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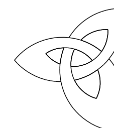

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Project identification

1. Defra Project code: SE3271
2. Project title: Development and field validation of a rapid immunomagnetic separation-lateral flow (IMS-LF) test for detecting *Mycobacterium bovis* infection in badgers and/or badger setts.
3. Contractor organisation(s): Queen's University Belfast
   School of Biological Sciences
   97 Lisburn Road
   Belfast BT9 7BL
4. Total Defra project costs (agreed fixed price): £ 316,855.00
5. Project: start date .... 1 January 2012
   end date .... 30 June 2013

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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.

This project addressed the Defra research requirement in relation to development of a rapid method for identifying *Mycobacterium bovis* (the causative agent of bovine Tuberculosis) infection in badgers, or identifying setts which contain infected badgers. The aim of this project was to develop a rapid diagnostic assay which could be used in the field and which did not involve capture of the animals (i.e. was a non-invasive test). Infected badgers are known to excrete *M. bovis* in their faeces so we proposed to develop an immunochromatographic-based, lateral flow assay and combine this with immunomagnetic separation (IMS) to detect *M. bovis* in badger faeces samples.

Phase 1 of the project was concerned with producing a prototype lateral flow device (LFD, a test format not dissimilar to a pregnancy test) incorporating *M. bovis* antibodies or peptides produced during a previous Defra funded project (SE3262) at Queen's University Belfast (QUB) that was both sensitive (able to detect low numbers of *M. bovis* added to badger faeces) and specific (would only give a positive result when *M. bovis* was present). Working in conjunction with Forsite Diagnostics Limited, York, the binders produced previously were assessed for their suitability for use as the capture and/or detector reagents. The capture and detector binder are usually the same binder, however, they can be different. Maximum sensitivity and specificity was achieved when two different binders were used in the LFD format: a polyclonal antibody produced to *M. bovis* as the capture reagent on the test (T) line and an *M. bovis*-specific IgG monoclonal antibody as the detector reagent. Various LFD membranes were also evaluated before the prototype LFDs were finally produced. The prototype LFD was found to be specific for *M. bovis* and had a limit of detection of 4.1×10^4 *M. bovis* cells per ml in buffer. Subsequently, the novel LFD test was combined with an immunomagnetic separation (IMS) technique, to be used as an IMS-LFD assay to test for presence of *M. bovis* in badger faeces samples. IMS permits selective capture and concentration of the target bacterium from dirty, complex test samples such as badger faeces that contain many other bacteria, and the LFD permits rapid and specific confirmation of the presence of *M. bovis* captured on the beads. An IMS method, developed previously at QUB, was adapted to allow the paramagnetic beads with *M. bovis* attached to move along the LFD. Optimisation of the field IMS-LFD test included developing a suitable sample preparation technique which was rapid and simple with minimal equipment that could be performed in the field. This involved investigations of faeces matrix effects, dilution factors, bead volumes and bead retrieval times required for optimal capture of *M. bovis*. By the end of phase 1 of the project an *M. bovis*-specific IMS-LFD assay had been developed, which possessed sufficient detection sensitivity to detect *M. bovis* in badger faeces, and a standardised protocol for its application in the field had been finalised.

In Phase 2 of the project the novel IMS-LFD assay was evaluated in the field to determine its ability to detect *M. bovis* infection in badger faeces. A field sampling officer was appointed and trained in application of the field IMS-LFD procedure, locating badger setts and identifying badger faeces in the field. A total of 441 badger faeces samples were collected from latrines at 110 badger setts throughout Northern Ireland (NI) between August and December 2012. GPS coordinates were recorded at each collection site to enable retrospective association with the bovine tuberculosis prevalence in cattle herds within a 5 km radius between 2008 and 2011 (information kindly supplied by Department of Agriculture for Northern Ireland). Faeces samples were processed in the field and approximately 6-8 ml of clarified, faecal homogenate subjected to the IMS-LFD test. Residual faecal homogenate was taken back to the laboratory where 1 ml was subjected to automated IMS (AIMS) and then the bead sample was split between MGIT broth culture and real-time qPCR. This allowed comparison of the results obtained with the IMS-LFD field test with those obtained with established methods for isolation (MGIT culture) and detection (qPCR) of *M. bovis*. Evidence of *M. bovis* contamination was obtained for 78 (18%), 61 (14%) and 140 (32%) of 441 NI badger faeces samples by IMS-LFD, AIMS-MGIT culture and AIMS-qPCR tests, respectively. The AIMS-qPCR test had greater detection sensitivity than the IMS-LFD test, which was not unexpected given the respective limits of detection of the two tests (1.7×10^4 and 2.8×10^5 CFU *M. bovis*/ml faeces homogenate). In essence, the field IMS-LFD test is able to detect high *M. bovis* shedding badgers, and whilst this is clearly a limitation of the newly developed test, it does not mean that the test could not find potential application as a surveillance tool to detect highly shedding badgers in the field.

This research project was primarily carried out using Northern Ireland badger faeces. However, given that badger ecology in NI differs from the Great Britain (GB) mainland in a number of respects (landscape, hydrology, land use, field size and topography), in order to ensure that the newly developed IMS-LFD test was equally applicable in the GB context, 100 faeces samples from badgers in the Woodchester Park study area, of known *M. bovis* infection status (i.e. prior TB test results for the badgers available), were obtained from the Food and Environment Research Agency (FERA) for IMS-LFD testing at QUB. Additionally, 30 faeces samples from TB-free badgers were obtained from Animal Health Veterinary Laboratory Agency (AHVLA), Weybridge, to enable a further check on IMS-LFD specificity for *M. bovis*. All 130 GB badger faeces samples were subjected to the field IMS-LFD test and the two laboratory tests (AIMS-MGIT culture and AIMS-qPCR) at QUB. For the 100 samples obtained from Woodchester Park that had associated prior *M. bovis* test history, the field IMS-LFD tested positive in 10% of cases whilst AIMS-qPCR and AIMS-MGIT culture tested positive in 56% and 41% of samples, respectively. The IMS-LFD test had high specificity relative to either AIMS-qPCR (Sp = 0.9545) or AIMS-MGIT culture (Sp=0.915), however sensitivity relative to both lab-based tests was low (Se of 0.1429 and 0.122 for AIMS-qPCR and AIMS-MGIT culture, respectively), as was found for the NI badger faeces results. LFD readings for the GB IMS-LFD positive samples were lower than obtained for the NI IMS-LFD positive badger faeces samples, suggesting that numbers of *M. bovis* being shed by the GB badgers was lower than by the NI badgers. Distinct differences in terms of faecal consistency and composition were also noted between GB and NI badger faeces samples by the personnel carrying out the testing at QUB. Statistical analysis indicated that there was no association between the results of the field IMS-LFD test and any of the contemporaneous or previous tests carried out by FERA (StatPak, Gamma interferon and culture). When the *M. bovis* infection status of the badger indicated by the FERA tests was taken into consideration, the AIMS-qPCR test was the best of the three tests in terms of diagnostic sensitivity and positive and negative predictive values, whereas the field IMS-LFD test had greatest diagnostic specificity but a very low diagnostic sensitivity. However, it should be remembered that the FERA tests were principally blood tests measuring immune response, whereas the QUB tests were carried out on badger faeces and were measuring potential excretion of *M. bovis* cells. QUB test results for the 30 truly *M. bovis* negative GB badger faeces samples provided by AHVLA, Weybridge, indicated that all samples were field IMS-LFD negative and also tested negative via AIMS-qPCR and AIMS-MGIT culture. Hence, no false positives were obtained with the field IMS-LFD test if *M. bovis* infection was not present, which once again confirmed the specificity of the novel field IMS-LFD test.

