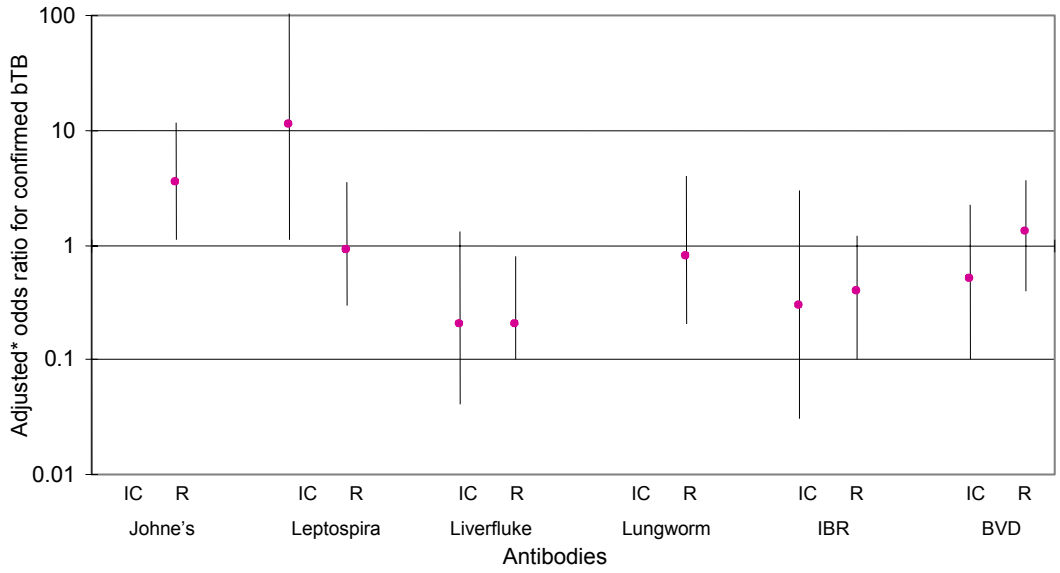
In non-dairy animals, intra-mammary injections were rare in both in-contacts and reactors. Leptospira vaccination was slightly higher in in-contacts, but there was no difference in the proportions of animals with antibodies to Leptospira. Flukicide medication was more common in reactors, but there was no difference in the prevalence of animals with antibodies to liver fluke. Anthelmintic medication was also more common in reactors compared to in-contacts. Antibodies to Johne's disease were more common in reactors compared to in-contacts but the difference was not as great as that observed in dairy animals. Antibodies to infectious bovine rhinotracheitis were generally higher in in-contacts than in reactors.

Table 10. Disease, vaccination and serology in dairy and non dairy in-contacts and reactors

	Dairy				Non-dairy			
	In-contacts (n=85)		Reactors (n=82)		In-contacts (n=115)		Reactors (n=118)	
	n	%	n	%	n	%	n	%
Disease during past 12 months								
Diarrhoea	1	1.2	0	0.0	1	0.9	2	1.7
Ill-thrift	0	0.0	1	1.2	5	4.3	2	1.7
Respiratory problems	1	1.2	2	2.4	4	3.5	4	3.4
Mastitis in females	15	17.6	10	12.2	0	0.0	1	0.8
Lameness	6	7.1	12	14.6	3	2.6	2	1.7
Infertility	2	2.4	6	7.3	0	0.0	2	1.7
Medication/vaccination in last 12 months								
Flukicide	16	18.8	11	13.4	8	7.0	19	16.1
Intra mammary in females	39	45.9	26	31.7	2	1.7	1	0.8
Bovine viral diarrhoea vaccine	22	25.9	14	17.1	1	0.9	2	1.7
Leptospira vaccine	36	42.4	19	23.2	7	6.1	4	3.4
Anthelmintic	19	22.4	25	30.5	58	50.4	75	63.6
Injected antibiotics	11	12.9	8	9.8	5	4.3	6	5.1
Salmonella vaccine	0	0.0	4	4.9	0	0.0	0	0.0
Vaccinated ever against								
Johne's disease	0	0.0	0	0.0	0	0.0	0	0.0
Lungworm	28	32.9	23	28.0	9	7.8	4	3.4
Infectious bovine rhinotracheitis	0	0.0	6	7.3	0	0.0	2	1.7
Antibodies measured in blood sample drawn at recruitment								
Johne's disease	5	5.9	16	19.5	27	23.5	35	29.7
Leptospira	36	42.4	18	22.0	34	29.6	33	28.0
Liverfluke	69	81.2	61	74.4	76	66.1	74	62.7
Lungworm	72	84.7	65	79.3	82	71.3	83	70.3
Infectious bovine rhinotracheitis	22	25.9	27	32.9	25	21.7	11	9.3
Bovine viral diarrhoea	53	62.4	50	61.0	53	46.1	63	53.4

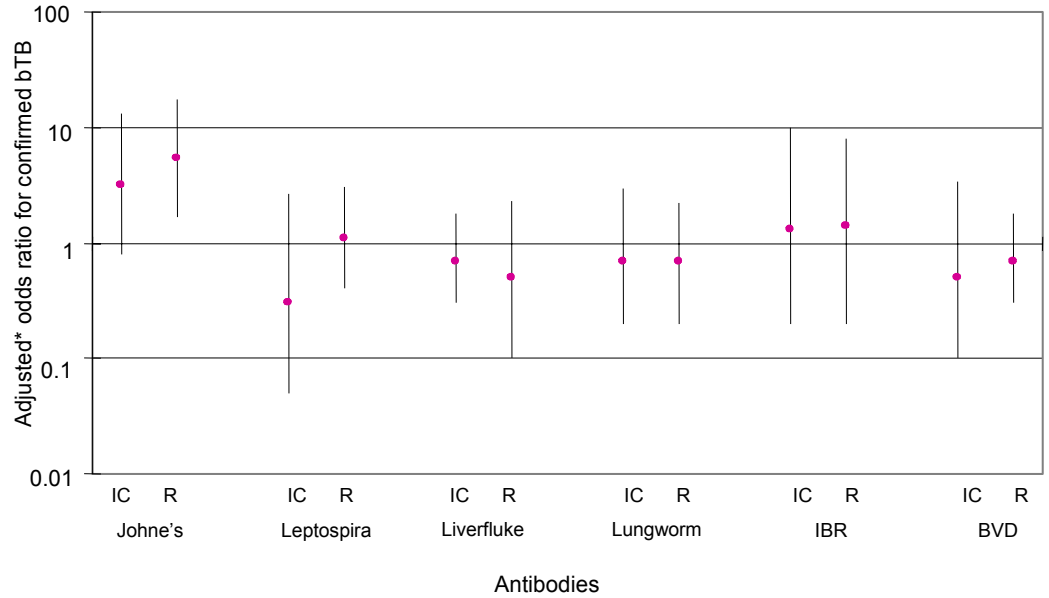
Next, the effect of antibody status on bTB confirmation was examined (see figures 19a and 19b). Having antibodies to Johne's disease was associated with having confirmed bTB in both dairy (aOR 3.6, 95%CI 1.1-12.8, p=0.038) and non-dairy reactors (aOR 5.4, 95%CI 1.7 -17.6, p=0.005) and the association in non-dairy in-contacts was in the same direction, although not as statistically significant (aOR 3.2, 95%CI 0.8-13.0, p=0.110). Having antibodies to Leptospira was strongly associated with bTB confirmation in in-contact dairy cattle (aOR 12.3, 95%CI 1.1 – 136.0, p=0.041) but if anything there was the opposite association in in-contact non-dairy cattle (aOR 0.2, 95%CI 0.2-2.7, p=0.265). There was no association between antibodies to Leptospira in either dairy or non-dairy reactors. Having antibodies to liverfluke was associated with a lower risk of confirmed bTB in all four groups and most statistically significant in dairy reactors (aOR 0.2, 95%CI 0.1 – 0.7, p=0.016). If the analysis was confined to dairy breeds rather than production class (see table 1) an even stronger negative relation emerged between antibodies to liverfluke and bTB confirmation (aOR 0.04, 95%CI 0.1-0.5, p=0.013).

