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Department for Environment Food & Rural Affairs

Evidence Project Final Report

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Project identification

1. Defra Project code: SE3289

2. Project title:
   A study to comparatively assess diagnostic methods for detection of *M.bovis* in badger faeces

3. Contractor organisation(s):
   Animal and Plant Health Agency (APHA)
   Woodham Lane
   New Haw
   Addlestone
   Surrey
   KT15 3NB

4. Total Defra project costs (agreed fixed price): £ **360,065**

5. Project: start date 01/06/14

   end date 30/06/15

6. It is Defra's intention to publish this form.

   Please confirm your agreement to do so.....**YES** ☒ **NO** ☐

   (a) When preparing Evidence Project Final Reports 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.

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   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.

Background

Bovine tuberculosis (bTB) continues to represent a major animal health problem in Great Britain. Badgers can act as a wildlife reservoir for the causative agent, *Mycobacterium bovis* (*M. bovis*) and are implicated in transmission to cattle. Experience from Great Britain and elsewhere demonstrates that the elimination of bTB in domestic livestock is problematic where such a wildlife reservoir exists. Consequently, control of infection in badger populations in regions of high bTB incidence is important for disease control in cattle.

Over the last 7 years Defra has invested in the development of diagnostic tests to detect *M. bovis* in badger faeces. Because badgers tend to deposit faecal samples in latrines close to their home sett, such tests have the potential to allow identification of TB infected badger setts without the need for trapping animals. The use of such tests could contribute to future disease control strategies and policies.

Potentially applicable PCR-based tests have been proposed by Warwick University, LGC Ltd and BadgerCare Ltd. Such tests measure the presence of M. bovis DNA in a given sample. Queen's University Belfast has developed a lateral flow device (LFD) test, similar to a pregnancy test, which detects M. bovis bacteria present in a given sample.

In December 2013, the Diagnostic Programme Advisory Group (DPAG) of the Defra bTB Science Advisory Body reviewed the research undertaken in this area to date. DPAG's judgement was that it was not currently possible to ascertain which, if any, method was suitable for potential routine deployment. They advised that an inter-laboratory comparative study of the currently available methods be undertaken, to allow an objective assessment of the diagnostic performance of the assays. Defra subsequently set out the performance critieria against which the performance of the tests were assessed.

An inter-laboratory comparative study was designed to assess and compare the performance of the currently available methods for detection of *M.bovis* in badger faeces

The inter-laboratory comparative study involved four participants testing a panel of badger faecal samples with their diagnostic test method. Each participatant received a replicate of the same sample panel although the order of the samples was randomised and participants were blind to the *M.bovis* status of the samples or make up of the sample panel. One participant, with Defra's agreement, tested the same sample panel with two different tests. One participant with Defra's agreement, received two sets of

samples and used a separate single test on each panel. So in total 6 tests were assessed in the comparative study. These 6 tests are referred to as Tests A to F in this report. Details of tests and participants carrying these tests out are detailed in Table 1.

Table 1 Summary of the tests used, participants and Test ID in SE3289

Test ID	Test type	Participant	Brief technical description
A	PCR	LGC	Commercial DNA extraction kit (Qiagen QIAamp Fast DNA stool minikit) combined with LGC designed real time PCR for *M. bovis.*
B	PCR	Warwick University	Commercial DNA extraction kit (FastDNA Spin kit for Soil from MPBiomedicals) combined with real time PCR for *M. bovis*. Method previously evaluated in Defra funded project SE3280.
C	PCR	Warwick University	Commercial soil high throughput format extraction kit (FASTDNA™-96 SOIL MICROBE DNA KIT 96 extraction from MPBiochemicals) combined with real time PCR for *M. bovis.*
D	Direct LFD	Queen's University Belfast	Immunological detection based lateral flow device
E	IMS LFD	Queen's University Belfast	Immunological detection based lateral flow device (as in test D) with immuno-magnetic separation step prior to LFD detection
F	PCR	BadgerCare	PCR testing using the Cepheid Xpert MTB/RIF test system (automated PCR test platform which combines DNA extraction and PCR amplification into one instrument) Method previously evaluated in Defra funded Project SE3282

