

**Bovine tuberculosis:
a review of diagnostic tests for *M. bovis*
infection in badgers**

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1. Executive summary

1.1. General

There is relatively little peer reviewed literature available on the comparative value of different approaches to the diagnosis of *M. bovis* infection in badgers. This review has focussed on studies carried out in badgers but of necessity reference has also been made to studies in other species where this may be illustrative.

There is a general consensus that due to the close proximity of badgers to the soil and the nature of their primary food sources, badgers have a high likelihood of exposure to environmental mycobacteria. This is likely to affect the characteristics of a number of tests due to immunological cross reactions leading to a reduction in the specificity and possibly sensitivity of a number of these.

Significant practical issues exist in assessing the infection status of badgers and efforts to develop improve diagnostic tests. These include the need for Home Office / DHSS&PS approval under the Animals Scientific Procedures Act (ASPA) for any experimental work and the protected status of the species, with the requirement for NIEA (or equivalent) licences. Other practical constraints include the need for extensive survey work to identify setts; the practicalities of cage and other trapping methods, and the lack of any meaningful test that can be performed on the conscious badger i.e. current tests require the animal to be anaesthetised to obtain the relevant samples.

Compared to human and cattle tuberculosis diagnostics, there has been relatively little commercial interest in developing reagents or assays for immunological tests in badgers. No currently available test has a specificity comparable to that of the single intradermal comparative skin test in cattle.

The possible use of 'road traffic accident' (RTA) badgers to provide disease information has been assumed to be outside of the intended remit of this review and for that reason has not been considered further or included in the options outlined below. It is probably worth noting however that RTA surveys are capable of providing useful information and have been used in some scenarios e.g. as part of the evidence base used by the Welsh Assembly Government in relation to the intensive action area (Veterinary Laboratories Agency, 2007)

1.2. Diagnostic tests available to assess the *M. bovis* status of badgers

1.2.1. Environmental sampling

The sampling of the badger environment, including badger setts and latrines, to identify the infection status of individual setts has obvious attractions as an alternative to animal testing. Currently available techniques (including PCR)

however lack sufficient sensitivity and are not currently recommended as a viable alternative to animal tests.

1.2.2. Intradermal skin testing

Experimental work in the 1980s indicated that various forms of the tuberculin skin test in badgers had very limited sensitivity. In addition, due to the need to read results a number of days after the initial injection of antigen (i.e. due to the need to re-capture animals to read the test), skin testing in badgers is not regarded as a feasible option and subsequent research has focussed on alternative, mainly blood based assays.

1.2.3. Bacterial culture of samples from the live animal

Bacterial culture of a range of samples, notably faeces, urine, bite wounds and tracheal aspirates is possible from the live animal. However, with the exception of the badgers that defaecate, and possibly those that urinate in the cage trap, collection of these samples requires the animal to be anaesthetised. While specificity is extremely high, the sensitivity of culturing such samples is low in comparison to immunological tests and / or the culture of lymph node and other tissues collected post mortem. In addition, TB culture can take up to 8 weeks resulting in significant time delays between sample collection and the availability of results.

1.2.4. Blood based tests

Both cell mediated tests (e.g. interferon-gamma) and humoral (serological tests for antibodies) have been developed for use in badgers. However, of the tests developed to date, the only animal side test currently available is the Brock TB Stat-Pak i.e. all others are laboratory based tests.

It should be noted that most reports of sensitivity and specificity of blood based tests have been made relative to the culture of post-mortem samples. While the specificity of culture is very high, sensitivity is imperfect and variable depending on the techniques used and the range of tissues examined. It would therefore be expected that comparisons between blood based tests and culture will under-estimate the true specificity of blood based tests and possibly over-estimate their sensitivity.

An interferon-gamma test has been developed for use in badgers. However the test requires the use of badger specific reagents, which are only currently available in certain research institutes. The test has the highest reported sensitivity and specificity of any live test currently available (estimated to be approximately 80.9% and 93.6% respectively relative to post mortem culture). The assay requires blood to be tested at a dedicated laboratory within a short period of sampling (typically less than 8 hours) and so is dependent upon careful coordination between field sampling and laboratory testing.

The two most promising antibody based tests for which published information is available are the Brock TB Stat-Pak and the Dachs TB ELISA. As with antibody tests for TB detection in other species, these tests have relatively low overall sensitivities. However, those animals that do have detectable antibody responses are considered to be those with more advanced disease and are therefore more likely to be infectious. It is therefore likely that animals that give a positive serological test pose a greater risk of disease transmission.

The Brock TB Stat-Pak is the only test currently available which can be used as an animal side test requiring the application of a few drops of blood to a pre-loaded plastic cassette. In one study, the test had an estimated sensitivity of 49.2% and a specificity of 93.1%. However it did not appear to be able to detect a significant number of additional infected badgers compared to the badger adapted IFN-g test.

The Dachs TB ELISA is a conventional immunoassay which measures the amount of antibody to 4 *M. tuberculosis* complex specific antigens in blood serum. Using post mortem bacterial culture as the 'gold standard' the test had an estimated sensitivity of 61% and a specificity of 82%. Newer antibody tests (such as Enferplex) are likely to supersede the Dachs TB ELISA test. However these would require to be adapted using badger specific reagents and there is also currently no available published data on the sensitivity and specificity of these tests in badgers.

1.2.5. Post Mortem Examination

The sensitivity of post mortem examination of badgers is entirely dependent upon the extent and thoroughness of the examination, and the quality and quantity of any ancillary testing carried out. Many infected badgers have limited or no gross pathological changes. The submission of visible lesions alone for further examination using either histopathology or bacteriology is likely to lead to an underestimation of the true disease status of animals. Therefore any post mortem protocol must include the bacteriological examination of a range of tissues irrespective of whether pathology is visible or not.

The sensitivity of tissue culture for definitive diagnosis is dependent upon a number of factors, most importantly the number of tissues submitted for individual culture, the aseptic technique used for removing tissues and the methods used for culture including the number and type of media used, the degree of decontamination used to avoid contaminant overgrowth, and the length of culture time.

The thorough post mortem examination of badgers with multiple tissues submitted for bacteriology is likely to give the greatest overall accuracy in estimating the presence or absence of *M. bovis* infection. However a number of significant disadvantages exist with this approach. These include the cost, the expertise required to carry out the post mortem examinations and ancillary

tests, and in the case of trapping and euthanizing badgers, the risk of badger population disruption leading to the so-called perturbation effect.

1.3. Application of these tests in differing scenarios

1.3.1. Non-capture

The environmental sampling of badger setts, latrines and other sites is currently regarded as lacking sufficient sensitivity and is not currently recommended as a viable alternative to animal tests.

1.3.2. Live badgers trapped and released

Blood sampling, to obtain the quantities currently required, and any meaningful clinical examination requires the animal to be anaesthetised. Sampling of live badgers trapped and not anaesthetised is currently restricted to the bacterial culture of faeces and possibly urine from badgers that may defaecate / urinate in the cage trap. The sensitivity of culturing such samples is too low to provide meaningful information.

1.3.3. Live badgers trapped, anaesthetised and released

A much wider range of samples can be taken from badgers under anaesthesia than in the conscious animal. This includes blood samples for both serological and IFNG tests, and tracheal aspirates, bite wounds and faeces for bacterial culture. Notably however, the Brock TB Stat-Pak is the only one of these that is currently available as an animal side test.

1.3.4. Badgers trapped, euthanized and examined post-mortem

The combination of post mortem examination (including the bacterial culture of samples collected post mortem) and blood based tests provides the greatest range of samples and the most sensitive and specific approach to the diagnosis of infection. It is however the most costly and involves all of the issues and dangers associated with badger removal.

1.4. Means by which a better understanding of bovine tuberculosis infection in the local badger population can be achieved.

Post mortem examination and the bacterial culture of associated samples provides the most sensitive and specific approach to the diagnosis of *M. bovis* infection in badgers. It also has the advantage of providing a source of isolates for strain type comparisons with *M. bovis* isolates from cattle. The approach however is the most expensive due to the need to culture a wide range of samples. It is also the most invasive, with strong evidence in GB at least that the associated badger disturbance can lead to increase bTB occurrence in cattle herds.

Blood based tests have the significant advantage of being non-lethal and the associated capture and release of badgers has also no known perturbation effect. These tests however lack the sensitivity and particularly the specificity of post-mortem culture. Statistical techniques to adjust for the imperfect test performance of serological tests are increasingly being used in other scenarios. Additional statistical work to model the possible outcomes of post mortem and serological approaches would, however, be indicated to predict their comparative outcomes.