Figure 19a. Association between antibodies to specific diseases and confirmed bTB in reactor and in-contact dairy cattle



IC= in-contacts R=reactors *Adjusting for age, medication, vaccination, number of reactors in herd and SICTT testing interval. Not possible to calculate two odds ratios: None of in-contacts without antibodies to lungworm had confirmed bTB and none of in-contacts with Johne's antibodies had confirmed bTB.

Figure 19b. Association between antibodies to specific diseases and confirmed bTB in reactor and in-contact non-dairy cattle



IC= in-contacts R=reactors
*Adjusting for age, medication, vaccination, number of reactors in herd and SICTT testing interval.

3.10 Trace nutrients

There was little evidence for a difference by bTB status in copper or vitamin B12 measured in blood drawn on farm at recruitment (see table 11). By contrast, levels of the enzyme GSHPx, a surrogate for plasma selenium, varied significantly between in-contacts and reactors and indicators of bTB status.

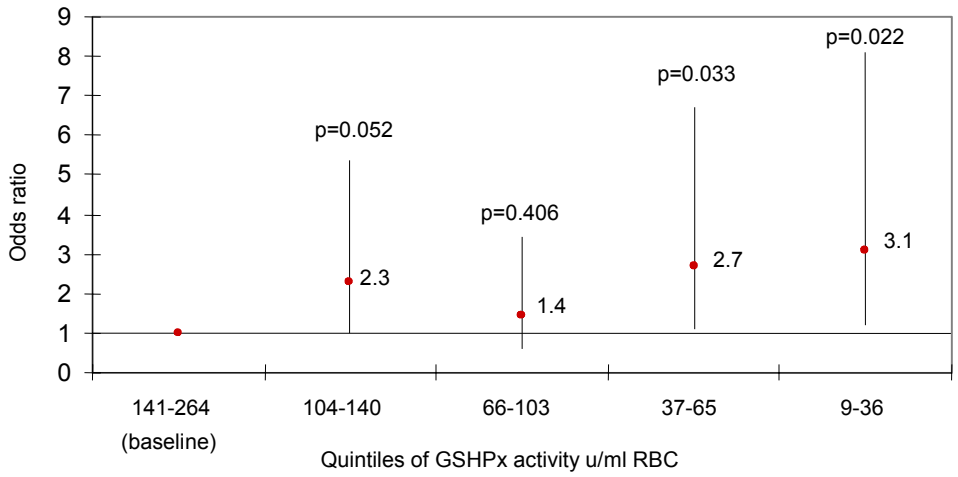
Table 11. Trace nutrient levels in blood by bTB status

Group At recruitment	n	Median	IQR	p value for difference in means*
GSHPx (u/mL RBC)				
In-contacts	199	94.4	48.8-138.5	
Reactors	198	74.2	37.5-126.7	0.027
bTB unconfirmed	266	94.3	48.0-140.3	
bTB confirmed	131	63.5	35.4-114.2	0.0004
No visible lesions	258	93.7	48.5-138.5	
Visible lesion	138	66.3	36.4-117.8	0.003
Vitamin B12 [pmol/L]				
In-contacts	199	170	132-213	
Reactors	198	173	132-214	0.984
bTB unconfirmed	266	174	132-215	
bTB confirmed	131	167	127-209	0.611
No visible lesions	258	174	132-213	
Visible lesion	138	169	133-215	0.988
Copper [µmol/L]				
In-contacts	199	15	13.2-17	
Reactors	198	14.7	12.8-17	0.045
bTB unconfirmed	266	14.9	12.9-16.9	
bTB confirmed	131	14.9	13.0-17.5	0.333
No visible lesions	258	14.9	12.9-16.9	
Visible lesion	138	14.9	12.9-17.7	0.279

*Geometric means for GSHPx and vitamin B12
† 98% of selenium in blood is converted to GSPHx during erythropoesis (Koller and Exon 1986)
‡ bTB confirmation by culture or histology (see Figure 4)
±Vitamin B12 is a marker for cobalt levels

In a logistic regression model adjusting for age, production class and other factors, animals in the lowest two quintiles for selenium exposure (levels below 65 u/mL RBC) had an approximately threefold higher risk of having *M. bovis* detected by culture ((aOR 2.85 (95%CI 1.21-6.71) see figure 20).

Figure 20. Adjusted odds ratio for bTB confirmation by quintiles of GSPHx activity



bTB confirmation=Culture and/or histology positive (see Figure 4). P values are for odds for quintile compared to baseline quintile. Odds ratios are adjusted for age, number of reactors in herd, testing interval, PM laboratory and animal production class. Number of animals in each quintile~80.

Trace nutrient levels were measured twice in in-contact animals: At recruitment and at the end of the seven week holding period. Overall, levels of GSHPx in blood increased in animals bTB confirmed and unconfirmed at slaughter (see table 12).