The test panel consisted of 569 samples: 205 negative samples (collected from captive animals known to be TB free), 119 putative positive samples (taken from setts which based on previous diagnostic testing on live animals, were known to contain TB-infected animals) and 245 spiked positive faecal samples (samples to which known levels *M.bovis* were added in the laboratory). This was prepared by APHA, blinded and distributed to participating organisations. Participants tested the samples and subsequently submitted their test results for statistical analysis.

The statistical analysis of the results was undertaken by an independent statistician, who was blind to the participant identity and test type. APHA staff (who were not involved in the preparation of samples or overall management of the project) also carried audits of the participating laboratories during testing to ensure adherence to submitted testing protocols. Required standards for test performance were established independently by Defra.

This study was intended as a comparative study primarily designed to compare the performance of the different tests and identify the best performing test of those currently available. It was not intended to determine the performance of the tests in the field or to interpret/decide how the tests could be used as part of Defra policy.

Results

No test fully met all the pre-defined criteria for test performance, although Test B (Warwick PCR) was the best performing test overall

- Test B (Warwick, PCR) was the best performing test overall. It met three criteria and was close to the required standard (borderline) for two others.
- All the other tests (A, C, D, E and F) failed to meet at least one criterion.
- Three tests (D, E and F) failed to meet two or more criteria.

The results of this inter-laboratory comparative study provide objective data on the relative performance of the diagnostic methods assessed. Only one test appears to be potentially suitable for taking forward to routine use. However, its borderline performance against some criteria highlight areas which may need further assessment and validation to fully understand the performance characteristics and utility of the test and hence determine if, and how, it could be best applied in the field. The potential practical use of a badger faecal test will also depend on future Defra policy. To support such decision making a cost benefit analysis maybe useful to provide more information on the cost of this approach relative to other options.

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 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 Exchange).

Project objectives & the extent to which objectives have been met

1. Collection of field samples from Woodchester Park and putative negative samples from TB free areas (APHA National Wildlife Management Centre). Completion by: 31/06/14

2. Collection of TB negative samples from APHA Weybridge and Sand Hutton captive badgers (APHA Weybridge & APHA National Wildlife Management Centre). Completion by: 31/06/14

3. Design of comparative study and calculation of sample numbers required (Royal Veterinary College with input from APHA Weybridge and National Wildlife Management Centre). Completion by: 31/06/14

4. Work up and development of laboratory homogenisation & spiking protocol for preparation of comparative study samples (APHA Weybridge). Completion by: 31/06/14

5. Design of comparative study labelling scheme, database and population of database with sample details (APHA National Wildlife Management Centre and APHA Weybridge). Completion by: 31/08/14

6. Preparation of comparative study samples and dispatch to participating laboratories (APHA Weybridge). Completion by: 11/11/14

7. Carry out audits of the participating laboratories (APHA Weybridge). Completion by 16/01/15

8. Submission of test results by participating laboratories (Participating Laboratories). Completion by 26/01/15

9. Analysis of results submitted by participants (Royal Veterinary College). Completion by: 08/05/15

10. Preparation and submission of draft project report (APHA Weybridge with input from Royal Veterinary College and APHA National Wildlife Management Centre). Completion by: 22/05/15

All objectives have been met.

Methods & Approach

Design of the comparative study

The design of the comparative trial was completed in collaboration and agreement with Defra. The design was in response to Defra's specification for the ring trial (Appendix 1). The document describing the design of the ring trial and detailed rationale behind sample size calculations to achieve the statistical values required by Defra in terms of test performance (e.g sensitivity and specificity at an individual sample and sett level) is attached in Appendix 2. The design of the trial was subjected to additional statistical review by a statistician not connected to the project.