The main outcomes of the project are: a newly developed IMS-LFD test specific for *M. bovis*; a standardised protocol for application of the IMS-LFD test in the field to test for the presence of *M. bovis* in badger faeces; statistical analysis of the IMS-LFD results to determine how well the new field test performed relative to existing lab-based methods of detecting *M. bovis* in the NI and GB contexts; and new information on the location of TB infected badger setts in NI, identified as such in course of this study.

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:
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- 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).

Development and field validation of a rapid immunomagnetic separation-lateral flow (IMS-LF) test for detecting *Mycobacterium bovis* infection in badgers and/or badger setts.

The aim of the project was to develop a rapid diagnostic assay for the detection of *Mycobacterium bovis* infection in badgers which could be used in the field and which did not involve capture of the animals (i.e. was non-invasive test). Badgers are known to excrete *M. bovis* in their faeces so we proposed to develop an immunochromatographic based assay and combine this with immunomagnetic separation (IMS) to detect *M .bovis* in badger faeces samples.

Specific objectives were:
1. To produce a prototype lateral flow device (LFD), incorporating either a monoclonal antibody or specific recombinant peptides, capable of sensitively detecting *M. bovis* (Forsite and QUB).
2. To combine *M. bovis*-specific immunomagnetic separation (IMS) and the LFD to produce a novel IMS-LFD assay capable of detecting low numbers of *M. bovis* cells in badger faeces (QUB and Forsite).
3. To compare performance of the new IMS-LFD test applied in the field to test badger faeces and latrine samples for evidence of *M. bovis* infection with results of parallel laboratory testing using IMS in conjunction with MGIT culture and PCR. (QUB only)
4. To verify that the new IMS-LFD test is applicable to badger faeces samples from England and Wales. (QUB and FERA)

Objective 1 - To produce a prototype lateral flow device, incorporating either a monoclonal antibody or specific recombinant peptides, capable of sensitively detecting *Mycobacterium bovis* (QUB and sub-contractor Forsite Diagnostics Limited, York)**.**

The first part of the project involved the production of a prototype lateral flow device (LFD) which would sensitively and specificially detect *M. bovis*. The LFDs were produced by Forsite Diagnostics Limited (Sub-contractor) and incorporated binders supplied by QUB which had been produced as part of DEFRA project SE3262. LFDs are immunochromatographic assays which are easy to use, cheap to produce, and provide a rapid result (within 15 min) so can be readily used in the field. The basic requirement of any immunoassay for detection of a bacterial target is the availability of high-affinity, target-specific binders. As part of a recently funded DEFRA project QUB had produced a panel of polyclonal and monoclonal antibodies and phage-display derived peptides using gamma irradiated *M. bovis* cells as the immunogen which had been evaluated for binding to *M. bovis* and cross-reactivity with other *Mycobacterium species*. Cross-reactivity studies indicated that the binders were specific for *M. bovis*. The best *M. bovis* binders in terms of specificity and sensitivity were selected with further studies identifying the optimal combination of the ligands required to achieve maximal capture of *M. bovis* cells from bovine tissue samples by immunomagnetic separation (IMS, Stewart et al., 2012) which was subsequently found to improve detection of *M. bovis* from bovine lymph node tissue when compared to culture (Stewart et al. 2013). IMS served to selectively capture and concentrate the bacterial target from the matrix providing a sample preparation technique which negated the need for the harsh decontamination steps required for mycobacterial culture and effectively cleaned up the sample. IMS has the advantage of either being performed manually, in which case the sample volumes can be varied simply by using the appropriate magnetic rack, or automated IMS which uses a preset 1 ml volume of sample. The aim of this study was to assess if these binders could be used to produce an immunochromatographic assay which would sensitively and specficically detect *M. bovis*. A sample preparation technique would then be developed incorporating IMS which would selectively capture and isolate *M. bovis* from badger faeces samples. Combining the IMS with the LFD would produce a rapid

diagnostic assay for the capture, concentration and identification of *M. bovis* from badger faeces which is in a easy to use format and that can be performed in the field.

Milestone 1. Production of prototype lateral flow devices by Forsite Diagnostics Limited (Sub-contractor) using monoclonal antibodies or phage-derived peptides supplied by QUB.

Production of stable antibody-gold nanoparticle conjugate (detector reagent). Initial studies indicated that it was not possible to achieve a stable gold nano-particle conjugate with any of the IgM antibodies. It was thought that this may be as a result of the 'sticky' characteristic of IgMs that may result in one IgM molecule being adsorbed onto different nanoparticles simultaneously causing their aggregation. Consequently, alternative binders were investigated. An anti-*M. bovis* IgG monoclonal antibody (Mab IgG) and an anti-*M. bovis* polyclonal antibody (Pab), were successfully conjugated to the gold nanoparticles and the final conjugates (Mab-IgG-Gold and Pab-Gold) were found to be stable.
Selection of nitrocellulose membrane for LFD production. Different nitrocellulose membranes were investigated for immobilising the binders. The potential binders (Mab 11G3, the N-terminal amidated peptide EEAR302 and the Pab R43) were immobilised on the Test (T) Line position on the membranes and a cocktail of commercial anti-mouse-IgM IgG, anti-mouse-IgG IgG and anti-rabbit-IgG IgG was immobilized in the Control (C) Line positions. A two-step wet assay was performed to evaluate the recognition of *M. bovis* whole cells using the different membranes and the gold conjugates (Mab IgG-Gold and Pab-Gold) previously prepared. The results showed that the gold conjugates (Mab-IgG-Gold and Pab-Gold) were able to detect *M. bovis* whole cells when the polyclonal antibody R43 was immobilized at the T-line, with more intense T-lines obtained when Mab-IgG-Gold was used as detector reagent. Two membranes CN140 and HF135 were found to produce the most consistent and most intense T lines.