1.5. Practicality of a test and release (test negative–release: test positive-cull) approach.

The only test currently available as a sett-side test (the Brock-Tb StatPak) has limited sensitivity, although this is higher in badgers with more advanced disease and those presumed to be infectious. Importantly there have been no field trials anywhere in the UK or Ireland to assess the likely outcome of a ‘trap-test-cull-vaccinate’ approach. Mathematical modelling work, undertaken for the Welsh Assembly Government indicates that the likely effect of such an approach was heavily dependent on assumptions surrounding perturbation. Where perturbation was assumed to occur the model predicted that such an intervention would actually make the TB situation in cattle worse (i.e. lead to increased confirmed cattle herd breakdowns). Where perturbation was assumed not to occur the model predicted that trap-test-cull-vaccinate would be only marginally better than cull only and vaccinate only approaches. All three approaches gave very similar results with small reductions in the rate of confirmed cattle herd breakdowns. The authors concluded that “given the large uncertainty, we cannot accurately predict the outcome of a combined trap-test-cull-vaccinate strategy at present”.

There has been no equivalent disease modelling work undertaken in NI, to predict what effect such an approach might have here or indeed the possible effect of alternative trap-test-cull approaches such as one based on whole-sett removal.

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Objectives of this review:

A review of assays available to test badgers for infection with *Mycobacterium bovis*. The review will have particular reference to published work, or work nearing completion, to identify tests that could be conducted on blood or other samples collected from badgers, under the following conditions:

- (a) live badgers trapped and released
- (b) live badgers trapped, anaesthetised and released
- (c) badgers trapped, euthanized and examined post-mortem

The review should provide the means by which a better understanding of bovine tuberculosis infection in the local badger population can be achieved. It will also identify the efficacy of specific tests or combinations of tests, indicate the practicality of a test and release (test negative–release: test positive–cull) approach and provide a comparative assessment of the likely results of such testing between a lethal and non lethal intervention.

[DARD Animal Health and Welfare Branch 2010]

Methods:

This review was written following extensive key-word based searches of the Web of Science and PubMed data bases. Appropriate peer reviewed publications were identified and used to contribute to the review process. A number of websites that hold government reports were also used. Ms Sheila George (QUB PhD student) was consulted to help inform the procedures by which badgers can be trapped and sampled. Also, staff at University College Dublin and the Veterinary Laboratories Agency (now ‘Animal Health and Veterinary Laboratories Agency’) Weybridge were consulted on certain aspects of badger diagnostics.

The following relevant areas have been discussed:

- Blood based tests which include those based upon either cell mediated or antibody immune responses
- Post mortem examination together with ancillary tests.
- Other technologies that are currently in development and an assessment of their current status.
- The relative merits and demerits of the various tests currently available for each proposed badger sampling strategy.

2. Introduction

This review attempts to summarise the methods for the diagnosis of tuberculosis in badgers. As background to this a brief summary of *M. bovis* infection in badgers is made followed by a discussion of the diagnostic tests currently or potentially available. The tests fall into two broad categories, the direct detection of infection using post mortem examination and associated tests on animal tissues (histopathology and bacteriology) and the indirect detection of infection using blood based immunological tests which identify and measure the animals immunological response following exposure to the *M. bovis* organism. This latter category can be further divided into those tests which are based upon the cellular immune response and those based upon antibody responses. There is considerable overlap in how the various tests could be implemented e.g. any trapped and anaesthetized animal for post mortem examination could also be blood sampled. Finally an overall summary of the advantages and disadvantages of the various approaches to determining disease status of badgers is made.

There is comparatively little published information on the pathogenesis of tuberculosis in badgers compared to that of humans or cattle. Where possible, information on TB in badgers has been reviewed. However in some cases knowledge from cattle and other infection models has been used to inform on current knowledge and available technologies. However it is clear that there are significant differences in host species' responses to the *M. bovis* organism and in the likely levels of transmission of infection and progression of disease. For example the repertoire of *M. bovis* antigens that badgers preferentially respond to immunologically appear different from those of cattle (Lesellier et al., 2008), the prevalence of TB in badgers is likely to be significantly different from that of cattle within N. Ireland (Abernethy, 2011) and the pathology seen in badgers is somewhat different from that seen in cattle (Corner et al., 2010). Therefore caution needs to be exercised in drawing comparisons across species.

It should be noted that the performance of any diagnostic test is also influenced by a number of factors other than the test itself. These include the disease prevalence within the population of animals being tested, the stage of disease tested animals are at, the robustness of the test in terms of repeatability and reproducibility and the laboratory expertise to carry out any tests. These factors are discussed in more detail in 'Bovine tuberculosis: A review of diagnostic tests for *M. bovis* infection in cattle.'

3. *Mycobacterium bovis* infection in badgers

Mycobacterium bovis belongs to the *Mycobacterium tuberculosis* complex and infects a very broad host range (O'Reilly, 1995) including the European badger (*Meles meles*). The bacillus is relatively small with a wax-like mycolic acid cell wall that confers a degree of protection from host defence mechanisms such as degradation within the macrophage phagocyte. The primary route of infection in badgers is through the inhalation of infectious particles within an aerosol droplet (Corner, 1994; Neill et al., 1994). The evidence for this comes from several studies involving the detailed dissection of badger carcasses followed by extensive bacteriology which has indicated that TB in badgers is primarily a respiratory disease (Fagan, 1993; Gallagher and Clifton-Hadley, 2000; Murphy et al., 2010). The badger is now considered to be a wildlife maintenance host for *M. bovis* (Clifton-Hadley, 1993; Griffin et al., 2005).

Both the route of infection and the pathogenesis of disease are a major determinant of the development of the immune response in badgers, with implications for diagnostic methods to correctly identify *M. bovis* infected animals. In cattle for example, tuberculosis induces a spectrum of immune responses dominated by the cell mediated response to intracellular infection (Pollock and Neill, 2002; Welsh et al., 2005). If the disease progresses within the host, the balance of the immune response shifts from a Th-1 type (dominated by T-cells) to a Th-2 type (dominated by B-cells which produce antibody). It appears that something broadly similar happens in badgers. In one study, badgers infected with *M. bovis* were found to mount an immune response dominated by cell mediated responses (lymphocyte transformation, [LTA]) by month 3 post infection (Mahmood, 1987). Antibody responses were virtually absent during this stage but as disease progressed, antibody levels increased as the LTA response fell.

There is a wide spectrum of disease severity seen in infected badgers. Most are sub-clinically infected with a relatively small proportion showing more advanced signs. Most infected badgers are mildly diseased with only a small proportion showing advanced pathology (Murphy et al., 2010). This spectrum of disease has important implications for performance of different diagnostic test. It would, for example, be expected that most animals with mild or pre-clinical infection would be less likely to have measurable antibody responses (Th2 type) and that the relative sensitivity of antibody assays would be highest in those with advanced disease. Similarly animals in the initial stages of infections or with mild disease are likely to have very limited pathology so any tests based upon post mortem examination and bacteriology will have to be thorough in order to have a reasonable sensitivity. Similarly these animals are not likely to be shedding significant numbers of bacteria in to the environment so any live badger bacteriology based upon the culture of faeces, urine, tracheal aspirates or wounds is likely to be very insensitive. It is likely however that badgers with advanced disease represent the greatest risk to of transmission to cattle. The sensitivity of antibody, post-mortem and culture tests based tests all would be expected to be highest in this subgroup. Therefore while many diagnostic tests are likely to be insensitive in detecting sub-clinically or latently infected animals

those that are more likely to be shedding bacteria because of more advanced disease will have a greater likelihood of detection.

4. Immunological Assays

4.1 Assays based on cell mediated immune responses

There are broadly three approaches to measuring the cellular immune response. The first measures the proliferation of cells following stimulation with mycobacterial antigens, the second measures the release of cytokines (for example interferon gamma [IFN γ]) from memory T-cells stimulated with antigen and the third is the conventional intradermal skin test. The first two methods are laboratory based and require a high degree of laboratory expertise. Each take a number of days to complete and each are relatively expensive.

Notably most reports of sensitivity and specificity of blood based tests have been made relative to the culture of post-mortem samples. It should be borne in mind that while the specificity of culture is very high (assuming no cross contamination), the sensitivity of culture is imperfect and variable depending on the techniques used and range of tissues examined. It would therefore be expected that such comparisons would under-estimate the true specificity of blood based tests, as a proportion of those samples regarded as false positive (i.e. blood positive / culture negative) would actually be true positives but incorrectly classified due to the limited sensitivity of culture.