In summary, a panel of 570 samples was planned for testing by the participating laboratories. The panel was to comprise of:

a. 200 negatives (from captive badgers and from a region of England known to be TB-free). The inclusion of these samples allowed the specificity of assays to be assessed.

b. 120 samples from putative positive groups at Woodchester Park (10 samples per sett, 12 groups). The inclusion of faecal samples taken from setts from which badgers are regularly trapped and blood tested (and hence for which there is existing data on the TB status of the setts) allows correlation of PCR results with other test data.

c. 250 spiked samples (making them known positives) – These were spiked with known levels of *M.bovis*. The inclusion of these samples allowed the sensitivity of assays to be assessed.

Because of practical and sample availability issues the final composition of the panel was slightly different from that planned; however, the numbers of samples obtained still allowed for a statistically significant assessment of the diagnostic methods used (details below).

Collection of badger faecal samples for use in the comparative study

Putative positive field samples were collected from 12 badger social groups from the Woodchester Park study area. These groups were targetted based on previous trapping and blood sampling results that strongly suggested infection was present within those groups. Putative negative samples were collected from a non-enemic TB area in the UK. Faecal samples were also collected from known TB negative captive animals housed at APHA Weybridge and Sand Hutton. Samples were frozen and transported to APHA Weybridge.

Development of laboratory methods for homogenisation & spiking of badger faecal samples

Preparation of homogenous faecal samples containing the same levels of bacteria was crucial for this project. Producing homogenous faecal samples is challenging. Experiments were carried out spiking BCG into negative faeces to work up a practical method of faecal sample spiking and subsequent homogenisation. Following development of a method which produced consistent results, the method was demonstrated using *M.bovis*.

Initial experiments employing a stomacher demonstrated this method was unsuitable due to sharp debris present in badger faecal samples (stones etc) puncturing the stomacher bags. As a result, a simple method involving mixing samples in a plastic beaker using a spatula or spoon was adopted. To test the method initially, 150g of negative faecal material was spiked with BCG by adding 20 mL of buffer containing known levels of BCG.

The sample was mixed in a plastic beaker for 5 min (timed) using a spoon/spatula. Five individual 1 g aliquots were then removed from the mixture and frozen in 2 mL microfuge tubes. DNA was then extracted from the samples and PCR amplified using an assay that targetted BCG. The PCR results demonstrated that the homogenisation method used was successful, with consistent quantitative values (termed Ct values) for all the aliquots (within expected experimental variance) demonstrating that the bacteria were evenly distributed throughout the samples (data not shown).

These results demonstrated that it was possible to prepare consistent spiked faecal samples with near identical loads of mycobacteria for subsequent testing by several laboratories. In addition, the samples were subjected to a freeze/thaw procedure prior to testing (to mimic freezing and transport to participants) demonstrating that samples were still suitable for use. The results were encouraging as the Ct values are similar to those expected from the majority of real positive samples (Ct >30; based on results from SE3280) This is because the most challenging samples to homogenise successfully (and ensure even distribution of bacteria through the sample) would be those samples with lower concentrations of spiked bacteria (which subsequently result in high Ct values). Following the successful use of BCG, similar work was carried out with *M. bovis* (strain 2122/97). The resulting quantitative PCR results (Figure 1 and Appendix 3) demonstrate consistent results from aliquots taken from the same *M. bovis* spiked sample and at bacterial concentrations (and hence Ct values) similar to those expected in field samples.

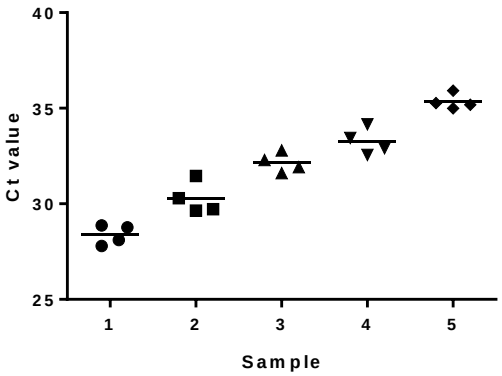

Figure 1. Quantitative PCR results (Ct value) from replicate sub-samples (n=4) taken from badger faeces spiked with *M. bovis* at five biologically representative concentraions (samples 1 to 5).