Milestone 1b. Evaluation of the different prototype lateral flow devices for ability to detect *M. bovis* cells in spiked broth/buffer samples and directly from badger faeces samples.

Specificity and sensitivity of LFD. Preliminary investigations showed that when Pab was immobilized on the T-Line as the capture reagent and Mab-IgG-gold conjugate was used as the detector reagent this assay was able to detect *M. bovis* and there was no cross-reactivity detected with *Mycobacterium avium* subsp. *avium*. The results demonstrated that for the production of an LFD for the detection of *M. bovis* whole cells the optimal combination was found to be membrane CN140 (membrane 1), with Pab R43 immobilised at the T-Line and Mab-IgG used in the gold conjugate. These components were therefore employed to produce complete LFD prototypes. Prototype LFDs were also produced using membrane HF135 (membrane 2) with the Pab immobilised on the T-Line and Mab-IgG-gold conjugate, and both prototype devices were sent to Queen's University Belfast for further evaluation.
Cross-reactivity of LFDs. Thirteen different strains of *Mycobacterium spp*. suspended in PBS: *M. bovis* BCG NCTC 5692, *M. avium* subsp. *avium* NCTC 13034, *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. fortuitum* NCTC 10394, *M. intracellulare* NCTC 10425, *M. kansasii* NCTC 10268, *M. xenopi NCTC* 10042, *M. terrae* NCTC 10856, *M. scrofulaceum* NCTC 10803, *M. marinum* NCTC 2275, *M. smegmatis* mc^2 155, *M. gordonae* NCTC 10267 and a field isolate of *M. hiberniae* were assessed using the LFDs. Using membrane 1 positive T lines were obtained with cell suspensions containing high numbers (10^6 CFU/ml) of *M. bovis* BCG, *M. kansasii* NCTC 10268, *M. marinum* NCTC 2275 and *M. gordonae* NCTC 10267. Using membrane 2 only *M. bovis* BCG gave a positive reaction on the LFD (Figure 1A and B). Subsequently, we supplied membrane 1 LFDs to AFBI-VSD, Stormont, who tested them with *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv. Colonies of each organism were emulsified in PBS and 80 µl tested on the LFD. The results showed that *M. bovis* was positive and *M. tuberculosis* was negative indicating that the LFD (Figure 1C) was potentially capable of differentiating between these two closely related species. This is a significant finding as zoonotic TB is indistinguishable clinically or pathologically from TB caused by *M. tuberculosis,* and with a 99% similarity at the genome level and identical 16S rRNA sequences differentiation on a molecular basis is difficult and requires sophisticated laboratory methods. LFD devices are available which can differentiate *M. tuberculosis* complex (MTBC) organisms from non-MTBC species but this is the first report of an LFD able to differentiate between two MTBC species. Other species within the MTB complex were not available to test.

In order to confirm that the LFD was not capable of detecting just *M. bovis* AF2122/97, which is spoligotype 140, six other spoligotypes of *M. bovis* commonly isolated in N. Ireland were tested on the LFD. The results (Figure 1D) showed that all six spoligotypes were detectable using the prototype membrane 1 LFDs.

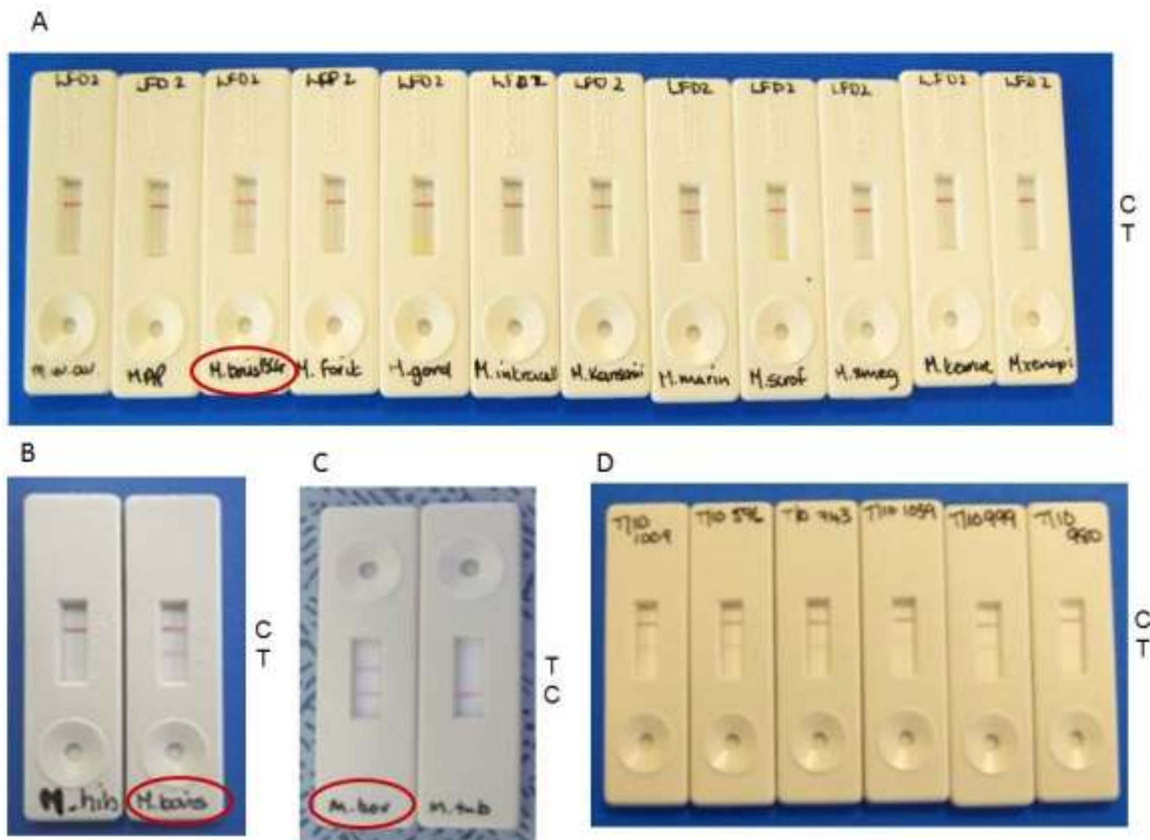

Figure 1. Specificity of membrane 1 prototype LFD for *M. bovis* confirmed by testing concentrated suspensions (ca. 10^6 CFU/ml) of a range of *Mycobacterium* species (A, B and C) and six different *M. bovis* spoligotypes (D). Only *M. bovis* and *M. bovis* BCG ever gave rise to a positive T line.