4.1.1 The Skin Test

The skin test is the central ante mortem diagnostic test applied to the diagnosis of tuberculosis in cattle. The test itself is described in more detail in the associated review 'Bovine tuberculosis: A review of diagnostic tests for *M. bovis* infection in cattle.' It measures the delayed type hypersensitivity response to components of mycobacteria injected intradermally. In the UK and Ireland it is used as a comparative test, the single intradermal comparative cervical test (SICCT) using the tuberculin reagents PPD_b and PPD_a. An infected animal is classified as infected if there is a net larger response to PPD_b than PPD_a with several potential cut-offs applied depending upon the disease history of the animal being tested. Using the normal cut-off in cattle it has a high specificity (>99%) but only a moderate sensitivity (approx 50-60%).

Much less is known of the performance of the test in badgers although it appears most evidence suggests its performance is poorer in badgers than in cattle. One study failed to detect any reaction to tuberculins injected into the thighs of naturally infected badgers (Little et al., 1982) while another recorded three skin test positive badgers from 45 naturally infected animals (Pritchard et al., 1986). Another recorded only small increases in skin thickness in tuberculin-injected sites, without evidence of erythema, palpable oedema or induration (Higgins, 1985). The general consensus on the skin test applied to badgers is that the delayed hypersensitivity response to infection was inconsistent and not an accurate measure of immunity. This is supported by the failure to detect cell mediated responses in badgers to ESAT-6 and other antigens commonly recognised by humans and cattle (Buddle et al., 2001; Mustafa et al., 1998) in badgers experimentally infected with *M. bovis* (Lesellier

et al., 2008). These findings suggest that the badger immune system responds differently to infection by *M. bovis* compared to other species.

A number of independent cattle-based studies have been carried out to improve the specificity of reagents that provoke the delayed type hypersensitivity reaction by using a mixture of defined reagents, principally recombinant proteins (Pollock et al., 2003) and defined peptides (Whelan et al. 2010). The results from these studies are promising but would benefit from wider application in greater numbers of naturally infected cattle (Pollock et al., 2003). A novel method based on infrared thermography was developed to replace the need for skin thickness measurement using callipers (Johnson, 2008). However to date, these novel approaches have not been tested in badgers, either under experimental conditions or in naturally infected animals.

In addition to the apparent poor test performance the skin test is logistically difficult to perform in wild badger populations. Skin thickness measurements require the animal to be anaesthetised and the thickness of the skin measured on two occasions 72 hours apart. This time between skin measurements requires the animal to either be held captive for that length of time or quickly re-captured. Re-capture is likely to fail for a large proportion of the tested badgers and holding a wild animal captive would be resource intensive and may be ethically unacceptable.

For these reasons the intradermal skin test is not currently regarded as a useful test for applying to a wild population of badgers to assess their disease status.

4.1.2 Lymphocyte proliferation

A sub-population of viable immune cells (memory T cells) has the ability to divide if they are exposed in vitro to antigens that they have previously been exposed to. The lymphocyte proliferation assay uses this by measuring the proliferation of blood immune cells after exposure to defined components of mycobacteria e.g. PPD_b (purified protein derivative bovis) (Pollock et al., 1994; Stuart et al., 1988). Normally, a radiochemical is used to label clonally expanding cells through the incorporation of radiolabelled nucleotides into newly formed cellular DNA. This technique provides an accurate measure of the immune response as measured by cellular proliferation and as such can be quantitative. One study assessed the technique using both PPD_a and PPD_b and defined a positive test result where there was a significantly greater response using PPD_b compared to PPD_a (Dalley et al., 1999). Using culture positive animals as the comparative 'gold standard' test this study found the test to have a better sensitivity compared to the Brock ELISA test (87.5% compared to 62.5%) but a poorer specificity (84.6% compared to 100%). However a number of significant practical drawbacks to the test have precluded its widespread development or use. The use of a radiochemical compound poses a safety hazard to laboratory staff and requires storage and disposal in keeping with national legislation. Also the technique can take up to five days to complete and is very costly in terms of staff time and reagents required. It

requires the presence of viable T-cells, therefore, blood samples must be delivered to the laboratory with minimal delay. Taken together these factors have largely made the technique redundant and it is not currently offered as a routine diagnostic test for tuberculosis in any animal species.

4.1.3 The IFN- γ test

Cytokine release assays, particularly the IFN- γ test, are being increasingly used internationally for the diagnosis of tuberculosis in animals (Schiller et al., 2009) due to the ready availability of commercial reagents (Bovigam test kits, Prionics, Switzerland). Since the introduction of the IFN- γ assay for bovine TB in 1998 (Rothel et al., 1990; Wood et al., 1990) a number of studies have demonstrated its value as an ancillary test for the diagnosis of TB in cattle (de la Rua-Domenech et al., 2006; Schiller et al. 2010).

The interferon-gamma assay is described in more detail in the associated literature review 'Bovine tuberculosis: A review of diagnostic tests for *M. bovis* infection in cattle.' The assay has the advantage that various test parameters can be changed to suit particular test circumstances. For example different antigens can be used, the cut-off values used to define positive test results can be varied and the performance of the IFN- γ detection component of the test can be accredited (e.g. to the 17025 standard) thereby giving assurance about the reliability of the test and those who perform it. However, one of the most significant practical disadvantages of the assay is the need to test blood which has been sampled less than 8 hours previously. As the T-cell population ages following blood sampling, the number of cells able to respond to antigen diminishes. In addition, the cost of the test and the equipment and personnel needed to undertake the test may preclude its widespread use. One other notable badger-specific disadvantage to the test is the greater propensity for collected badger blood to clot compared to bovine blood. This requires the addition of extra heparin (anticoagulant) to blood collection tubes used for sampling badgers (Dalley et al., 2008).

The actual test characteristic is dependent upon the format of the test, the antigens used for the test and the population the test is applied to. The most extensive work attempting to measure the test's characteristics in badgers estimated it to have a sensitivity of 80.9% and a specificity of 93% (Dalley et al., 2008) using post mortem culture as the 'gold standard.' In this study the use of tuberculin (PPDs) was superior to the use of the TB specific antigens both in terms of sensitivity and specificity. In comparison to the Brock (antibody) test the IFN- γ assay was able to detect significantly more non-visibly lesioned animals. This probably indicates that antibody positive animals are likely to have more advanced disease. Interestingly in this study only one culture positive animal missed by the IFN- γ assay was detected using the Brock assay suggesting that the use of both of these tests will not increase significantly the overall sensitivity of detecting infected badgers using the IFN- γ assay alone. This is in contrast to a more recent study which attempted to

identify the optimal combination use of culture from live animals, serology (using the Stat-Pak) and the IFN- γ assay using a Bayesian approach (Drewe et al. 2010). This study estimated that the IFN- γ assay had a sensitivity of 79.9% and a specificity of 95.0%. Interestingly in this study the estimated diagnostic accuracy of the IFN- γ assay in culture negative animals was increased if combined with the Stat-Pak (75% of animals correctly identified based on a positive IFN- γ result alone and 81% of animals correctly identified based on both a positive IFN- γ and a positive Stat-Pak result), suggesting that running these two tests in parallel is of value.

Alternative platforms have been developed for use in the interferon-gamma test. For example the Luminex multiplex assay uses colour coded beads (microspheres) which can be coated with a specific reagent for example anti-interferon-gamma antibodies. These are then used as the means of measuring the interferon-gamma released following blood stimulation. Using an optimized cut-off following blood stimulation with PPDa and PPDb the assay was assessed during DEFRA project SE3228 and performed better than the conventional interferon-gamma assay with an estimated specificity of 95.2% and a sensitivity of 86.4% (see table 1).

Another platform tested in badgers during the same project was the BioVeris assay which used anti interferon-gamma antibodies with chemical labels which emit light following electrochemical stimulation. When compared to the conventional interferon-gamma assay this platform did not appear to perform significantly better with an estimated specificity of 96.1% and a sensitivity of 68.1% (see table 1).