Design of comparative study labelling scheme and project database and population of database with sample details

Sample data (of the samples making up the panel of faecal samples) were initially entered into Microsoft Excel (2010). Three separate data tables were created to hold sample data. This included (1) Table sample (sample designation and social group where appropriate), (2) Table pool (ID of samples contributing to pooled material) and (3) Table link (to link aliquot and sample IDs). Excel tables were organised in a relational database which was maintained in Microsoft Access (2010) by the Data Systems Group, APHA Weybridge. Laboratories were supplied with a Microsoft Excel spreadsheet, pre-populated with sample details, to record their test results. The database Relationship Diagram and Data Dictionary are available.

Advice on source of tube labels was sought from APHA proficiency Testing Unit at APHA Sutton Bonington. All sample numbers were allocated randomly within sample sets and were blinded. Differents sets (for different participants) were colour coded to reduce the chances of sample set mix-up (for example, sample set 1, 2 and 3 were colour coded blue, yellow and green with sample numbers 1-600 (participants did not receive all the numbers). However, samples numbers were allocated randomly so the sample number for the same homogenised sample were different in different sets of samples (for example, blue 140, yellow 4, green 346).

Preparation of comparative study samples and dispatch to participating laboratories

In discussion with Defra it was agreed that APHA aimed to produce 20 sets of 570 samples. This would allow samples to be sent to the current participants whilst at the same time preparing other sets of samples which could be stored and sent out for future testing. This required preparation of 11,400 individual samples. The samples were 1g of faeces presented in screw capped microcentrifuge tubes.

Spiked samples were prepared from 150g of pooled faecal sample as described above. These spiked and homogenised 150g samples were then used to prepare 100 (5 × 20) individual samples. So all spiked samples were prepared as replicates of 5, which were included in each set of 570 samples. Preparation of the 250 spiked samples required for the study therefore required preparation of 50 × 150g spiked samples, each one resulting in 100 individual samples (5000 samples in total). Five of the “spiked” samples in each sample set were prepared as controls – “spiked” with buffer containing no bacteria or nothing added (prepared in same laboratory facilities as spiked samples to check for cross contamination). Preparation of these controls was carried out by sub-aliquoting one of the 150g pools used for preparing spiked samples. As a result, the number of spiked samples known to contain *M. bovis* was 245 in each panel of samples. The control samples were used as additional negative samples in the study (total number 205).

To ensure a variety of faecal "background" in the spiked samples (to account for factors such as faecal consistency and presence of inhibitors) faecal samples used to prepare the 150g spiked samples were sourced from a variety of sources. Of the 50 × 150g spiked samples, 25 were from captive badgers from APHA Weybridge, 12 from captive badgers from APHA York, 10 faecal material from Woodchester Park and 3 from putative natural negative faecal samples (sourced by APHA field teams).

Bacterial dilution series were prepared in 7H9 culture buffer media containing 0.05% Tween 80 which were then used to spike sequential 150 g samples. Bacterial concentration ranged from approximately 10^5 to 10^1 CFU/g faeces. The levels of bacteria added to the spiked samples attempted to balance the need to provide a variety of spiking levels with prior knowledge that positive natural samples tend to have low levels of bacteria. In addition, it was important to challenge the tests and use concentrations of bacteria that were at or below the likely limit of detection.