Table 12. Levels of GSPHx measured in in-contacts at recruitment and at slaughter

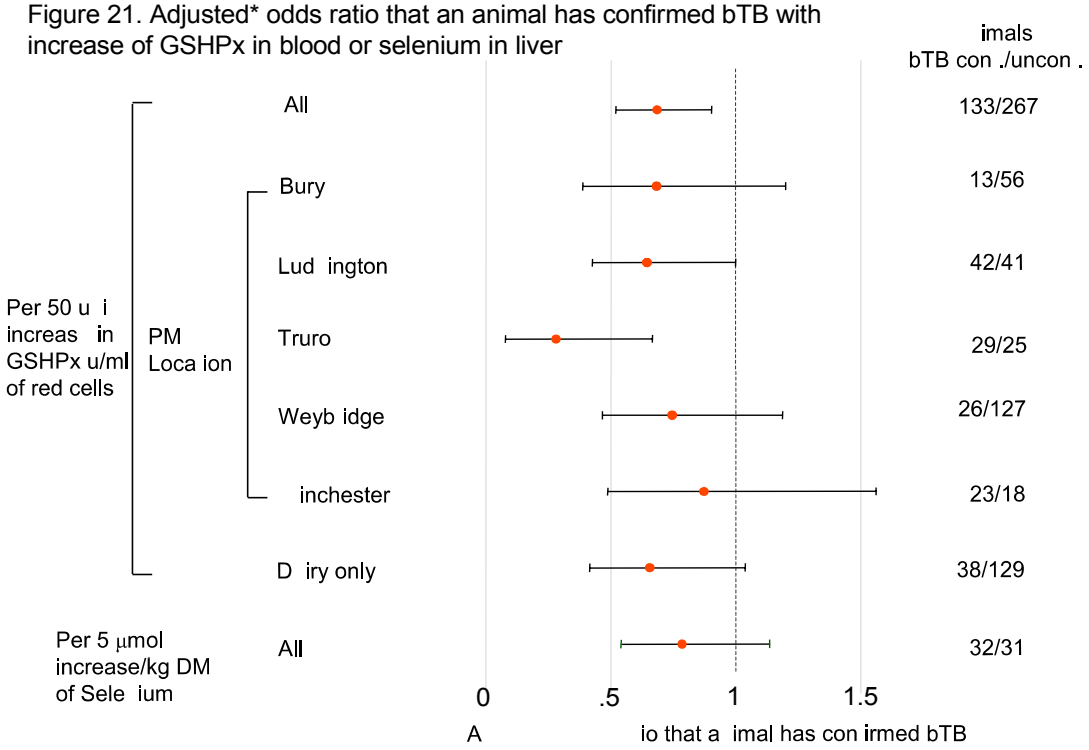
		n	Median	IQR	p value*
All in-contacts	At recruitment	199	94.4	48.8-138.5	
	7 weeks later at slaughter	200	103	64.4-142.7	0.006
Confirmed bTB	At recruitment	22	108	65.9-125.5	
	7 weeks later at slaughter	23	114.5	92.6-132.2	0.219
Unconfirmed bTB	At recruitment	177	93.7	47-139.4	
	7 weeks later at slaughter	177	101.1	63.3-144	0.012

*Paired T test for difference in geometric means

Levels of GSHPx tended to be higher in dairy animals compared to other production types. The median level in dairy animals was 131 u/mL RBC (IQR 101 – 157) compared to 54 in non-dairy animals (IQR 31 - 84) (geometric p value for difference<0.001). However a negative association between GSPHx and bTB confirmation could still be observed when the association was examined within dairy animals and when calculated for each PM laboratory separately (see Figure 21).

There was also some evidence that a lower level of liver selenium was associated with an increased the risk of being a reactor. The median level in in-contacts was 16 µmol/Kg DM (IQR 14-19) compared to a median of 8 in reactors (IQR 5-17). Lower liver selenium was associated with an increased risk of bTB confirmation although the association was not statistically significant (OR 0.95 per µmol/Kg DM (95%CI 0.9-1.0) (see figure 21)

Figure 21. Adjusted* odds ratio that an animal has confirmed bTB with increase of GSHPx in blood or selenium in liver



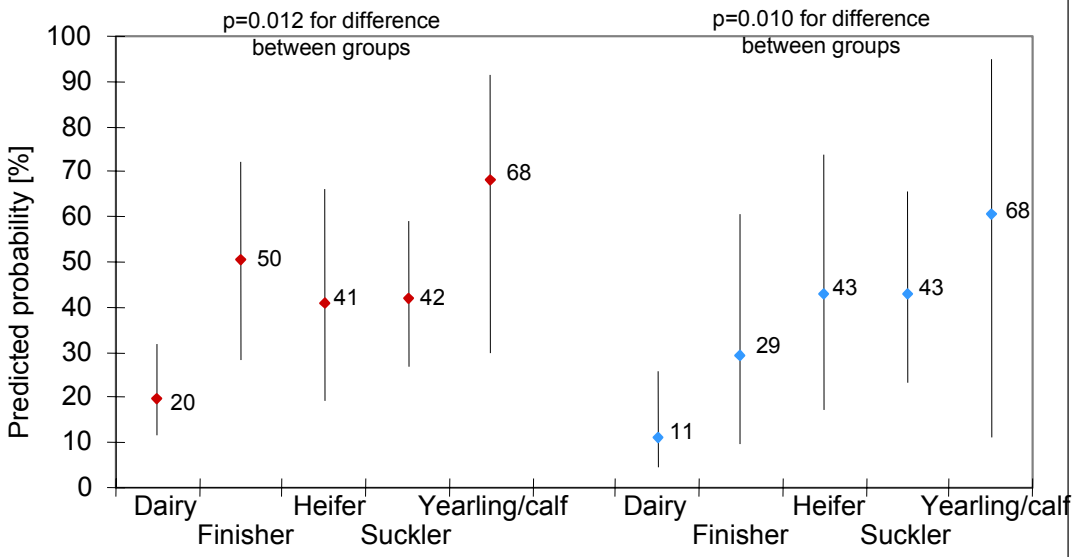
*Adjusted for age, number of reactors in herd, testing interval, PM laboratory and animal production type as relevant.

3.11 Animal Production class

The percent of animals with lesions and confirmed bTB was lower in dairy animals compared to other production types (see table 5). Logistic regression was used to model the relation between confirmed bTB and production class whilst adjusting for age, testing interval and for the level of immunological response in the animals (measured by difference in bovine and avian response to the SICTT or difference in IFN-γ response to bovine and avian PPD).

Figure 22 shows the adjusted percent risks derived from the logistic regression model adjusted to the immunological thresholds used to defined reactor status. Adjusting for selenium levels or presence of anti-bodies to liverfluke had little effect on differences between classes. However, adjusting for both factors reduced differences and the level of significance (p=0.431).