4.1.4 The EliSpot Assay

The EliSpot assay is a method of visualising and counting individual immune cell types usually from blood samples that have been labelled with one or more specific markers. It allows for the identification of a wide range of cell populations based upon their cell function and their production of specific immune messenger molecules (cytokines). In tuberculosis diagnosis this assay has been used as a research tool to detect cell populations that are releasing IFN- γ following exposure to mycobacterial antigens. In this way the number and type of cells responding to *M. bovis* antigen specific stimulation can be identified. This assay has been evaluated for a number of animal species including the badger (Lesellier et al, 2006). However while the assay provides useful information regarding the immune response it is a laboratory based technique requiring considerable capital investment and expertise. It is relatively cumbersome taking some time to complete for each sample tested and so does not easily allow for a high throughput of tests. These considerations make the assay more useful as a research tool than a rapid diagnostic test.

4.2 Assays based on antibody responses

The Enzyme Linked ImmunoSorbant Assay was first introduced in the 1970's (Engvall and Perlmann, 1971) and has become a very widely used tool to measure a wide range of soluble molecules including antibodies to a wide range of diseases (Voller and Bidwell, 1988). In its simplest form a protein or range of protein antigens are bound to the wells of a plastic plate. Dilute serum is then added to the antigen coated wells and any antigen specific antibody binds to the antigen coated plate. These antibodies can then be measured using one or more subsequent steps. The test format has a number of significant advantages. Only one serum sample is required to carry out a test and serum samples can be stored, retested or used as a reference reagent. This test format is relatively cheap, the capital outlay for equipment is, for the most straightforward versions, modest and the assays can be easily automated to allow rapid sample throughput and a short turnaround period for results. Significant advances in the ELISA test have included the introduction of recombinant protein technology, providing greater assay robustness and the introduction of alternative technology platforms such as multianalyte assays in which up to 6 or more antigens can be detected in one well of an adapted ELISA plate (Whelan et al. 2010).

4.2.1 The Brock ELISA

The earliest ELISAs used for the diagnosis of TB in badgers used PPD_b as the antigenic target. In one study only 22 out of 33 known badgers were identified using this assay (Morris et al., 1979). More seriously a significant number of negative badgers (i.e. badgers from non-TB endemic areas and showing no bacteriological or pathological evidence of TB) displayed some reactivity suggesting that these badgers had antibodies that cross-reacted with PPD_b. In order to address this specificity concern subsequent ELISAs have focussed on using defined antigenic targets believed to be more specific to the *M. tuberculosis* complex. One of the first such immunological assays to be developed and evaluated in badgers was an ELISA based on a 25 kDa antigen purified from cultures of *M. bovis* (MPB83). MPB83 was known to be immunodominant in infected badgers (Goodger et al., 1994) and present in all field strains of *M. bovis* tested. This initial study concluded that the test had a specificity of 98% and a sensitivity of 37%. This is broadly in line with a subsequent study which concluded that the test sensitivity is modest with only approximately 40% of badgers with post mortem evidence of infection being detected by the assay (Clifton-Hadley et al., 1995). In this case the test specificity was estimated to be 94.3%. As might be expected with an antibody test the test was more successful in detecting animals with visible post mortem lesions. As with any test the sensitivity of detecting infection increases with repeated testing of the animal. One study estimated that repeat testing the same animal 3 times using a test with the characteristics described above would increase the sensitivity of the test to 79.5% but reduce the specificity of the test to 83.1% (Forrester et al., 2001). However this assumes that each test

is independent which they may not be as the infected status of the animal could change between samplings or the animal could sero-convert (i.e. become antibody positive) between samplings. Fundamentally such an approach, involving multiple trappings (and anaesthesia) of each animal, is likely to significantly limit its applicability. In a subsequent comparison with the Dach TB ELISA the Brock test had an estimated sensitivity of 46% and a specificity of 82% (Kampfer, 2003). In two more recent studies test sensitivity and specificity were estimated to be 52.9% and 90.7% (Sawyer et al., 2007) and 48.9% and 93.6% (Dalley et al., 2008) respectively.

4.2.2 DACHS TB ELISA

In an attempt to improve the performance of the Brock ELISA, the DACHS TB ELISA was developed by introducing additional antigenic targets to the assay. In total the assay used MPB83, MPB70, CFP10 and the 16kD antigen. The test was evaluated using serum samples collected during the ISG RBC badger cull (Kampfer, 2003). The study concluded that the DACHS test was superior to the Brock ELISA with sensitivity estimated at 61% compared to the 46% for the Brock ELISA if the specificity cut-off was set to the same for both tests (82%). Adjusting the DACHs test cut-off so that it had a similar sensitivity to the Brocks test led to the DACHs test having an estimated specificity of 97%. In addition it had superior positive and negative predictive values (81% and 63% respectively) than those of the Brock test. However subsequent to this there appeared to be technical difficulties with the production of the assay which meant that during a DEFRA funded research project the test appeared to under perform. When the specificity for the test was set at 93-94% the estimated test sensitivity was 57% (compared to 54% for the Brock ELISA using the same specificity criteria) (DEFRA project SE 3215). There does not appear to have been any subsequent work done on the DACHs assay so while in principle it appears it may be superior to the Brock ELISA due to the addition of extra antigenic targets the authors are not aware of any subsequent development or validation work carried out using it.

4.2.3 Multi Antigen Print Immunoassay (MAPIA)

MAPIA technology was initially developed to test human serum samples against a panel of specific *M. tuberculosis* antigens. The principle of the test is similar to ELISAs except that using this technique up to four antigens are printed directly onto a nitrocellulose membrane. Test serum is then added to the membrane and the presence of antibody detected using methods similar to that used for conventional ELISAs (Lyashchenko et al., 2000). The advantages of MAPIA based tests are that they are an efficient and cost-effective method to screen for antibody against an antigen panel and are a very rapid assay platform. This method has been further developed commercially (Chembio, New York, USA) by using a coloured latex-based lateral flow technology. This combination has allowed an animal side test to profile antibody responses to infection in a range of wildlife species including badgers (Vet TB Stat Pak and the Brock TB Stat Pak) (Lyashchenko et al., 2008) and has confirmed the immunodominance of MPB83.

4.2.4 Brock TB Stat-Pak

The Brock TB Stat-Pak was developed to test for antibodies in badger serum. As with the Brock ELISA the dominant target antigen is MPB83 although the assay includes ESAT 6 and CFP 10. In common with the other test variants, the assay has a simple operation giving rapid results for little expense (Lyashchenko et al., 2008) allowing it to be the only true animal side test currently available. However, in common with many of the antibody detection systems it has only a modest sensitivity (46 to 55%) (Chambers et al. 2010). In the largest study evaluating the test it was estimated to have a sensitivity of 49% and a specificity of 93% (Chambers et al., 2008). In a subsequent study using a Bayesian approach to estimating test parameters, the test had an estimated sensitivity of 50.4% and a specificity of 96.9% (Drewe et al. 2010).

Interestingly it has also been evaluated as a post mortem test using thoracic blood from badgers found dead (Chambers et al. 2010). In this study the test had a specificity of 99% and a sensitivity of 35%, when measured against post mortem examination and bacteriology, suggesting that it could be used as a screening test at post mortem where only test negative animals need go forward for intensive post mortem investigation and bacteriology. This is probably due to the degradation of thoracic blood following death meaning that only animals with very high levels of antibody could be detected using this assay i.e. animals very likely to be truly infected.

4.2.5 Fluorescent Polarisation Assay

Fluorescence polarisation assays (FPA) use a tracer (the target antigen or part of it) with a fluorescent molecule bound to it which is added to serum. If antibody is present in the serum it binds to this and the measurable fluorescent polarisation increases due to the increased size of the combined antigen-antibody complex (Jolley et al., 2007; Surujballi et al., 2002). The technology on which this technique is based is simple with modest capital outlay and is rapid, with assays completed within four hours. This technique in particular is suited to the detection of antibodies in multiple species without the requirement for multiple reagents. One significant advantage of the test is its simplicity with very few steps making it therefore very robust and comparatively free from test to test variation. In common with all antibody tests, the fluorescent polarisation assay is fundamentally dependent upon the antigens used (e.g. MPB70, MPB83). The assay was assessed as part of a DEFRA research project evaluating BCG vaccine in badgers. An initial screen of a commercial FPA supplied by Diachemix using the MPB83 antigen indicated the test to have a sensitivity of 94% but a very poor specificity of 38%. When sera were used at a lower dilution the specificity increased to 78% and the sensitivity reduced to 54%. Taken together this work suggested that in its current form it is unsuitable for the diagnosis of TB in badgers.