Limit of detection of LFD. The limit of detection ($LOD_{50\%}$) was calculated for the two LFD membranes (without prior IMS) using 10-fold serial dilutions of a stock of irradiated *M. bovis* (containing 5.3 x 10^6 CFU/ml) in PBS. Four replicate samples at each of four dilutions containing 10^5, 10^4, 10^3 and 0 CFU/ml were tested. The presence or absence of a T line was assessed visually after the sample had been run on the LFD for 10 min. The $LOD_{50\%}$ for both membranes was estimated using the generalized Spearman-Kärber $LOD_{50\%}$ calculation for 4-level spiking protocols to be 1.68 x 10^4 CFU/ml, although noticeably less intense T lines were obtained with membrane 2 LFDs.

Subsequently, a Forsite LFD reader was purchased which permitted verification of the presence of T lines and enabled a standard curve for *M. bovis* detection by the membrane 1 prototype LFD to be constructed and the lowest limit of *M. bovis* that could reliably be detected by the LFD was determined from the standard curve to be 4.1 x 10^4 CFU/ml (Figure 2).

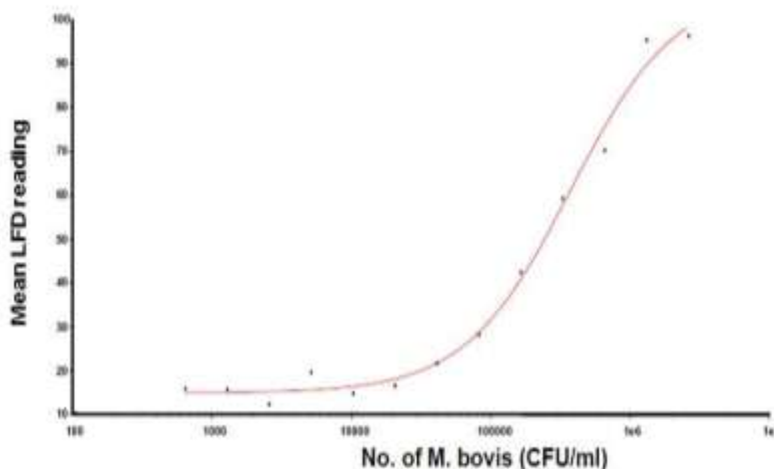

Figure 2. Standard curve generated using LFD reader for *M. bovis* detection on membrane 1 prototype LFD.

Status of Objective 1: Successfully completed with the production of a unique prototype LFD, incorporating two different *M. bovis*-specific binders; a rabbit anti-*M. bovis* polyclonal antibody as the capture reagent and a mouse anti-*M. bovis* monoclonal antibody as the detector reagent. This LFD is specific for *M. bovis* and had sufficient sensitivity to be used as a field test.

Objective 2 – To combine *M. bovis*-specific immunomagnetic separation (IMS) and LFD to produce a novel IMS-LFD assay capable of detecting low numbers of *M. bovis* cells in badger faeces.

Milestone 2. Impact of presence of magnetic beads on detection of *M. bovis* cells by lateral flow devices assessed.

Evaluation of smaller Ademtech magnetic beads. The MyOne tosylactivated Dynabeads used in the previous Defra study for immunocapture of *M. bovis* from bovine lymph node tissue are 1 µm in diameter. Preliminary observations suggested that these beads were too large to run along the LFD. Hence, there was a need to identify magnetic beads of diameter < 1 µm that would be able to pass along an LFD but still have similar capture capability for *M. bovis* to the MyOne Dynabeads. Carboxylated magnetic beads, available in three smaller sizes (200, 300 and 500 nm), were purchased from Ademtech, France, for evaluation.

Coating of beads. Ademtech beads of 200, 300 and 500 nm were coated using the antibody and the peptide separately and also using a mixture of the peptide and the antibody (dually coated). 10-fold serial dilutions (10^{-1} to 10^{-5}) of irradiated *M. bovis* AF2122/97 (OD 4.0, 10^{6}-10^{7} CFU/ml) were prepared and 1 ml of each dilution subjected to IMS using the different sized/coated Ademtech beads, suspending the beads after IMS in 100 µl Tris-EDTA (TE) buffer. After extraction of DNA the samples were analysed using *M. bovis* touchdown PCR and visualised using agarose gel electrophoresis. As controls, and to enable comparison of detection sensitivities achievable, MyOne Tosylactivated Dynabeads previously evaluated and a dilution series of irradiated *M. bovis* AF2122 in TE buffer not subjected to IMS (= Before IMS) were included. The results showed that, like the MyOne Tosylactivated beads, the dually coated Ademtech beads (i.e. using both the peptide and the antibody) produced the best capture results. The dually coated 300 nm beads and the dually coated 200 nm beads produced similar capture capability.

***M. bovis* capture capability of dually coated 200 and 300 nm Ademtech beads**. The range of *M. bovis* dilutions in PBS for IMS was extended down to 10^{-7} and the experiment repeated using the dually coated 200 and 300 nm beads. PCR products were visible down to 10^{-6} dilution using 300 nm dually coated beads and 10^{-5} dilution using 200 nm dually coated beads.

Assessment of matrix effect on capture of *M. bovis* by coated beads. A 1:5 dilution of autoclaved bovine faeces was prepared in sterile distilled water, spun at 300g for 3 min to sediment debris and supernatant aliquoted into 900 µl amounts and spiked with 100 µl of dilutions of *M. bovis* AF2122/97. The original MyOne Dynabeads and samples diluted in TE buffer with no IMS were used as controls. The results showed that there was no significant effect of the faeces matrix on recovery of *M. bovis* by either of the alternative beads. It was concluded that both the smaller diameter magnetic beads (200 and 300 nm) showed good recovery of *M. bovis* from both buffer and spiked faeces and that either could be used with the LFD. The smaller beads had to be used in conjunction with the LFD, however, it was decided, in order to maximise the recovery of *M. bovis* during the culture and PCR based comparative studies undertaken when the faeces samples were returned to the laboratory, that the original MyOne Dynabeads would be used.