Figure 22. Probability of confirmed bTB adjusted to the same immunological response, for age and bTB testing interval



Adjusted to bovine-avian difference in skin test of 4mm

n=200

Adjusted to mean bovine-avian PPD OD of 0.1 & mean bovine PPD ODD of –0.1

n=168, see table 7

4.0 Discussion

4.1 External validity of study sample

The study population samples comprised animals naturally exposed to bTB. The two samples selected were characteristic of two major epidemiological groups of concern in GB herds today: animals from herds with new outbreaks of bTB and animals from herds with persistent breakdowns where it has not been possible to resolve infection.

Reactors were randomly selected from virtually all regions of England and Wales in which bTB is endemic. The distribution of spoligotypes in the reactor sample was very similar to the overall distribution of spoligotypes in herds in England and Wales. However, a stratification technique was employed in the selection of reactors ensuring that single-reactor herds were under-represented compared with British herds in general. This is likely to mean that the weight of infection in the herds selected for the study was greater than the average weight of infection in herds with new breakdowns in GB during the study time period. Three percent of the cattle came from herds with more than one *M. bovis* spoligotype isolated in other reactors during the breakdown. However, since a tissue sample from all reactors in a breakdown is not routinely sent for culture and spoligotyping, the breakdowns may have contained other *M. bovis* spoligotypes.

For logistic reasons, in-contacts were selected from a smaller number of regions. The selection criteria were designed to ensure that animals selected were representative of "dangerous contacts" routinely identified by VOs as potentially infected and a possible source of further spread of bTB. In general the herds from which in-contacts were selected were larger and contained more reactors than those herds from which reactors were selected. This is likely to have been a function of the purposive selection for herds where in-contacts were most likely to be exposed to bTB infection i.e. herds with more reactors.

4.2 Lesion distribution

Gross lesions typical of bTB were commonly observed in the thorax and head of both reactors and in-contact animals, and particularly the thorax. Other studies of cattle naturally infected with *M. bovis* have

also reported a predominance of thorax and head involvement (Lepper and Pearson 1973; Whipple and others 1996) and respiration of infected aerosols is recognised as the principal route of infection in contemporary cattle (Buddle and others 1994; Griffin and Dolan 1995). In reactors, infection in the abdomen was a strong predictor of infection in the head and thorax, but infection in the thorax and head were not predictors of infection in the abdomen. This is consistent with the hypothesis that infection is spread to the abdomen via infected saliva or sputum coughed up from the lungs.

Lesion distributions resulting from intra-nasal and intra-tracheal inoculation with *M. bovis* have been examined in longitudinal experimental studies. Cattle infected intra-nasally are more likely to have lesions in the tonsils and upper respiratory tract (Cassidy and others 1999), whereas the profile resulting from intra-tracheal infection is similar to the profile observed in this study (Buddle and others 1994) with a predominance of lesions in the thorax following low-dose inoculation.

Studies before the implementation of widespread tuberculin testing and milk pasteurisation indicated the alimentary tract as an important route of infection (Neill and others 1994; Stamp 1944).There was more involvement of abdominal lymph nodes in the infected in-contact animals compared to reactors, although the finding was based on very small numbers. The finding, if real, could be accounted for by one or both of the following hypotheses: i) that in-contact animals are at greater risk of infection through the oral route than reactors i.e. are more likely to be exposed to infected milk or other feedstuffs including pasture; and/or ii) the pathogenesis of disease differs between *M. bovis* subtypes and the pathogenesis of subtypes found in in-contact animals includes more abdominal involvement.

4.3 Abattoir inspection procedures in GB

An attempt was made to determine whether the level of abattoir post mortem inspection currently required under UK legislation is adequate. One concern has been that animals are missed because the range of lymph nodes and tissues examined is insufficient. In this study, bTB-like lesions in animals with confirmed bTB were only observed in sites already specified in the Fresh Meat Hygiene Regulations and lesions were not found in other sites. Unsurprisingly, the study showed that not all cattle infected with *M. bovis* had gross lesions typical of bTB visible in tissues examined at PM. Bovine TB was confirmed in seven in-contacts (3.5%) without visible lesions and in 12 reactors (6%) without visible lesions.

An Australian study reported that the abattoir inspection procedure detected an estimated 47% of lesions compared to a detailed post-mortem (Buddle and others 1994). By contrast, we found a strong association between the notional TB50a classification and classification of animals based on the detailed post mortem. However, the TB50a part of the PM inspection did not demonstrably follow usual abattoir inspection practice and was not conducted in an abattoir under conditions of limited time and space.

In short, the study indicated that appropriate tissues are examined during abattoir inspection conforming to the Meat Hygiene Regulations in GB but confirmed that even a detailed PM will miss infected animals; tissues from animals without visible lesions were culture or histology positive. Agreement between bTB confirmation by culture and histology was extremely high at the animal level (see figure 4) confirming that both are reliable methods of bTB confirmation but also showing that a minority of cases will be preferentially diagnosed by both.

4.4 Immunological profile of disease

4.41 VLA

Comparison of IFN-γ responses in reactors that were grouped according to disease severity suggests little or no response to the defined antigens (ESAT6/CFP10 peptides or ESAT-6 protein) in reactors where disease was not confirmed by culture or histopathology (figure 10). A simplistic interpretation of this observation could be that these defined antigens are more specific diagnostic reagents and that the positive reaction to PPD in the skin test, and to a lesser extent the IFN-γ test, are false positive responses. However, it is important to consider that these defined antigens lack the sensitivity of PPD and that these animals may be at an earlier stage of disease and thus PPD is representing a sensitivity

advantage. In experimentally infected cattle it has been demonstrated that the strength of ESAT-6 responses correlate with pathological severity[1] (Lyashchenko and others 2004; Vordermeier and others 2002). Therefore, it is more likely that only the most sensitive diagnostic antigen, i.e. PPD, will detect animals in very early stages of disease. Concomitant with the idea that immune responses increase with disease severity and bacillary load (Lyashchenko and others 2004), the strength of MPB83 serological responses also increased with increased 'ranking' of disease severity (see figure 11).

4.42 IAH

Differences in a number of immune parameters were noted between groups of animals classified by disease status. Overall the number of in-contact animals in these groups was low and, with large animal to animal variation observed in many of the immunological tests, data interpretation was difficult. However, in in-contact animals, progression to disease (confirmed bTB) was associated with reduction in antigen specific T cell responses, particularly in $CD8^+$ and $WC1^+$ T cell subsets. A potential loss of cytotoxic T cell activity would result in enhanced survival of *M. bovis* within the host and faster progression towards disease. Experimental depletion of $CD8^+$ and $WC1^+$ T cells from cattle has been shown to result in increased disease severity (Kennedy and others 2003; Villarreal-Ramos and others 2006)indicating an important role in control of bTB. Further analyses of these parameters could provide useful information regarding disease progression and pinpoint appropriate areas for targeted vaccination strategies.