4.2.6 The SeraLyte-Mbv (PriTest Inc) assay

This novel assay uses magnetic ferrite iron bound to MPB83 to detect serum antibodies. Following a number of washing steps and reagent additions, a chemiluminescent reaction indicates the presence and quantity of antigen specific antibody. A pilot trial in infected cattle has shown that the test has some promise (Green et al., 2009). In badgers, the test was evaluated during the DEFRA funded VLA project SE3228. An initial assessment of the test suggested that the test had a sensitivity of 86% and a specificity of 94%. This estimate was based upon badgers confirmed infected using bacteriology and badgers suspected to be infected but not confirmed using culture. In a smaller follow-up assessment using 47 samples the test had a sensitivity of 96% and a specificity of 95%. Again this was based upon badgers either confirmed infected by bacteriology or having histopathology consistent with TB. Because of this criteria caution should be exercised in comparing the SeraLyte test with other antibody tests as in many cases the other test performances were assessed against infection confirmed by bacteriology. Using culture confirmed badgers as the 'gold standard' the optimised SeraLyte method performed by the best operator had an estimated sensitivity of 55.3% and a specificity of 95.5% giving it the best overall characteristics of all the serological tests evaluated in this study. For ease of comparison the results for the serological and cell mediated immunological tests used during the SE3228 project are summarised in table 1 using as the selection criteria the test cut-off giving the highest test specificity.

<i>Test</i>	<i>Cut Off Used (>/=)</i>	<i>Estimated Sensitivity</i>	<i>Estimated Specificity</i>
Stat Pak	1	44.7	97.2
Brock Test	1.5	40.4	95.0
Seralyte (CCJ Operator)	6.88	55.3	95.5
Fluorescent Polarisation Assay	118.3	7.7	97.8
Conventional IFN-g Assay	0.49	72.3	95.5
Luminex Platform	58.4	86.4	95.2
BioVeris Platform	89	68.1	96.1

Table 1 From DEFRA Project SE3228. A comparison of test characteristics where the test cut-off was selected to maximise specificity and using bacteriological confirmation as the gold standard.

4.2.7 Future tests

The Enferplex assay (Whelan et al., 2008) exploits a relatively new multiplex chemiluminescent technology and was developed primarily to detect bovine antibodies to a variety of *M. bovis* antigenic targets. With this platform, up to 9 individual protein antigens are imprinted onto the wells of a 96 test plate format and the antibody binding pattern, using a single serum sample, is analysed to discriminate between responses to each antigen. This antibody profile is used to describe the immune response of that particular animal and to give an accurate diagnosis. This is a very rapid laboratory assay which can be automated. Despite the sensitivity (93.1%) and specificity (98.4%) of this assay (Whelan et al., 2008) it remains unclear what influence recent comparative skin tests using PPDa and PPDb might have on test result outcome. In vivo inoculation of tuberculins are known to boost the antibody response in animals that are infected with *M. bovis* (Lightbody et al., 1998). Therefore these test characteristics remain to be fully validated in cattle and thus it would be premature to extrapolate these results to badgers. However it is likely that this test will be trialled in badgers within a number of years especially with studies carried out in the Republic of Ireland and in the UK which may demonstrate its potential usefulness as a future serological test. Similarly other serological tests currently in development such as the Idexx ELISA test remain to be validated in badgers of known disease status.

With increasing knowledge of how the immune response to TB develops and improved availability of defined reagents, there is a concerted effort to define an immune signature for tuberculosis infections (Hussain et al., 2007; Meade et al., 2008; Seth et al., 2009). This signature will entail the measurement of a number of cytokines including IFN- γ , the expression ratios of certain cytokines as well as other biomarkers that are active during the immune response to infection. Technology has advanced significantly in the past 5 years and will permit multiple analyte measurements simultaneously, from a single sample source (Pinel et al. 2010; Whelan et al., 2008). In effect, significantly greater information can be retrieved from a single assay, refining diagnosis and improving accuracy. This principle, when applied to the diagnosis of tuberculosis will enhance diagnostic capability. However to date there is no published information on the application of these technologies to the diagnosis of TB in badgers.

5. Direct detection of *M. bovis*

5.1 Post Mortem Examination

The pathology of tuberculosis in badgers is described in detail elsewhere (Corner et al., 2010). The disease is primarily respiratory although with progressive disease the infection can disseminate to other tissues throughout the body. Infection is most frequently found in the thoracic cavity with the most common extra-thoracic sites being the head and body lymph nodes. In one Irish study 61.5% of *M. bovis* positive badgers were found to be infected in the lungs and thoracic cavity with most animals not having visible lesions suggesting a predominance of latent infection (Murphy et al., 2010). The distribution of infection in badgers in GB was found to be very similar (Gallagher and Clifton-Hadley, 2000) with a predominance of respiratory infection and the likelihood that dormancy or latency was a common feature.

Most surveys find a small number of skin lesions which are presumed to originate from bite wounds inflicted by infected and bacteriologically shedding badgers. However it is difficult to compare different pathological surveys in badgers as all will use different methodologies and be based upon different background populations of badgers, for example in a population where culling has been extensive it might be expected that typical pathology would be less advanced than in infected populations where culling has not been undertaken or has been less thorough.

Post mortem examination of animals normally comprises two stages, dissection of tissues together with the description of the gross or visible pathology found and the ancillary testing of tissues, either tissues showing gross pathological changes or the elective submission of tissues regardless of pathological change. The ancillary testing of tissues is normally histopathology where tissues are examined microscopically to identify pathology consistent with tuberculosis and bacteriology with a confirmatory step to definitively identify any mycobacteria isolated. It is worth noting that routine histopathology alone cannot be used to definitively confirm tuberculosis infection as infection with other non-tuberculosis complex organisms can cause similar pathological change although most typical lesions are confirmed as tuberculous (Chambers et al. 2010).

Reliance of gross pathology alone will lead to a significant underestimation of infection status. In one study two thirds of infected badgers had no visible pathology following routine pathology with infection only detected following elective pathology of pooled animal tissues (Murphy et al., 2010). Importantly a number of lesions found during gross necropsy were subsequently found not to be tuberculous. In another study, of 205 badgers examined by a standard protocol, 120 were classified as visibly lesioned. However when additional tissues were examined a further 3 animals were found to have visible lesions (Crawshaw et al., 2008). In the same study, and taking account of the enhanced overall protocol where more extensive gross pathological examination was done and additional tissues submitted for histopathology and bacteriology

compared to a standard protocol, the enhanced method revealed an additional 24 infected animals, 17 of which were revealed by bacteriology alone.

Frequently, only single lesions are found in naturally infected badgers (Corner et al., 2010) and these may be very small and not associated with tissue enlargement. During the randomised badger culling trial in England only 5.6% of badgers (out of a total of 1166) had extensively disseminated disease involving four or more body compartments (Jenkins et al., 2008). It is clear therefore that the thoroughness of the post mortem procedure is critical in maximising the likelihood of detecting tuberculosis. This will be both time consuming and expensive (Murphy et al., 2010). In this study gross lesions (confirmed by culture) were detected in only 12.1% of badgers examined by a comprehensive post mortem examination (compared to 36.3% of badgers confirmed infected using tissue bacteriology).

During post mortem examination the assiduous examination of tissues is required to enhance accuracy. Detection of tuberculous lesions in the respiratory system can be challenging due to the size and nature of the upper and lower respiratory tracts. One review highlighted the need to slice each lymph node completely, as thinly as possible, to significantly increase the lesion detection rate (Corner, 1990). Disclosure of small lesions in the lungs is improved if each lobe is finely sliced (5 mm) and the cut surfaces on both sides for the sliced tissue palpated for respiratory lesions (Gavier-Widen, 2009). Additionally, slicing lung tissue that had been first fixed in formaldehyde, revealed macroscopic lesions which were not observed in the fresh tissue (Gallagher et al., 1998).

Consistently a small but significant proportion of badgers have bite wounds infected with *M. bovis* (Fagan, 1993; Gallagher et al., 1998) although these wounds may be difficult to see due to the presence of thick hair. Infection may be more easily detected if those superficial lymph nodes that drain superficial sites were also examined. There is a clear association between bite wounds (5% of badgers) and a higher prevalence of visible lesions with a different distribution pattern of infection compared to respiratory acquired infection (Jenkins et al., 2008). This group speculated that *M. bovis* infected bite wounds were more likely to take longer to heal than uninfected wounds and that they were acquired through aggressive encounters between different social groups.

5.2 Culture of Post Mortem Material

The direct detection of *M. bovis* using culture methods to isolate the bacterium from post mortem samples is generally regarded as the most sensitive and therefore the 'gold standard' for determining infection status (Corner et al., 2010; Crawshaw et al., 2008; Murphy et al., 2010). However the sensitivity of tissue culture is greatly influenced by a number of factors most notably the number of tissues examined, the aseptic methods used to take tissues, the processing of tissues prior to culture and the methods and materials used for culture including the number and type of media used and the length of incubation time.