Specificity of coated Ademtech beads. Specificity of the 200 and 300 nm coated beads was assessed by IMS experiments involving the following range of *Mycobacterium spp.*: *M. bovis* BCG NCTC 5692, *M. avium* subsp. *avium* NCTC 13034, *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. fortuitum* NCTC 10394, *M. intracellulare* NCTC 10425, *M. kansasii* NCTC 10268, *M. xenopi* NCTC 10042, *M. terrae* NCTC 10856, *M. scrofulaceum* NCTC 10803, *M. marinum* NCTC 2275, *M. smegmatis* mc2 155, and *M. gordonae* NCTC 10267. Stationary phase broth cultures of each species were diluted to 10^{3}-10^{4} CFU per ml in Middlebrook 7H9/OADC broth. 100 µl of the dilutions was spread onto Middlebrook 7H10/OADC agar plates to determine accurate CFU/ml before IMS and 1 ml of each dilution was then subjected to automated IMS using each of the coated beads. Following IMS, beads were resuspended in 1 ml Middlebrook 7H9/OADC broth and 100 µl spread onto Middlebrook 7H10/OADC agar plates and incubated for an appropriate time at an appropriate temperature, dependant on *Mycobacterium sp*. Experiments were carried out in duplicate and colonies after IMS were counted and the mean count expressed as a percentage of the number of CFU present in the original suspension to indicate the degree of non-specific binding by *Mycobacterium spp*. other than *M. bovis*. The results showed non-specific binding of <3% for both beads with all the mycobacteria tested.

Milestone 3. An IMS-LFD protocol for application in the field finalised.

A number of factors in relation to application of IMS in the field needed to be considered before arriving at a finalised IMS-LFD protocol:

Volume of diluent needed to homogenise faeces sample. Autoclaved cattle faeces was used for preliminary studies. 5 g faeces was weighed and placed into a BagPage filter bag (pore size 280 µm,

Lynchwood Diagnostics Ltd, Peterborough). 5 ml, 10 ml or 15 ml of sterile water was added and the bag sealed and rollered. The supernatant was poured off and assessed for suitability for IMS. The results showed that 15 ml sterile water was needed to homogenise the faeces sample to produce a useable suspension for IMS.

Matrix effect on bead retrieval (a) Time needed to retrieve beads. 50 µl beads was added to 10 ml of the prepared diluted faeces sample and put onto the magnet for 10 min. A 1:10 dilution of the sample was also made and 50 µl of beads added to this and put onto the magnet and the time noted for maximum number of beads to be recovered. Results showed that beads could be retrieved from both the diluted and undiluted samples, but in the undiluted samples 10 min was needed on the magnet to recover the beads.

(b) Dilution factor required. Cattle faeces samples (5 g) were spiked with 10^{-1} dilution *M. bovis* AF2122/97 (1 ml) mixed and added to the BagPage filter bag. 15 ml of PBS was added and the bag rollered. The supernatant was removed and 50 µl beads added to this neat sample and 1:10 dilution of this sample was made and 50 µl beads added to this. Samples were incubated on the bench for 30 min and put on the magnet for 10 min. They were then washed twice with 5 ml PBS-T and resuspended in 100 µl TE buffer and checked by PCR. The results showed that the neat samples worked just as well as the diluted samples, so no need for dilution after rollering.

Quantity of beads required. To determine the optimal quantity of beads required cattle faeces samples were spiked with 1 ml of 10^{-1} dilution *M. bovis* AF2122/97 and prepared as before. 10 µl, 20 µl and 50 µl beads were added to each sample. IMS was performed as before. Results showed that 20 µl beads per 10 ml sample was optimal bead concentration. It would also be more economical to use the lowest volume of beads per sample tested.

Preliminary trials with badger faeces. We found that the filter bags did not work very well with the badger samples as the samples were of a more formed consistency, were less digested and were 'grainy' when homogenised. The filter bag was not optimal for removing unwanted sample constituents (probably because pore size was too large) resulting in matrix interference when retrieving the beads during IMS. We investigated using cell strainers with smaller pore sizes to filter the samples after initial dilution and homogenisation by vigorous shaking. BD Falcon nylon cell strainers with 70 µm and 100 µm pore sizes which fitted neatly into a 50 ml centrifuge tube were tested. We reduced the volume of sample tested to 1 g and adjusted the dilution factor by adding 9 ml PBS. The sample was shaken vigorously and then strained into a 50 ml centrifuge tube. This method worked well and removed more of the gross particulates from the badger faeces samples than the BagPage filter bag had been able to.

Effect of nylon strainers. To determine if the nylon strainers had any adverse effects on the recovery of *M. bovis* (i.e. by retaining cells rather than allowing them to pass through) badger faeces samples were spiked with dilutions of *M. bovis* and subjected to the revised field sample preparation technique. The samples were then subjected to automated IMS and field IMS and prepared for PCR. qPCR showed that there was no adverse effect of the nylon strainers on the recovery of *M. bovis* after both field IMS or automated IMS.

Combining optimised field IMS with the LFD. After field IMS the beads were resuspended in 200 µl PBS for analysis using the LFDs. However, it was found that uncoated beads, coated beads and the storage buffer recommended by Ademtech all produced a false positive T line on the LFD after IMS. To resolve this issue an extra step of quenching the activated unused carboxyl groups present on the bead surface using ethanolamine was introduced during coating of the beads, and various blocking solutions were investigated as alternatives to the Ademtech storage buffer for resuspending the coated beads in. Resuspension of beads after IMS in KPL Detector™ block, a commercially available blocking solution (KPL Inc., Gaithersburg, Maryland), was found to consistently produce negative T lines on the LFDs when *M. bovis* was not present.

Earlier results had indicated that PBS suspensions of three *Mycobacterium spp.* gave weak false positive T lines when applied directly to the LFD. However, when IMS was subsequently applied to suspensions of these mycobacterial species and beads resuspended in KPL buffer, negative T lines were obtained, i.e. the IMS-LFD assay was demonstrated to be specific for *M. bovis* only.

Limit of detection of field IMS-LFD. The $LOD_{50\%}$ determination for the field IMS-LFD was performed twice using two different *M. bovis* negative faeces samples as spiking matrix and using membrane 1 LFDs. Four replicate samples were spiked at four cell concentrations (10^5, 10^4, 10^3 and 0 CFU/ml) before IMS-LFD assay. The presence or absence of T lines was assessed visually. The 50% limit of detection (LOD_{50}) was estimated using the generalized Spearman-Kärber LOD_{50} calculation for 4-level spiking protocols. This was determined to be 2.79 x 10^5 CFU/ml faeces homogenate (1:10 dilution), so the detection sensitivity of the LFD was reduced when IMS preceded LFD detection, and faeces homogenates rather than PBS suspensions were being tested.