Levels of IFN-γ and IL-10 were highest in animals with confirmed bTB. Those in-contacts which were confirmed bTB at the end of the holding period had consistently higher IFN-γ responses throughout the entire holding period compared to non-TB^+ in-contacts. These elevated IFN-γ levels were observed even at the first blood sample suggesting that IFN-γ is a relatively early predictor of disease which may more accurately detect infected animals when compared to skin test. Measurement of IL-10 gave broadly similar responses with respect to disease classification/animal groups. However measurement of PPD-B specific IL-10 did not allow more sensitive or accurate diagnosis of bTB. The relative ratios of IL-10: IFN-γ indicated that measurement of dual parameters could potentially be indicative of a change in disease status; in-contact bTB animals had much lower IL-10: IFN-γ ratios than in-contacts without confirmed disease. This suggests a change in immunological profile that would enable growth/dissemination of *M. bovis* resulting in bTB. Overall, interesting data were generated regarding relative levels of IL-10 and IFN-γ, and T cell responses that warrant further investigation.

4.5 Alternative immunological tests for bTB

A particularly important outcome of this study is the confirmation that bTB infection can be missed by skin testing alone and that it may be possible to detect a large proportion of these animals by using blood based assays. Of 20 bTB confirmed in-contacts for which QC valid immunological results were available, 14/20 (70%) of them would have been detected by positive IFN-γ responses to ESAT-6/CFP-10 peptides and/or PPD (bovine - avian), or 9/20 (45%) with MPB83 serology alone. If serology and IFN-γ positive animals were combined, 16/20 (80%) of the skin-test negative in-contacts were detected. Therefore, the sensitivity benefits of using blood tests alongside the skin test has been demonstrated again in this study. Additionally, benefits can be obtained by combining blood tests (serology and IFN-γ), but cost-benefit analysis needs to be performed to determine if these benefits off-set the increased costs. It is also important to note that a further short-interval skin test (SIT) would not have detected the majority of animals detected by blood tests. Only 3/20 bTB confirmed in-contacts were skin test positive (on severe interpretation) at the follow up SIT and these three animals would have been detected by PPD in the IFN-γ assay.

The results from in-contacts were used to determine odds ratios for a skin test negative but blood-test positive animal having confirmed bTB (as defined by culture or histopathological). The most powerful predictor for bTB (OR = 76.0) was a positive response to both PPD (bovine - avian) and ESAT-6/CFP-10 derived peptides (Table 8). However, applying this interpretation will also result in decreased test sensitivity compared with the use of just PPD IFN-γ as a positive predictor (OR = 11.1). Therefore, a positive response to PPD and ESAT6/CFP10 peptides should only be used when specificity is a premier

concern, e.g. when attempting to resolve suspected chronic non-specific skin test reactions (NSR). Regardless, these data demonstrated that IFN-γ positive animals were many times more likely to be *M. bovis* culture positive compared to IFN-γ negative animals. This is in good agreement with a similar study performed in the Republic of Ireland that reported an odds ratio of 8.9 that a skin test negative but PPD IFN-γ positive animal would be a skin test reactor at the next SIT (Gormley and others 2006). Similarly, a study in Northern Ireland reported that a IFN-γ positive but skin test negative cow had an 18% risk of a becoming skin test positive within one year (Dr Jim McNair, personal communication).

Our results also showed that 9% of the skin test positive reactors with confirmed bTB did not have a positive IFN-γ response to Bovine-Avian PPD and 24% did not have positive responses to both Bovine-Avian PPD and ESAT6/CFP10 peptides at selection. However since immunological blood-based tests were not used to select the animals for the study we cannot determine how many additional bTB confirmed animals would be identified by the blood-based tests that were missed by the SICCT. These data further emphasise the utility of INF-y tests in combination with the SICCT.

4.6 Skin test negative animals as a source of infection

Bovine TB was confirmed by culture and/or histology in 23/200 (11.5%) of in-contact animals skin test negative at the time of selection. A further 13 animals (one of which was a reactor at standard interpretation at the end of the holding period) had gross macroscopic lesions typical of bTB. Nasal shedding of *M. bovis* was investigated as a possible significant route of infection and disease transmission, particularly in skin test negative animals since shedding, if it occurred, is more likely to represent an undetected source of infection and disease transmission in these animals.

Results from other studies of nasal mucus are conflicting. The external validity of experimental studies where cattle have been infected intra-nasally, and nasal shedding of *M. bovis* subsequently detected, is questionable. However, shedding of *M. bovis* was observed in one study of naturally infected cattle and appeared to be greatest soon after infection (McCorry and others 2005). In a larger experimental study (SE3033), currently ongoing, no *M. bovis* culture positive samples have been detected in approximately 1000 nasal samples taken from 40 reactors at varying time intervals.

M. bovis was not detected in any of the nasal mucous samples collected from either in-contacts or reactors in this project. Decontamination of samples was not conducted because earlier work at VLA Weybridge suggested that a deleterious reduction in sensitivity would occur in samples anticipated to be sparely infected. Some contamination did occur, but there were 1006 uncontaminated samples from 335 (84%) animals. The results suggest that large concentrations of *M. bovis* are not present in the nasal passages and that the shedding of *M. bovis* in nasal mucus, if it occurs, is rare in naturally infected GB cattle. However, transmission through mouth respiration and coughing was not investigated and these remain potential sources of disease transmission in infected skin test negative animals.

4.6 Inter-current disease

Clinical history and exposure to common cattle diseases were measured to determine if these factors might interact with the tuberculin test thereby reducing test accuracy. The performance of diagnostic tests for bTB is dependent on immune response which may be critically influenced by exposure to other diseases. Given the observational design of the study, we were aware that that observed effects might be related to confounding and selection bias. In analysing the data we attempted to reduce the effects from biases due to differences associated with cattle husbandry by stratifying analyses into a dairy and non-dairy group as well as controlling for other factors that might act as confounders. Furthermore, we examined whether similar patterns were observed in both reactors and in-contacts.

Higher incidence of false positives to the tuberculin test has been attributed to cross-reactivity between *M. bovis* with *Mycobacterium avium* subspecies paratuberculosis (Aranaz and others 2006). Our findings did not support this: we found no evidence of a positive association between past exposure to Johne's disease and unconfirmed bTB. In fact, we observed strong positive associations between past exposure to Johne's disease and confirmed bTB in non-dairy reactors and in-contacts and dairy reactors

(none of the dairy in-contacts with antibodies to Johne's disease had confirmed bTB). The positive association between having antibodies to Johne's disease and confirmed bTB suggests similar exposure patterns to *M. bovis* and *M. avium* subspecies *paratuberculosis* in cattle in GB. It is possible, therefore, that because of co-linear exposure to the bacteria we are unable to disentangle effects from cross-reactivity in this study.