During post mortem examination there is potential for cross contamination between tissues or between animals particularly where lesioned material, which can contain large numbers of the organism are being handled. This can only be countered by meticulous aseptic techniques and careful adherence to disinfection protocols (Corner et al., 2008). Therefore it is crucial that the operator is trained and skilled in the dissection of badgers.

It is important that bacteriology is supported by a recognised confirmatory step as other mycobacteria can be isolated using the normal bacteriological methods and that on occasion these mycobacteria can have a similar phenotype to *M. bovis* when examined microscopically. Using a molecular confirmation step such as spoligotyping means that the specificity of culture is very high (>99.99%) (Roring et al., 2002; Roring et al., 2004; Skuce et al., 2005).

The detection rate for infected animals is a function of the number of sites inspected as well as how carefully each of these tissues are examined (Gavier-Widen, 2009). This is borne out by several studies which have unambiguously demonstrated that the culture of multiple tissues greatly enhances the infection detection rate in badgers. For example one study (Murphy et al., 2010) found that the infection prevalence estimate was only 12.1% where visible lesions were confirmed by bacteriology but increased to 36.3% when added to this was the culture of pooled tissue samples. Similarly a GB survey (Crawshaw et al., 2008) found that the sensitivity of a standard protocol had a relative sensitivity of 54.6% compared to a more detailed protocol involving the culture of additional tissues.

M. bovis may be present in tissues in very small numbers, (ranging between 1 bacillus to more than 10^5 cfu per gram of tissue (Gallagher et al., 1998). The numbers of viable bacteria may be too few to culture, especially if chemical decontamination is needed to prevent overgrowth of contaminating microorganisms. The need for decontamination arises from the presence of non-mycobacteria in tissue samples taken at post mortem examination, or from the environment where the examination takes place. Since *M. bovis* is a particularly slow growing organism, faster growing non mycobacterial species can outgrow *M. bovis*, making the identification of *M. bovis* in samples impossible. To avoid this chemical decontamination is usually carried out to attempt to reduce or eliminate other contaminating organisms using oxalic acid, sodium hydroxide or hexadecylpyridinium chloride (HPC). While these agents are effective in controlling contamination they are also to some extent toxic to *M. bovis* (Corner and Trajstman, 1988; Corner et al., 1995) and a proportion of *M. bovis* can be lost using these chemicals. This is particularly important where there are few organisms present in the tissue such that decontamination can reduce the numbers of organisms present below the limits of cultural detection. However, toxicity can be reduced depending on the perceived risk from microorganisms by varying the concentration of decontaminant used (Corner et al., 1995).

Recovery of *M. bovis* can be optimised by using more than one culture medium (Corner and Nicolacopoulos, 1988), by extending the length of time that cultures are maintained for example from 42 days to 12 weeks and by

increasing the number of cultures inoculated with the original sample preparation (Leigh Corner, personal communication). One study (Jenkins et al., 2008) reported that doubling the number of culture tubes from 6 to 12 and extending the incubation period from 6 weeks to 12, significantly enhanced identification of infected badgers. When comparing a standard post mortem protocol with a more detailed one, another study reported a culture sensitivity of 70.7% for the detailed examination of visibly lesioned badgers, compared to a culture sensitivity of 55.9% using a standard protocol (Crawshaw et al., 2008). However these researchers also noted the cost implications of more detailed and intensive pathological and bacteriological investigations - 'There is clearly scope to improve the sensitivity of the standard protocol for detecting *M. bovis* infection in badgers, and similar improvements could probably be made to the protocols used in other species. However, the cost of such improvements needs to be considered in relation to the aims and objectives of any study.'

5.3 Culture of Live Animal Material

It is likely that the infectivity of any infected animal is related to the extent of infection in the animal with animals exhibiting advancing disease the most likely to be shedding bacteria into the environment (Gallagher et al., 1998). Given that most infected badgers do not appear to have gross pathological lesions (Crawshaw et al., 2008; Murphy et al., 2010) it follows that most infected badgers do not shed significant numbers of the organism and that the shedding is likely to be intermittent in many. In one study 61 badgers known to have excreted *M. bovis* on one occasion were serially trapped and sampled for faeces, urine, tracheal aspirates and bite wounds (Chambers et al., 2002). The sensitivity of bacteriology in these known infected badgers was 27.5%. In comparison 68% of these badgers were detected using the Brock ELISA test. In a previous study using lung lavages and urine taken post mortem, 65% of known infected badgers yielded detectable *M. bovis* (Gallagher and Clifton-Hadley, 2000). In this case, the use of a post mortem examination allowed for better sample collection and more recovered material. Therefore there seems only limited value in using live animal bacteriology as even in known infected animals that have excreted *M. bovis* in the past the sensitivity is poorer than routine blood based immunological tests.

5.4 Polymerase Chain Reaction assays

The Polymerase chain reaction (PCR) is a widely used technique to detect very small amounts of DNA from a variety of samples. In principle it is sensitive, rapid and inexpensive. It has been reported to have a sensitivity of at least 98% in human sputum samples where bacteria are detectable by microscopy ((DEFRA), 2010) although it's negative predictive value is poor. In human patients with clinical disease the sensitivity for faecal PCR was poor (86% in adults and 38% in children) ((DEFRA), 2010; Oberhelman et al. 2010)

In cattle, PCR methods based on IS6110 or IS1081, were found to have sensitivities ranging between 55% and 100%. In most studies conventional

culture was superior in correctly identifying infected animals, (Cardoso et al., 2009; Garbaccio and Cataldi; Mishra et al., 2005; Parra et al., 2008; Taylor et al., 2007). One study of Mexican cattle infected with *M. bovis* compared traditional culture methods and PCR to define infection status (Bermudez, 2010). They concluded that the use of PCR did not increase the detection rate of *M. bovis* compared with culture. Some attempts have been made to adapt PCR methods to detect infection with *M. bovis* in other animals but with no success in coyotes (Sangster et al., 2007), or limited success in white-tailed deer (O'Brien et al., 2004) due in part to the tissue treatment prior to PCR analysis.

One study has described the development of a PCR assay to detect the RD4 flanking region and used the assay to detect the presence of *M. bovis* at badger setts and latrines (Sweeney et al., 2007). This assay was specific for *M. bovis* and identified all setts sampled although there was greater variation in cell numbers found at setts compared to latrines. However, despite the apparent and perceived sensitivity of PCR methods in general, direct detection of *M. bovis* by PCR was not recommended by a DEFRA expert panel (July 2010). While the panel agreed there was potential for PCR with respect to the faecal and air (sample) approaches, the expert group concluded that PCR was not a test that could be usefully used for detecting TB in badgers based on the current state of knowledge, particularly in the field ((DEFRA), 2010). This is in agreement with an earlier review into bovine TB which concluded that 'the PCR technique is not yet able to perform as well as conventional bacterial culture in the detection of *M. bovis* in terms of sensitivity, specificity or reliability' (Wilsmore, 2008). To support this one study using an optimised PCR test applied to bovine post mortem tissues resulted in a test sensitivity of 61-65% compared to conventional pathology and culture (Parra et al., 2008).

Very little validation work has been carried out on the PCR technique in badger derived samples and there is currently no evidence to suggest that it currently offers any advantages over other diagnostic tests currently available for badgers. Therefore while the PCR technique holds great potential as a diagnostic test it cannot currently be recommended as a diagnostic test in badgers.

5.5 Other Tests

The presence of *M. bovis* cells can be demonstrated directly without recourse to culture methods. IMS-phage is a method adapted from the detection of *Mycobacterium avium* subspecies *paratuberculosis* (Foddai et al. 2010) in which specific antibodies are linked to magnetic micro beads and used to concentrate and recover bacilli from tissue samples. The bacteria are then identified using a mycobacteria specific virus (bacteriophage). This work is now being developed to identify *M. bovis* from bovine tissues and may in due course be a viable alternative to tissue culture. However to date it remains untested in badgers.

Imaging techniques such as magnetic resonance imaging (MRI) are now available to identify a variety of pathologies in animal tissues. In one study the

use MRI scanning of lungs from tuberculosis infected experimental animals was shown to have a high detection rate for lesions down to less than 1mm in diameter throughout the lungs (Kraft et al., 2004). However the usefulness of this technology as an ancillary aid to post mortem examination remains uncertain given the cost of equipment to carry out the imaging and the expertise required to interpret images generated. Also, it should be noted that there are other common lung pathologies seen in badgers caused by a variety of infectious and non-infectious agents which may complicate the interpretation of scans (Corner et al., 2010; Gallagher and Clifton-Hadley, 2000).