Homogenisation and aliquoting of the negative and putative positive faecal samples used individual samples (from one individual faecal deposit). Approximately 30g of faecal sample was homogenised and aliquoted into 20 x 1g aliquots. The same method of homogenisation was applied to the natural and negative samples as was used with the spiked samples to maintain consistency and ensure participants were not able to distinguish the different sample types. There were 6380 samples in total (200 negative samples and 119 putative positive Woodchester Park samples, aliquoted into 20 sets)*

Handling of all samples, apart from the samples from known negative captive animals, was carried out in Containment Level 3 laboratory conditions and appropriate biological safety cabinets, as all these samples could potentially contain *M.bovis* (or definitely did in the case of the spiked samples). Negative samples were handled and aliquoted in a Containment Level 2 laboratory not normally used for TB work to eliminate the possibility of cross contamination of samples with *M.bovis*.

Samples were dispatched to participants in 6 clip locked boxes (approx 100 samples per box) on dry ice under appropriate transport conditions appropriate for samples containing Containment Level 3 organisms. Samples were accompanied by instructions and background information.

*Because of difficulties collecting sufficient field samples (insufficient sample availability) from one putative positive sett at Woodchester Park only 9 samples could be collected, one less than planned. As a result the sample panel contained 569 samples.

Audits of the participating laboratories

Earlier projects investigating the use of tests for *M.bovis* in badger faeces focused on the development of the diagnostic tests, there have been incremental changes to testing methods and, as a result, some uncertainty about the final method that would be used in this study. To work towards field deployment and routine use, it was essential to ensure that the participating laboratories used the methods exactly as submitted. Therfore a requirement of taking part in the comparative study was submission of detailed protocols including data analysis, criteria for calling samples positive and negative and no "evolution" of methods during the study. In order to ensure that participant laboratories were following their methods, Defra requested “light touch” audits of the participating laboratories be carried out. These were carried out by experienced auditors from the Specialist Scientific Support Department (SSSD), part of the Research Directorate at APHA Weybridge. The auditors were not involved in the design or preparation of the ring trial or previous research work related to this area. Auditors examined the testing protocols submitted by the laboratories prior to auditing. Following their audits the auditors prepared short reports of their visits and findings (Appendices 4 -7). All audits concluded that the methodology used in the laboratory work observed was as described in the documentation and protocols submitted at the outset of the study.

Analysis of test results submitted by participants

The participating laboratories filled in their test results using the supplied Excel spreadsheet and returned to APHA for collation and unblinding. The members of staff involved in data collation and unblinding were not involved in laboratory preparation of samples or the statistical analysis phase. The results table was created in the project database, linked to the sample table via the Link table; a lookup table was used for the result type.

The final data report brought together the results from all 6 laboratories. Pooled samples were identified with their spike concentration, social group and latrine ID where applicable. A check was made to ensure a result was received from each laboratory for each sample. The number of each type of result on the results sheets was checked with the database table to ensure they matched. Cross checks were made on random samples in the report to ensure APHA Aliquot numbers and results matched the result sheets and database. The Detailed Pool table and full result sheets were also included.

The unblinded results were subsequently re-blinded and transfered to an independent statistician for analysis in a format which did not reveal to the statistican the identity of the participants or the tests (i.e. just the test results and sample identities were included). Analysis included calculation of sample-level sensitivity (using spiked samples and putative positive samples), sample-level specificity (using known negative samples), group-level sensitivity (using spiked, negative and putative positive samples) and group level specificity (using known negative samples). The methods and approach used by the statistician are described in Appendix 8. All the sample types used have advantages and disadvantages in terms of what the subsequent test results demonstrate about the performance of the tests. There is no perfect sample set with which to assess the performance of these tests and particular limitations have been highlighted in Appendix 8.

Results & Discussion

A summary of the results of the inter-laboratory comparative study is shown below (Table 1). A full analysis can be found in Appendix 8.

- Test B (Warwick PCR) was the best performing test overall. It meets three criteria and is on the borderline for two others: group-level specificity and group-level sensitivity (it achieves this latter criterion when spiked samples are analysed but is on the borderline when putative positive samples are analysed).
- All the other Tests (A, C, D, E and F) failed to meet at least one criterion.
- Three Tests (D, E and F) failed to meet two or more criteria.

Table 1. Comparative performance of each test against study criteria.