Finalised protocol for field IMS-LFD assay:

(1) Approximately 1 g of badger faeces is added to 9 ml PBS and the sample shaken vigorously by hand.
(2) When homogenous, the sample is filtered through a Falcon 70 µm cell strainer into a 50 ml centrifuge tube.
(3) 6-8 ml of the homogenised, clarified faeces sample is poured into a tube containing 20 µl of dually coated Ademtech 300 nm beads. The sample is incubated at ambient temperature for 30 min with shaking every 5 min.

(4) The tube is then placed in a magnetic rack for 10 min before sample is carefully poured off.
(5) Bead samples are washed three times using approx. 5 ml PBS-0.05% Tween 20.
(6) After the final wash the beads are resupended in 200 µl KPL Detector™ block solution and 80 µl added to the LFD.
(7) After 15 min record IMS-LFD result as positive if both C and T lines are visible or negative if only C line is observed and take digital photograph.
(8) The residual portion of each homogenised, clarified faeces sample is returned to the lab at QUB for automated IMS (AIMS) followed by RT-PCR and MGIT culture.

The field IMS-LFD procedure is summarised schematically in Figure 3.

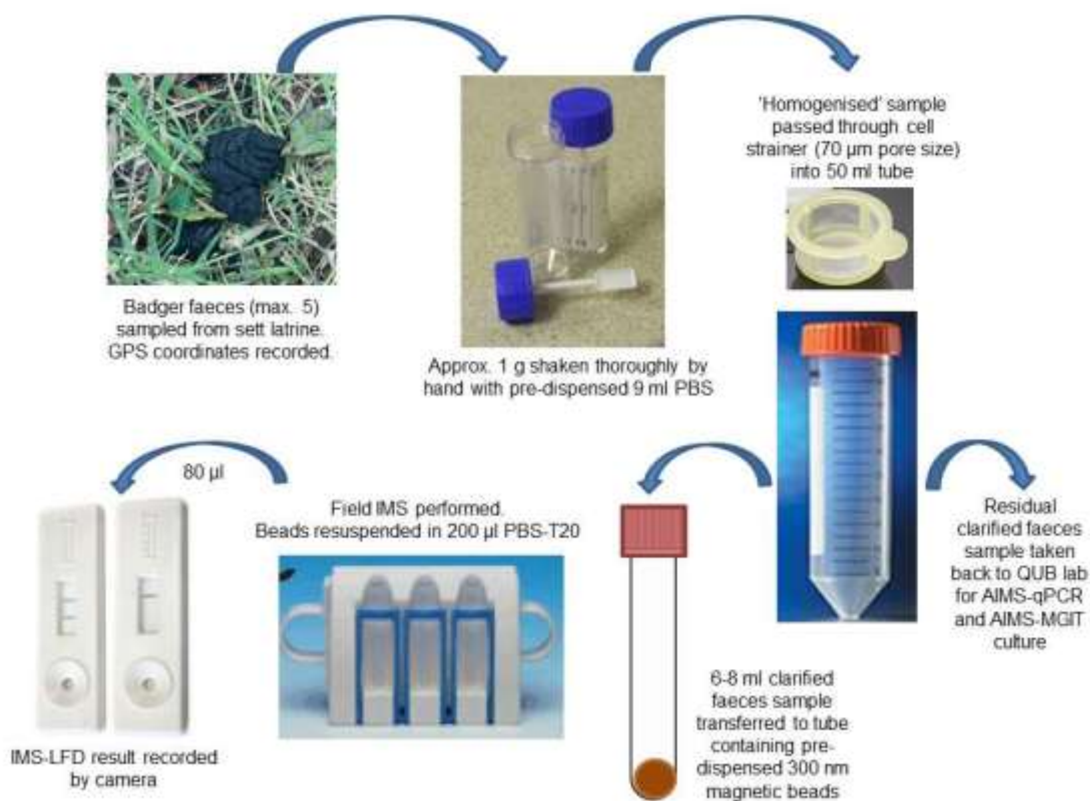

Fig. 3. Schematic of field IMS-LFD procedure.

Status of Objective 2: Successfully completed after resolving all issues encountered in relation to combining *M. bovis* IMS with *M. bovis* LFD. The IMS step removed faecal constituents and concentrated the cells which then enabled analysis of the sample using the LFD. The IMS-LFD test was, therefore, ready for evaluation in the field to detect *M. bovis* infection in badger faeces.

Objective 3 - Compare performance of the new IMS-LFD test applied in the field to test badger faeces and latrine samples for evidence of *M. bovis* infection with results of parallel laboratory testing using IMS in conjunction with MGIT culture and PCR (QUB only)

Milestone 4. Field sampling officer trained in application of new IMS-LFD test. A field sampling officer was appointed and commenced work on 31 July 2012. He was trained in application of the field IMS procedure and in locating badger setts and identifying badger faeces in the field.

Milestone 5. Survey of TB infected and non-infected Northern Ireland badger setts in order to validate new IMS-LFD test relative to existing laboratory methods of detecting *M. bovis*.

Selection of badger setts for sampling visits. Information on the bTB status of cattle herds near badger sett locations identified in the 2007/08 NI badger survey (Reid et al. 2011) was not obtained from the Department of Agriculture and Rural Development for Northern Ireland (DARD) until mid-October 2012, so we were unable to select sett sampling locations on basis of being TB infected or non-infected as originally anticipated. Instead, using maps generated during the 2007/08 NI badger survey (Reid et al. 2011), 1-km squares containing main badger setts were randomly selected to be visited. Badger faeces sample collection commenced in early August 2012 and ended in early December 2012. Sett and faeces sample locations were recorded using a handheld GPS, and 10-figure grid reference was recorded for each. To

ensure that all setts, latrines and individual deposits were located within each survey square, all linear features such as hedgerows, ditches, stone walls and habitat boundaries were walked. Latrines and individual faecal deposits were located along tracks leading away from setts and at crossing points e.g. gates, gaps in hedges between fields and stream crossings.

Faeces sampling at badger setts. Wherever possible fresh faecal material was sampled from the sett latrine and a maximum of five faeces samples were collected per independent sett to be processed for field IMS-LFD assay. Due to the fact that a working LFD only became available in mid-September 2012, faeces samples collected prior to this time (n= 232) were processed by the field IMS procedure in the laboratory and resulting bead suspensions stored at 4°C until LFDs became available, at which point all bead suspensions were applied to LFDs and IMS-LFD result recorded. From 1 October 2012 the full IMS-LFD assay was applied to all badger faeces samples in the field. Presence or absence of a T line on the LFD was assessed visually after 15 min incubation of bead sample on LFD and result recorded photographically. The LFDs were retained and returned to the laboratory and the presence of a T line subsequently verified with the LFD reader. The reader reports intensity of the T line as a percentage of C line intensity.