Consistent negative associations were observed between having antibodies to liverfluke (*Fasciola hepatica)* and having confirmed bTB. The effect was most significant in dairy reactors. This finding merits further investigation. It could be explained by some husbandry factor associated with exposure to liverfluke that is also associated with a factor that retards pathogenesis of bTB; possibly related to season of selection of cattle. Possibly infection with liver fluke modulates the inflammatory response leading to a lower positive predictive value of the SICTT in infected animals. Liverfluke antigens are potent stimulators of T-helper (Th2) responses (Dalton 1998). Prior or concurrent exposure to liverfluke antigens may modulate the cell-mediated response to tuberculin which is the basis for the SICTT.

4.7 Trace nutrients

The project included assessment of trace nutrients because deficiencies may affect the susceptibility of cattle to bTB by depressing immune function (Field and others 2002; Underwood and Suttle 1999) and hence contribute to the persistence and recurrence of bTB. Selenium, copper and vitamin B12 were monitored since deficiencies of all three trace nutrients can occur in the UK.

Glutathione peroxidase (GSHPx) is a red cell enzyme that contains four selenium atoms in each enzyme molecule. Activity of this enzyme correlates well with plasma selenium concentrations in the low and normal ranges (Kerr 2002; Koller and Exon 1986). The risk of clinical selenium deficiency increases progressively when red cell GSHPx declines below 30 u /mL RBC and deficiency typically presents as a muscular dystrophy known as "white muscle disease" in cattle and sheep (Kerr 2002; Koller and Exon 1986). Selenium deficiency has also been related to depressed immune function, including both humoral and cell mediated immunity and the control of free radicals (Field and others 2002; Koller and Exon 1986; Underwood and Suttle 1999).

Copper is an important enzyme co-factor for the synthesis of haemoglobin, melanin and elastin protein. Deficiency symptoms include generalised ill-thrift and failure to grow, anaemia and infertility. Lack of dietary cobalt leads to vitamin B12 deficiency in ruminants. Vitamin B12 deficiency typically presents as ill-thrift and if left untreated, deficiencies can lead to severe anaemia and potential nervous system damage (Underwood and Suttle 1999). In preliminary analyses, levels of copper and vitamin B12 did not appear to vary between animals with unconfirmed bTB and confirmed bTB. The results for copper are not conclusive however and require further investigation since deficiencies may be masked by inflammatory processes (Underwood and Suttle 1999).

Consistent negative associations were observed between GSPHx levels in blood and risk of bTB both in simple bivariate comparisons and in regression analysis controlling for possible confounding factors, indicating that with lower levels of GSHPx the risk of confirmed bTB increased. The odds ratio for confirming bTB was almost three-fold higher for animals with GSPHx levels less than 65 u/mL RBC than for animals with levels above. Levels of selenium in the liver samples of animals with confirmed bTB were also lower than in other animals although the association was not statistically significant. In logistic regression analyses we adjusted for a large number of possible confounders including animal production class and number of reactors in the herd. Levels of trace nutrients in blood or feed of the selected animals were unknown at the time of selection. However, a possible selection bias could only be excluded by a study where exposure to trace nutrients is allocated truly randomly.

It is also not possible to determine whether effects from selenium are causal since the study design does not enable us to distinguish whether the lower selenium levels are a risk factor for bTB or a result of the disease pathogenesis. The increase in GSPHx levels in diseased in-contacts over the holding period suggests that having bTB does not interfere with selenium absorption and metabolism, but these results

were based on very small numbers. However, since infected study animals are likely to be quite early in the bTB pathogenesis (Stamp 1944), it seems more likely that the effect of selenium is causal rather than an outcome of disease. Previous research has demonstrated that low selenium levels are associated with poorer health and lower milk yields (Kommisrud and others 2002). A case-control study of animal husbandry and bTB conducted in Ireland (Griffin and others 1993), found a failure to provide mineral licks to be strongly associated with case farms and it was postulated that this might be due to mineral deficiencies. None of the animals recruited in the study showed signs of clinical deficiency and could not have been recruited if such signs had been evident (Animals (Scientific Procedures) Act, 1986). Therefore the effects related to selenium, if causal, are operating at a sub-clinical level.

4.8 Novel hypotheses

Dairy reactors were less likely to have bTB confirmed than other production classes. This difference was unexpected and persisted even with adjustment for level of immunological response and testing interval. It could be explained by higher sensitivity of the SICTT and bovigam INF-γ tests in dairy animals identifying dairy animals that will eventually develop bovine TB being identified earlier in bTB pathogenesis. An alternative hypothesis is that, dairy animals exposed to bTB infection are more resistant to the disease and less likely to develop pathology. The fact that a similar relation was observed whether adjusting for skin test response or INF-γ response suggests that the differences are unlikely to be due to a feature of the test that varies between production types, such as skin thickness.

One possible factor, is husbandry differences between dairy and other cattle. Selenium levels measured by GSHPx were higher in dairy animals and higher GSPHx levels were associated with a lower risk of bTB confirmation. Antibodies to liver fluke were also negatively associated with bTB confirmation. Adjustment for selenium levels or antibodies to liver fluke, in a model of the relation between production class and bTB confirmation, made little difference to the size of differences between classes. However, adjustment for both selenium levels and antibodies to liver fluke reduced both the size of differences between classes and the significance of the difference between classes. Possibly these two factors are indicative of differences in animal production that have an effect on bTB pathogenesis.

Examination of the VETNET surveillance data for the time period of the study (2002 – 2005) supports the difference in bTB confirmation rates observed in dairy animals and other production classes observed in this study. Dairy animals were significantly less likely to have bTB confirmed by culture than beef and other production classes; both in herds with low bTB prevalence and high bTB prevalence (T. Goodchild, personal communication, see table 13). In conclusion the there appears to be a real difference in bTB confirmation rates between dairy and other cattle. This has implications not only for the potential identification of a factor or factors that might reduce pathology in non-dairy production classes but also for the interpretation of bTB diagnostic tests.

Table 13. Proportion of reactors in national herd with visible lesion or *M. bovis* confirmed by culture 2002-2005 inclusive.