6. Trapping strategies and diagnostic potential

Three possible scenarios where diagnostic tests could in principle be performed on badgers were outlined in the review objective. These are:

1. Live badgers trapped and released
2. Live badgers trapped, anaesthetised and released
3. Badgers trapped and euthanized.

A summary of the sampling that could be undertaken in each scenario is listed in table 2

Capture Scenario		
Scenario 1 Live trap / release	Scenario 2 Live trap / anaesthetised / release	Scenario 3 Live trap / anaesthetised / euthanized
Faeces ? Some basic Observations ¹	Faeces / Rectal swab Urine Tracheal aspirate/Broncho-alveolar lavage Whole blood Clotted blood Wound swab Observations ²	Faeces / Rectal swab Urine Tracheal aspirate/Lung lavage Whole blood (ante mortem) Clotted blood (ante mortem) Wound swab Observations ² Full post mortem with ancillary tests

Table 2. Samples available for collection under three testing scenarios.
Observations-¹: Visual examination, body condition, presence of wounds, size estimate.
Observations-²: Detailed examination, weight, sex, age, wounds / wound swabs.

6.1 Scenario 1: Live badgers trapped and released

In this scenario, badgers would be trapped, *not anaesthetised* and released. This method would only allow superficial examination of the animal including estimated body condition, sex (possibly) and general maturity. Given that the animal is wild there would be no feasible way of direct sampling. The only sample that may be available would be faeces if the badger defecates during capture. However this could only be collected from the trap and therefore poses the risk of cross contamination between animals. More importantly many badgers will not defaecate on capture and of those where a sample is obtained the sensitivity of the test is such as to preclude this approach as being in any way useful. Some badgers might urinate on capture but it is difficult to

envisage how this could be collected aseptically. Therefore this is not a feasible option for assessing the tuberculosis status of badgers.

6.2 Scenario 2: Live badgers trapped, anaesthetised and released

In this scenario badgers would be trapped, anaesthetised, examined, sampled and released. During the 15-30 minute window when the badger is anaesthetised, a whole blood sample could be taken for blood based testing. The only trap-side test (Brock TB Stat-Pak) for bovine TB in badgers could be performed while the animal is anaesthetised (approximately 20 minutes to complete). This is the only test option which would allow a decision on release/vaccinate/cull while the animal is initially anaesthetised. This assay has a limited estimated specificity (93-97%) but poor sensitivity (45-55%), with the likelihood of detecting animals only in the later stage of infection. If the primary aim of this scenario is to define the disease status of each badger, as rapidly as possible, the Brock TB Stat-Pak is the only option. Approximately 5% of positive badgers will be wrongly identified as infected and approximately half infected badgers will not be categorised as test positive. While this is likely to make the use of this test to decide whether to cull positive or vaccinate negative captured badgers unworkable due to the high rate of incorrect disease ascriptions it does not preclude the use of the test to estimate overall disease prevalence as this can still be calculated based on known test characteristics. (Although outside the scope of this review it is worth noting that vaccinating infected badgers may not have a detrimental effect on disease progression in badgers either at the individual or group level e.g. vaccinating infected but test negative animals (Derrick et al., 2008)).

All other live blood based tests would require the animal to recover and be housed for at least 24 hours while the tests are performed if there is to be further intervention such as a decision to cull test positive and release test negative animals. Using all tests a number of animals will be incorrectly identified whether as infected or uninfected. For the serological tests all appear to perform broadly similar to the Stat Pak apart from the fluorescent polarisation assay and it is unlikely that the currently available tests would add much to the Stat Pak. However interferon-gamma testing does appear to have a significantly increased sensitivity when compared to the serological tests and its use would be recommended as an ancillary test for screening badgers. A variety of platforms for this test are available with the Luminex system possibly out-performing other platforms currently available. However it must be noted that this test also has a specificity of approximately 95% and so a significant number of animals will be incorrectly categorised as infected using this test.

This scenario allows for the taking of faeces, respiratory tract samples and possibly urine. However for the reasons stated above these are likely to be highly insensitive and cannot be used as an animal side test whether the animal is held for several hours or not. As any animals excreting bacteria are likely to have relatively advanced disease they will most probably be detectable using other blood based tests. Therefore doing this type of testing will not add significantly to determining the infection status of animals.

6.3 Scenario 3: Live badgers trapped, euthanized and examined post mortem

In this scenario badgers will be trapped, anaesthetised, euthanized and examined post mortem. This approach gives the greatest opportunity of defining the infection status of trapped badgers as the sensitivity of thorough post mortem followed by multiple tissue bacteriology is the most sensitive method available to define disease status with a sensitivity of 1.2-2 times that of immunological based diagnostics (see table 1). Where infection status is defined by molecularly confirmed bacteriology the specificity of the test will be virtually 100%.

Post mortem examination will also help to inform the stage of disease progression within badgers. While it is likely that as with other studies most badgers in N. Ireland will have limited pathology and few will have measurable bacterial excretion either through the respiratory, alimentary or urinary tracts this remains undemonstrated. Additionally it will allow for the molecular characterisation of *M. bovis* isolates using for example VNTR to help inform the comparative diversity of isolates in badgers with cattle and where multiple tissues are cultured test whether it can be demonstrated that there are badgers infected with multiple *M. bovis* strains.

The use of post mortem examination allows for the comparative assessment of other currently available tests, combinations of tests and newer tests currently untested in badgers. This also affords the opportunity to assess the usefulness or otherwise of samples taken before and after death to assess if any could be usefully applied to road traffic accident badgers. For example it is recognised that, in the case of cell mediated responses, samples taken post mortem are compromised in terms of test performance although there is evidence that initial screening of post mortem material using the Stat Pak had a high specificity but low sensitivity potentially allowing for its use as an initial screen of road traffic accident animals where only test negatives are subjected to further testing (Chambers et al. 2010). This approach could be very valuable to improve the cost effectiveness of RTA badger screening. Therefore the testing of euthanized badgers allows for the further validation of several alternative test approaches using bacterial confirmation as the 'gold standard' measure.

7. The potential outcome of each test-based scenario

This review seeks to evaluate the three trapping strategies listed above against each of the following four criteria;

1. Provision of a better understanding of bovine tuberculosis in the local badger population.
2. The efficacy of specific tests or combinations of tests.
3. The practicality of a test and release (test negative – release; test positive – cull) approach
4. A comparative assessment between a lethal and non-lethal intervention.

7.1 Provision of a better understanding of bovine tuberculosis in the local badger population

Scenario one (live badgers trapped and released) will contribute little to this objective. There is no allowance for any of the more sensitive diagnostic tests to be carried out and little information of any value will be gained in terms of bovine tuberculosis in badgers.

Scenario two (live badgers trapped, anaesthetised and released) will be more productive since blood samples would be readily available for a variety of immunological tests. Provided the tests used have known characteristics, this would be sufficient to estimate the prevalence of bovine tuberculosis in badgers. Given this the most likely tests that could be employed in this scenario would be the Stat-Pak, the Brock ELISA, and the interferon-gamma assay. In the case of the latter test it would be important that the logistics of sampling sufficiently early to allow for the test to be run at the lab in the same day would need to be taken into account. In addition a limited number of physical measurements such as sex, weight, approximate age and body condition could be assessed and used for later analysis of test results.

Scenario three (live badgers trapped, euthanized and examined post mortem) provides the most comprehensive assessment of the disease status of any badger in addition to those options available in scenario two through post mortem examination and subsequent bacteriology. This approach will be the most sensitive and specific for defining the infection status of trapped badgers. It will provide data on the strain (molecular) type of *M. bovis* isolates and allow comparison with cattle isolates. However to maximise the sensitivity of this approach the methods employed for post mortem examination must be thorough with multiple tissues sampled for bacteriology (Murphy et al., 2010). This approach will therefore be the most expensive of all the scenarios under consideration and also involves all of the risks and issues associated with badger removal.

7.2 The efficacy of specific tests or combinations of tests

In the context of this review, efficacy is defined as the effectiveness of a test, or

combination of tests to accurately diagnose bovine TB in badgers.

Scenario one will not be able to contribute to this objective as no meaningful tests can be performed on conscious badgers.