Parallel laboratory testing. Upon return to the laboratory at QUB, 1 ml of each homogenised, clarified badger faeces sample which had been prepared in the field was subjected to automated IMS (AIMS) with dually coated MyOne tosylactivated Dynabeads. Following IMS, the beads plus any captured *M. bovis* cells were resuspended in 500 µl Middlebrook 7H9 broth which was then split 100 µl for quantitative real-time PCR (method of Sweeney et al. 2007) and 400 µl for MGIT culture. DNA was released from bead-bound *M. bovis* cells by boiling for 25 min and then purified by Zymoclean columns before qPCR was performed using an Illumina Eco-PCR instrument. The reaction volume was 25 µl comprising: 12.5 µl of TaqMan Gene expression master mix (x2, Life Technologies), 1 µl (20 pmol/µl) forward RD4 flanking primer (5' TGTGAATTCATACAAGCCGTAGTCG 3'), 1 µl (20 pmol/µl) reverse RD4 flanking primer (5' CCCGTAGCGTTACTGAGAAATTGC 3'), 1 µl (10 pmol/µl) RD4 hydrolysis probe (5' 6-FAM–AGCGCAACACTCTTGGAGTGGCCTAC–MGB 3'), 7 µl of nuclease-free sterile water and 2.5 µl of template DNA. The RD4 hydrolysis probe and qPCR primers were purchased from Life Technologies. Reaction conditions were: UDG at 50˚C for 10 min, enzyme activation at 95°C for 3 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Duplicate 2.5 µl aliquots of DNA were tested for each faeces sample. Both aliquots had to report positive or negative for a positive and negative AIMS-PCR result, respectively, to be recorded. In instances where there was disagreement between the duplicate results for any sample, the qPCR was repeated once. If disagreement between duplicate results still existed after second PCR then the sample was recorded AIMS-qPCR negative (this was the practice reported by Travis et al. 2011). A six point standard curve, generated using DNA from a dilution series (10^6–10 CFU/ml) of irradiated *M. bovis* AF2122/97, and no template control (water only) were run in duplicate in each qPCR run. The specificity of this RD4 probe-based qPCR method for *M. bovis* was checked using DNA from the following range of *Mycobacterium spp*.: *M. bovis* BCG NCTC 5692, *M. avium* subsp. *avium* NCTC 13034, *M. avium* subsp. *paratuberculosis* ATCC 19698, *M. fortuitum* NCTC 10394, *M. intracellulare* NCTC 10425, *M. kansasii* NCTC 10268, *M. xenopi* NCTC 10042, *M. terrae* NCTC 10856, *M. scrofulaceum* NCTC 10803, *M. marinum* NCTC 2275, *M. smegmatis* mc2 155, *M. gordonae* NCTC 10267, and a field isolate of *M. hiberniae*. The results showed that only *M. bovis* BCG NCTC 5692 yielded a positive qPCR result, so *M. bovis* specificity of the RD4 probe-based qPCR method employed in this study was confirmed.

The AIMS-MGIT culture protocol adopted for the badger faeces is outlined in Supplementary Figure 1 (Appendix 1). Beads after AIMS (400 µl) were initially inoculated into MGIT tubes supplemented with 10% OADC and PANTA antibiotic supplement (i.e. no chemical decontamination was employed) for samples from badger setts 1-79 (samples 1-303). However, (black) fungal contamination was observed in many of the MGIT tubes so it was later decided to decontaminate the samples after IMS for samples from badger setts 80-110 (samples 304-441). For these samples, after IMS the bead sample (400 µl) was decontaminated by transferring it to a sterile eppendorf tube and adding 400 µl 1N NaOH (4%) and leaving for 20 min. The sample was then neutralised by adding 400 µl KH_2PO_4 (14% w/v) containing phenol red (40 mg/l) and spun at 3000 x g for 20 min. The supernatant was discarded and the pellet resuspended and inoculated into the MGIT tube. The MGIT tubes were read manually over an incubation period of up to 12 weeks at 37°C. After 4 weeks incubation 100 µl of each of the MGIT cultures was removed, boiled and checked by Touchdown PCR (Zumarraga et al. 2005) targeting the IS6110 element and employing INS1 and INS2 primers to confirm the presence of *M. bovis*. Any samples which were found to be Touchdown PCR positive were decontaminated (400 µl of the sample was removed from the MGIT tube to a sterile eppendorf and decontaminated as above) and the pellet resuspended and inoculated onto Lowenstein-Jensen (LJ) slopes. Suspect colonies growing on the LJ slopes were sub-cultured onto 7H10 agar plates and all growth checked using the *M. bovis* specific LFD and real time PCR. DNA from six MGIT cultures which were considered to have the strongest positive bands following Touchdown PCR were selected and sent for spoligotyping. Spoligotyping results were not obtained for any of these samples, however, an hsp65-based mycobacterial speciation assay was positive for *M. hiberniae* in one of the samples. It was concluded that the level of *M. bovis* present in each of these samples was below the threshold number needed to obtain a spoligotyping result, rather than there being no *M. bovis* present necessarily.

If no growth occurred after 12 weeks and if the Touchdown PCR results were all negative then a negative AIMS-MGIT culture result was declared. *M. bovis* was isolated from one NI badger faeces sample

only. This sample had been LFD positive in the field, AIMS-qPCR negative, positive by Touchdown PCR at 4 weeks, but negative by Touchdown PCR at 8 weeks. Another sample was LFD positive in the field and was AIMS-qPCR negative but was Touchdown PCR positive at 4 weeks and 8 weeks and was also RT-PCR positive at 12 weeks but nothing grew on the LJ slope after decontamination. It had also been sub-cultured onto 7H10 agar but we failed to grow any colonies typical of *M. bovis*. All samples declared as 'AIMS-MGIT culture positive' in this report were MGIT cultures that tested TD- or q-PCR positive after incubation, not MGIT cultures from which colonies were isolated and successfully spoligotyped within the timeframe of the project.

In light of the fact that the LOD of the IMS-LFD assay is ~$10^{5\text{-}6}$ CFU/g faeces, a positive result with this assay should indicate the presence of sufficient *M. bovis* cells to be visible microscopically. In order to verify this, several of the residual bead suspensions that had tested *M. bovis* positive on the LFD were Ziehl-Neelsen stained. Acid-fast bacteria attached to the magnetic beads were observed, providing confirmation that high numbers of acid fast bacteria (assumed to be *M. bovis*) were indeed present when an IMS-LFD positive result was obtained.