	High incidence district >=0.125%			Low incidence district < 0.125%		
	Reactors [n]	mean [%]	95% CI	Reactors [n]	mean [%]	95% CI
Beef	24254	44.7	41.8 to 47.6	3791	28.3	23.9 to 32.7
Dairy	41128	32.1	28.9 to 35.3	8928	17.8	14.2 to 21.4
Other	3396	44.2	38.1 to 50.3	634	29.2	20.4 to 38.1

* Reactors with visible lesions or *M. bovis* in four years divided by four and total herd size
Proportions for disclosing tests and short interval tests were weighted equally.

4.9 Inter-net protocols

The project support system "PathMan" based on an internet bespoke design functioned extremely well. It ensured timely selection of animals, collection and processing of samples across several organisations, wide geographic distances and many research workers (Durr and Eastland 2004). As well as assisting in the management of the project, the system enabled data entry whilst the project was ongoing. Future systems could also incorporate cross-validation of data at interim points during the data collection

phase. Considerable investment has to be made, and was made, during the project lead-in developing and perfecting the project support system. The evident success in the collection of data for this project supports the utilisation of internet based database technology for complex multi-centre studies.

5.0 Conclusions and Implications

This project was designed to investigate the importance of cattle-to-cattle transmission in the persistence of bTB in GB cattle. A range of both confirmatory and novel findings have resulted. The study is also important in providing a backdrop of field data with which to compare results from experimental studies of bTB.

Confirmatory findings:

1. Detailed description of the pathology typical of bTB infection in naturally infected cattle confirming that granulomas are most commonly observed in lymph nodes in the lower respiratory tract.
2. A significant number of infected cattle that test negative to the tuberculin skin test may be detected by blood-based tests (BOVIGAM Interferon-γ and serology)
3. *M. bovis* is not commonly detected in nasal mucus.
4. Web-based systems are viable for project support in multi-centre veterinary studies

New findings requiring further investigation:

1. Animals with bTB infection concentrated in the abdomen may be less likely to be detected by the SICTT.
2. Low levels of GSPHx in blood, a surrogate for selenium levels is associated with an increased risk of an animal being infected with bTB.
3. Past exposure to liver fluke *Fasciola hepatica* is associated with a decreased risk of bTB infection.
4. Reactor dairy cattle are less likely to have *M. bovis* infection confirmed than other production classes.

Further work.

Papers are currently in preparation reporting the major findings from the study. A paper has already been published reporting the web-based project management system (Durr and Eastland 2004). Serological samples from the study have been given to Southampton University to pursue research into potential biomarker signatures of *M. bovis* infection in blood.

Acknowledgements

We thank all the staff who participated in the project at VLA Bury St Edmunds, Luddington, Truro, Shrewsbury, Weybridge, Winchester and Starcross; the Institute of Animal Health in Compton; the State Veterinary Service staff from Animal Health Offices in Cardiff, Carmarthen, Exeter, Gloucester, Stafford, Taunton, Truro and Worcester, and; ADAS UK Ltd.

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

The Fresh Meat (Hygiene and Inspection) Regulations 1995.

ANDERSON, P. H., BERRETT, S. & PATTERSON, D. S. (1979) The biological selenium status of livestock in Britain as indicated by sheep erythrocyte glutathione peroxidase activity. Vet Rec 104, 235-238

ARANAZ, A., DE JUAN, L., BEZOS, J., ALVAREZ, J., ROMERO, B., LOZANO, F., PARAMIO, J. L., LOPEZ-SANCHEZ, J., MATEOS, A. & DOMINGUEZ, L. (2006) Assessment of diagnostic tools for eradication of bovine tuberculosis in cattle co-infected with *Mycobacterium bovis* and *M. avium* subsp. paratuberculosis. Vet Res 37, 593-606

BOURNE, J., DONNELLY, C. A., COX, D. R., GETTINBY, G., MCINERNEY, J., MORRISON, I. & WOODROFFE, R. (2001) An epidemiological investigation into bovine tuberculosis. In Third report of the Independent Scientific Group on Cattle, DEFRA

BUDDLE, B. M., ALDWELL, F. E., PFEFFER, A., DE LISLE, G. W. & CORNER, L. A. (1994) Experimental *Mycobacterium bovis* infection of cattle: effect of dose of *M. bovis* and pregnancy on immune responses and distribution of lesions. N Z Vet J 42, 167-172

CASSIDY, J. P., BRYSON, D. G. & NEILL, S. D. (1999) Tonsillar lesions in cattle naturally infected with *Mycobacterium bovis*. Vet Rec 144, 139-142

COX, D. R., DONNELLY, C. A., BOURNE, F. J., GETTINBY, G., MCINERNEY, J. P., MORRISON, W. I. & WOODROFFE, R. (2005) Simple model for tuberculosis in cattle and badgers. Proc Natl Acad Sci U S A 102, 17588-17593

DALTON, J. P. (1998) Fasciolosis. New York, CABI *Publishing*

DURR, P. A. & EASTLAND, S. (2004) Use of web-enabled databases for complex animal health investigations. Rev Sci Tech 23, 873-884

FIELD, C. J., JOHNSON, I. R. & SCHLEY, P. D. (2002) Nutrients and their role in host resistance to infection. J Leuk Biol 71, 16-32

GALLAGHER, J. & HORWILL, D. M. (1977) A selective oleic acid albumin agar medium for the cultivation of *Mycobacterium bovis*. J Hyg (Lond) 79, 155-160

GOODCHILD, A. V. & CLIFTON-HADLEY, R. S. (2001) Cattle-to-cattle transmission of *Mycobacterium bovis.* Tuberculosis (Edinb) 81, 23-41

GORMLEY, E., DOYLE, M. B., FITZSIMONS, T., MCGILL, K. & COLLINS, J. D. (2006) Diagnosis of Mycobacterium bovis infection in cattle by use of the gamma-interferon (Bovigam) assay. Vet Microbiol 112, 171-179

GRIFFIN, J. M. & DOLAN, L. A. (1995) The role of cattle-to-cattle transmission of *Mycobacterium bovis* in the epidemiology of tuberculosis in cattle in the Republic of Ireland: A review. Irish Vet J 48, 228-234

GRIFFIN, J. M., HAHESY, T., LYNCH, K., SALMAN, M. D., MCCARTHY, J. & HURLEY, T. (1993) The association of cattle husbandry practices, environmental factors and farmer characteristics with the occurrence of chronic bovine tuberculosis in dairy herds in the Republic of Ireland. Prev Vet Med 17, 145-160

KAMERBEEK, J., SCHOULS, L., KOLK, A., VAN AGTERVELD, M., VAN SOOLINGEN, D., KUIJPER, S., BUNSCHOTEN, A., MOLHUIZEN, H., SHAW, R., GOYAL, M. & VAN EMBDEN, J. (1997) Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 35, 907-914