Scenario two allows the collection of blood samples for the variety of immunological assays. The relative test characteristics of the most commonly employed of these tests are already available and are summarised elsewhere. In summary antibody based tests have a relatively low sensitivity and sub-optimal specificity while the interferon-gamma assay has a similar specificity but better sensitivity. Despite these characteristics they are valuable and it is likely that the most accurate method of diagnosing infection in badgers would use a combination of antibody test (e.g. Stat-Pak or Brock test) plus the interferon gamma assay (possibly using a sensitive platform such as Luminex). For example Bayesian analysis has suggested that combining the Stat-Pak with the interferon-gamma assay led to an improved negative predictive value compared to using either test on its own (Drewe et al. 2010) (i.e. if tested negative using both tests there was an estimated 97% likelihood of freedom from infection). This study recommended the use of both tests in parallel with an animal defined as positive if testing positive by either test and negative if negative by both tests (in the absence of culture from faeces, urine, wounds or respiratory tract). In this particular study culture had an estimated sensitivity of 8% with most positive animals positive by one or other of the immunological tests. Only one animal (out of 305 tested) was positive by culture and negative by both other methods suggesting that culture from live animals was of very limited additional value.

Scenario three allows for the most accurate diagnosis of TB in badgers using a combination of post mortem examination and bacterial culture from tissues. Importantly recent work has suggested that the Stat-Pak could be used as an initial screening of euthanized animals prior to making a decision to undertake pathology (Chambers et al. 2010). In this study the Stat-Pak had an estimated specificity of 99% so that virtually all test positive animals were subsequently found to be culture positive. Therefore in this case only test negative animals needed to be examined further if the only outcome was to determine presence or absence of infection. This high specificity was probably due to the relatively poor quality of blood samples taken from dead badgers which will have degraded meaning that only animals with high titres of antibody present would have tested positive. Animals with high levels of antibody will be very probably truly infected.

7.3 The practicality of a test and release (test negative – release; test positive – cull) approach.

There are two potential options available to undertake this approach. The first is releasing (or culling) badgers after an initial anaesthesia, and the second is holding badgers in captivity for longer (perhaps 2 days) before test results are available. While this second option has been included for completeness, in

reality welfare considerations are likely to preclude holding badgers in captivity for this length of time. Alternatively it is possible that option 2 could be applied via a test-release-recapture approach whereby animals are released and re-captured if test positive, however the effectiveness of this approach would entirely depend on the re-capture rates achieved.

With the first option the only test currently available which will give a result quickly enough to allow a decision to be made before the animal is allowed to recover from initial anaesthesia is the Brock Stat-Pak. The sensitivity of detection of individual *M. bovis* infected badgers using this test is relatively low, approximately 50%, with a specificity of approximately 95%. The practical outcome of this approach would mean that approximately half of all infected badgers would fail to be detected and therefore be released, while approximately 1 in 20 uninfected badgers would test positive. Potentially this approach could be also applied at a sett level i.e. the decision to cull a sett could be based on the combined results of tests applied to all animals captured at the sett. In general terms the effect of this would be to increase the overall sensitivity of detection but reduce the specificity, the exact effect being dependent on the number of animals tested and the individual sett prevalence. While the overall sensitivity of the Brock Stat-Pak test is relatively low, it should be noted that animals that test positive are likely to be those with more advanced disease and therefore more likely to be infectious animals e.g. Chambers et al (2008) estimated sensitivity to be 78% in badgers classified in the paper as “super-excretors”.

The second option involves holding badgers in captivity for at least 2 days and / or undertaking to re-capture sampled animals to allow laboratory based tests to be performed. Of the other tests available the interferon-gamma assay is the most sensitive with a specificity broadly similar to the Stat-Pak. In this case it might be expected that an optimised assay would have a sensitivity of 70-80%. Therefore if this test was performed on its own approximately 1 in 4 infected badgers would be missed with a similar false positive rate to that of the Brock Stat Pak. In a Bayesian analysis of tests, for a badger population with an estimated infection prevalence of 20%, using the Stat Pak and the interferon-gamma assay in parallel led to an estimated positive predictive value (PPV) of 0.75 (i.e. 75% of animals testing positive by both tests were truly infected) and a negative predictive value (NPV) of 0.97 (i.e. 97% of animals testing negative by both tests were truly uninfected) (Drewe et al. 2010). In the same study using Stat Pak on its own had a PPV of 0.81 and a NPV of 0.88 while the interferon-gamma test had a PPV of 0.81 and a NPV of 0.95. Therefore using the tests in parallel allowed for a more accurate overall test although this did reduce the PPV of the test combination. This study included culture of live animal samples as one of the tests for determining animal disease status, which would not be available as part of a test/cull/release approach. In this case, in culture negative animals, the estimated PPV of the two tests where one or other is positive is 75%. Taken in its entirety this study recommended designating animals as negative only where both tests were negative.

A similar modelling exercise has not to the authors knowledge been undertaken for other serological tests and so it remains uncertain what benefits might come

from using other test combinations but in principle it is likely the greatest gain would come from using a combination of assays measuring cellular and antibody responses. However given the logistical constraints of the interferon-gamma test and the length of time taken to get a result, adding this test would significantly increase the cost of any testing program where badgers had to be held before a decision was reached to cull or release.

The overall effect of a “trap-test-cull-vaccinate” approach will depend on the efficiency of removing infectious animals relative to other approaches (e.g. proactive culling) and how this is counter-balanced by any detrimental effects, principally those due to badger perturbation. Importantly, and unlike proactive culling and to a lesser degree reactive culling, there have been no reported field trials of using a “trap-test-cull-vaccinate” approach.

In the absence of experimental field data, the best that can be used to try to predict the possible effect of such an approach is mathematical modelling. Such models have been used extensively as part of the evidence base for the intensive action area by the Welsh Assembly Government. One of these models (Wilkinson, 2009) specifically considered the likely effect of a trap-test-cull-vaccinate approach relative to no intervention, proactive culling and vaccination approaches. The model assumed the use of the Brock-Tb StatPak test with a specificity of 95% and variable test sensitivity of 34% for infected badgers, 42% for infectious badgers and 78% for super-infectious badgers. The model was run with and without a perturbation effect, in view of the uncertainty as to the impact perturbation might have with this culling approach. The model predicted that relatively few animals would be removed with less than half the social groups having badgers culled in any one year assuming a trapping efficiency of 70% (Wilkinson et al., 2009) and very few social groups would have more than one badger removed. Importantly the effect of a trap-test-cull-vaccinate on TB prevalence within both badgers and cattle was heavily dependent on the level of expected perturbation. In a situation where perturbation was assumed to occur, the trap-test-cull-vaccinate approach led to a significant increase in the rate of confirmed cattle herd breakdowns relative to no intervention. Trap-test-cull-vaccinate assuming no perturbation was only marginally better than cull only and vaccinate only approaches, all of which gave very similar results with small reductions in the rate of confirmed cattle herd breakdowns. The authors state that “given the large uncertainty, we cannot accurately predict the outcome of a combined trap-test-cull-vaccinate strategy at present”.

There has been no equivalent modelling work undertaken in NI, to predict what effect such an approach might have here or indeed the possible effect of alternative trap-test-cull approaches such as one based on whole-sett removal.

7.4 A comparative assessment between a lethal and non-lethal intervention.

Thorough post mortem examination combined with the culture of a wide range of tissues is the most specific and probably the most sensitive method currently

available to assess the TB disease status of individual badgers. It allows for a complete pathological investigation and for some samples improved collection over anaesthetised animals, e.g. lung flushes, urine/faeces collection. It also has the further advantage of providing a source of isolates to compare with the strain types found in cattle. Given the limited excretion of *M. bovis* from most badgers, culture of samples from live (anaesthetised) badgers will only provide limited information.

While outside the scope of this review it is important to highlight that live capture and euthanasia is the most invasive approach and the one most likely to have an ecological impact. The risk of badger perturbation and the effect this may have on bTB transmission to cattle needs to be fully considered prior to a lethal badger intervention being embarked upon.

While all non-lethal interventions are likely to lead to a less accurate assessment of each individual animal, prior knowledge about the tests' characteristics can be taken account of when estimating overall disease prevalence. So for example with unbiased trapping it would be expected that the true number of infected animals would be approximately twice the number of Stat-Pak test positive animals. Therefore where only an estimate of disease prevalence is required a trap, anaesthetise and test approach would be preferable. As stated above this could be based upon a single animal side test or involve more than one test. Using more than one test is likely to lead to a more robust estimate of disease status per animal.

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