	Test A LGC PCR	Test B Warw PCR 1	Test C Warw PCR 2	Test D Qu LFD	Test E Qu IMS LFD	Test F BC PCR
1. Comparatively the most sensitive test at the individual sample level?	Borderline (93.5%)	Yes (97.6%)	No (88.2%)	No (3.3%)	No (0.0%)	No (60.8%)
2. At least 98% specific at the individual sample level (80% specific at the sett [group] level*)	No (79%)	Borderline (97.1%)	No (90.7%)	Yes (100.0%)	Yes (100.0%)	No (91.2%)
3. At least 50% sensitive at the sett (group) level [laboratory samples]?**	Yes (96.4%)	Yes (73.4%)	Yes (84.8%)	No (3.2%)	No (0.0%)	Yes (77.8%)
4. At least 50% sensitive at the sett (group) level [field samples*]?**	Yes (100.0%)	Borderline (41.7%)	Borderline (41.7%)	Yes (75.0%)	No (16.7%)	No (16.7%)
5. Able to detect low *M. bovis* concentrations**?**	Borderline	Yes	Yes	No	No	No
Overall interpretation of each test's performance	Insufficiently specific.	**Best test overall.** Borderline specificity. Good ability to detect low levels of *M. bovis.*	Insufficiently specific.	Poor sensitivity with spiked samples but good performance on field samples.	Poor sensitivity.	Insufficiently specific. Poor ability to detect low levels of *M. bovis.*

Green highlights indicate criteria which are achieved, red highlights indicate criteria which are not achieved, and yellow highlights indicate that a test is on the borderline of achieving that criterion (where confidence intervals overlap the pre-set criterion). LGC, LGC Ltd; Warw, University of Warwick; Qu, Queen's University Belfast; BC, BadgerCare Limited.

*Group-level specificity calculated assuming 10 samples are tested per group and a cutpoint of 1 animal required to test positive in order to consider the badger social group to be infected. (These criteria were agreed in the design phase of the trial.)
**Laboratory samples refer to spiked and known negative samples. N.B. Quoted values are group-level sensitivity estimates at an infection prevalence of 10%. Group sensitivity estimates decline with prevalence.
***Field samples refer to putative positive samples from Woodchester Park. This measure of group-level sensitivity is based on a small sample size (12 groups) but it does represent the use of each test in the field.
****Able to detect less than 100 CFU/g in spiked faecal samples with at least 50% sensitivity. N.B. This was not a predefined trial criterion but is included here for additional information it provides.

Possible Future Work & Any action resulting from the research

The results of this inter-laboratory comparative study provide objective data on the relative performance of the diagnostic methods assessed. Only one test appears to be potentially suitable for taking forward to routine use. However, its borderline performance against some criteria highlight areas which may need further assessment and validation to fully understand the performance characteristics and utility of the test and hence determine if, and how, it could be best used practically. The potential practical use of a badger faecal test will also depend on future Defra policy and how the use of this test would fit alongside other interventions and control policies. To support such decision making a cost benefit analysis maybe useful to provide more information on the cost of this approach relative to other options.

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.

These papers have recently been accepted in the scientific press, describing the results of previously Defra funded work on the detection of TB in badger faeces:

King Hayley C., Andrew Murphy, Phillip James, Emma Travis, David Porter, Jason Sawyer, Jennifer Cork, Richard J. Delahay, William Gaze, Orin Courtenay, Elizabeth M. Wellington. The environmental reservoir of *Mycobacterium bovis* shed by wild European badgers. (Scientific Reports)

King Hayley C., Andrew Murphy, Phillip James, Emma Travis, David Porter, Jason Sawyer, Jennifer Cork, Richard J. Delahay, William Gaze, Orin Courtenay, Elizabeth M. Wellington. Performance of a Non-Invasive Test for Detecting *Mycobacterium bovis* Shedding in European Badger (*Meles meles*) Populations (Journal of Clinical Microbiology)