KENNEDY, H. E., WELSH, M. D., CASSIDY, J. P., BRYSON, D. G., FORSTER, F., MCNAIR, J., GANGADHARAN, B., HOWARD, C. J. & POLLOCK, J. M. (2003) The role of WC1(+) gamma delta T-cells in the delayed-type hypersensitivity (DTH) skin-test reaction of *Mycobacterium bovis*-infected cattle. Vet Immunol Immunopathol 93, 169-176

KERR, M. G. (2002) Veterinary Laboratory Medicine

KOLLER, L. D. & EXON, J. H. (1986) The two faces of Selenium - deficiency and toxicity - are similar in animals and man. Can J Vet Res 50, 297-306

KOMMISRUD, E., PAULENZ, H., SEHESTED, E. & GREVLE, I. S. (2002) Influence of boar and semen parameters on motility and acrosome integrity in liquid boar semen stored for five days. Acta Vet Scand 43, 49-55

KREBS, J., ANDERSON, R., CLUTTON-BROCK, T., MORRISON, T., YOUNG, D. & DONNELLY, C. (1997) Bovine Tuberculosis in Cattle and Badgers Report to The Rt Hon Dr Jack Cunningham MP. In MAFF Publications. Ed I. S. R. GROUP, Crown Copyright

LEPPER, A. W. & PEARSON, C. W. (1973) The route of infection in tuberculosis of beef cattle. Aust Vet J 49, 266-267

LYASHCHENKO, K., WHELAN, A. O., GREENWALD, R., POLLOCK, J. M., ANDERSEN, P., HEWINSON, R. G. & VORDERMEIER, H. M. (2004) Association of tuberculin-boosted antibody responses with pathology and cell-mediated immunity in cattle vaccinated with *Mycobacterium bovis* BCG and infected with *M. bovis*. Infect Immun 72, 2462-2467

MCCORRY, T., WHELAN, A. O., WELSH, M. D., MCNAIR, J., WALTON, E., BRYSON, D. G., HEWINSON, R. G., VORDERMEIER, H. M. & POLLOCK, J. M. (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

MONAGHAN, M. L., DOHERTY, M. L., COLLINS, J. D., KAZDA, J. F. & QUINN, P. J. (1994) The tuberculin test. Vet Microbiol 40, 111-124

MORRISON, W. I., BOURNE, F. J., COX, D. R., DONNELLY, C. A., GETTINBY, G., MCINERNEY, J. P. & WOODROFFE, R. (2000) Pathogenesis and diagnosis of infections with *Mycobacterium bovis* in cattle. Independent Scientific Group on Cattle TB. Vet Rec 146, 236-242

NEILL, S. D., BRYSON, D. G. & POLLOCK, J. M. (2001) Pathogenesis of tuberculosis in cattle. Tuberculosis (Edinb) 81, 79-86

NEILL, S. D., POLLOCK, J. M., BRYSON, D. B. & HANNA, J. (1994) Pathogenesis of *Mycobacterium bovis* infection in cattle. Vet Microbiol 40, 41-52

ROTHEL, J. S., JONES, S. L., CORNER, L. A., COX, J. C. & WOOD, P. R. (1990) A sandwich enzyme immunoassay for bovine interferon-gamma and its use for the detection of tuberculosis in cattle. Aust Vet J 67, 134-137

STAMP, J. T. (1944) A review of the pathogenesis and pathology of bovine tuberculosis with reference to practical problems Vet Rec 56, 443-446

UNDERWOOD, E. J. & SUTTLE, N. F. (1999) The mineral nutrition of livestock, CABI Publishing

VILLARREAL-RAMOS, B., REED, S., MCAULAY, M., PRENTICE, H., COFFEY, T., CHARLESTON, B. C. & HOWARD, C. J. (2006) Influence of the nature of the antigen on the boosting of responses to mycobacteria in *M. bovis*-BCG vaccinated cattle. Vaccine 24, 6850-6858

VORDERMEIER, H. M., CHAMBERS, M. A., COCKLE, P. J., WHELAN, A. O., SIMMONS, J. & HEWINSON, R. G. (2002) Correlation of ESAT-6-specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. Infect Immun 70, 3026-3032

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. Clin Diagn Lab Immunol 8, 571-578

VORDERMEIER, M., GOODCHILD, A., CLIFTON-HADLEY, R. & DE LA RUA, R. (2004) The interferon-gamma field trial: background, principles and progress. Vet Rec 155, 37-38

WHIPPLE, D. L., BOLIN, C. A. & MILLER, J. M. (1996) Distribution of lesions in cattle infected with *Mycobacterium bovis*. J Vet Diagn Invest 8, 351-354

WILESMITH, J. W. (1983) Epidemiological features of bovine tuberculosis in cattle herds in Great Britain. J Hyg (Lond) 90, 159-176

Standard Operating Procedures

BAC0100: Bovigam IFN-γ Elisa.
BAC0204: Bioassay to measure bovine IL-2 in culture supernatants.
BAC0209: Cell culture for IFN-γ EIA and IL-2 assay.
BA.274: *Mycobacterium paratuberculosis* (Johnes) Antibody detection by enzyme linked immunosorbent assay (commercial kit).
BA.386: TB Diagnosis - Examination of Bovine & Cervine Cultures
BI.039.A: Glutathione Peroxidase (GSHPx): Determination of in Whole Blood on Olympus AU400 Chemistry Analyser
BI.208: Copper in plasma, serum and whole blood: estimation of by graphite furnace atomic absorption.
BI.210: Multi-elemental analysis of tissues, feeds and environmental samples by flame atomic absorption spectroscopy.
BI.212.B: Vitamin B12: estimation in serum/plasma by Bayer Immuno-1 analyser.
BI.218: Vitamin B12: estimation on serum/plasma and Liver by Bayer ACS: 180 SE.
CBU0245: Spoligotyping Method
HA.001: Differential White Blood Cell Counts
HA.002: Haematological examination of veterinary blood samples using the Sysmex F-800/F820 analysers.
HA.003: Determination of Microhaematocrit.
SE.223: *Fasciola Hepatica* (Liverfluke) Antibody Detection in bovine serum by enzyme linked immunosorbant assay.
SE.227: *Leptospira hardjo* ELISA for the detection of antibodies in bovine serum.
PA.052: *Dictyocaulus viviparous* (bovine lungworm) antibody detection by Enzyme linked immunosorbant assay.
VI.027: Bovine Viral Diarrhoea (BVD) ELISA
VI.035.A: Infectious Bovine Rhinotracheitis (IBR/BHV-1) Indirect Antibody ELISA.