Statistical analysis. The location of 1 km squares from which badger faeces were collected were mapped relative to the prevalence of bTB in cattle herds between 2008-11 within a 5 km buffer around each sampling square (provided by the Department of Agriculture and Rural Development or DARD). Missing spatial data for bTB prevalence in cattle were interpolated using the Kriging function in the Spatial Analyst package for ArcGIS 10 (ESRI, California, USA).

Descriptive statistics were used to clarify trends in sampling and results. Pairwise receiver operating characteristic (ROC) curves were constructed between the results of each test. A ROC curve illustrates the performance of a binary classifier (in this case each test) as its discrimination threshold is varied. It is created by plotting the fraction of true positives out of the positives (sensitivity) versus the fraction of false positives out of the negatives (specificity), at various threshold settings. The area under curve (AUC) is equal to the probability that a classifier will rank a randomly chosen positive result higher than a randomly chosen negative result. Cross-tabulation was used to populate a Venn diagram to illustrate similarity in the results obtained from each test.

Variation in the results of each technique (field IMS-LFD, AIMS qPCR and AIMS culture) were examined using Generalized Linear Mixed Models (GLMMs) assuming a binomial error structure and a logit link function were disease status (positive or negative) was fitted as the response variable. In each case, whichever test results were not the dependent variable were fitted as fixed factors to examining correlation between the results of each test. The mean prevalence of bTB in cattle within a 5km buffer of each sampling location between 2008-11 and during 2011 only were fitted as covariates but in separate models as these parameters were collinear. To account for multiple measures of disease status (i.e. multiple faecal samples from each sett) in each sampling square, the nested term "Square_ID(Sett_ID)" was fitted as a random factor. All statistics were performed using SPSS 20.

Milestone 6. Project Review Point - DEFRA to review progress and results after testing of 30 NI badger setts (approx 150 badger faeces samples).

Field testing results. A total of 441 faecal samples were collected from 110 setts within 58 x 1 km squares (Figure 4) throughout Northern Ireland. Thus, a mean of 4.0 faecal samples were collected per sett at 1.9 setts per square. The majority of squares sampled were within the County Down bTB 'hotspot' in cattle (Figure 1). Using the survey square as the unit of variance, mean prevalence was 35% (range = 0-100%) for the field IMS-LFD, 63% (range = 0-100%) for the AIMS-qPCR and 29% (range = 0-100%) for the AIMS-MGIT culture.

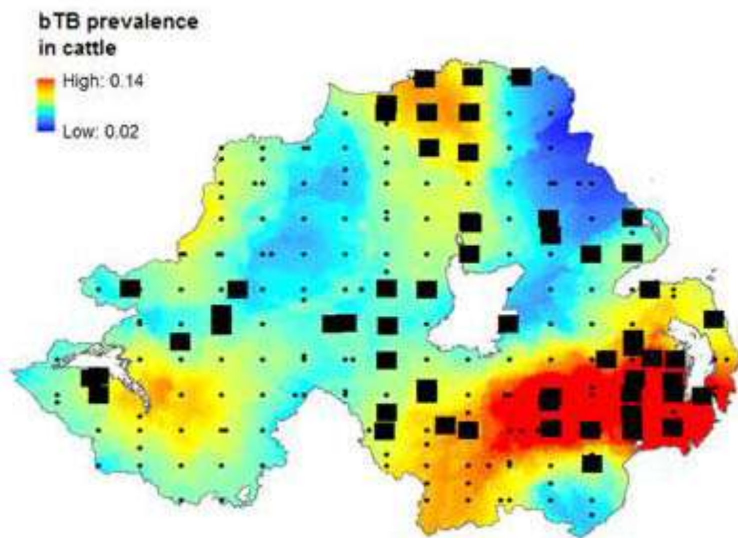

Fig. 4 Distribution of 58 x 1-km squares (black squares) in Northern Ireland from which 441 badger faeces samples were collected from 110 setts relative to the prevalence of bTB in cattle from 2008-11 (data were supplied for each dot on map by DARD and interpolated for missing areas using Spatial Kriging in ArcGIS)

When considered on an individual faeces sample basis, a total of 78 badger faeces samples (18%) tested positive with the field IMS-LFD test, with 21 (5%) also testing positive by AIMS-qPCR and 11 (2%) also testing positive by AIMS-MGIT culture. Only 3 faeces samples (1%) tested positive by all three tests (Fig. 5). A total of 140 samples (32%) tested positive using AIMS-qPCR suggesting it was the most sensitive test, whilst 64 (14%) tested positive using the AIMS-MGIT culture suggesting it was the least sensitive test.

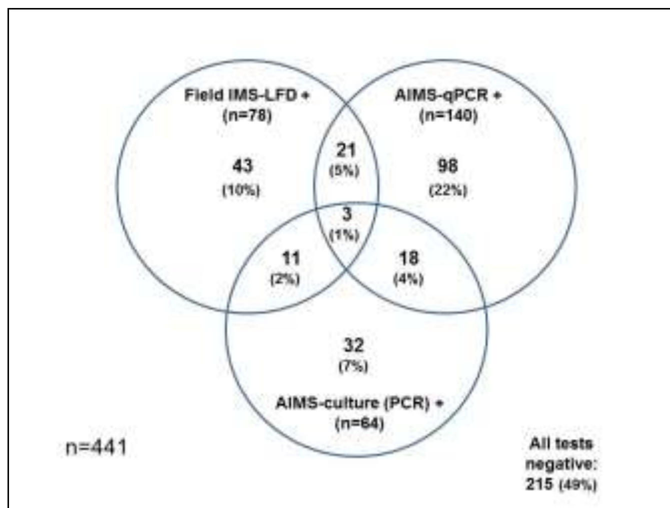

Fig. 5 Venn diagram showing the numbers of NI badger faeces samples (representing a percentage of the total *n* = 441) that tested positive by field IMS-LFD, AIMS-qPCR and AIMS-MGIT culture. Areas of overlap indicate positive test results in common.

Results were subjected to Generalised Linear Mixed Model (GLMM) analysis using SPSS 20. There was found to be no significant association between the results of any one test with the results of any other (Table 1).

Table 1. GLMM results for the prevalence of bTB in badger faeces using three different tests: **a)** field IMS-LFD, **b)** AIMS-qPCR and **c)** AIMS culture (*n*=425 unless otherwise stated) with two models each: one containing bTB prevalence data from **i)** 2008-11 and **ii)** 2011 only.

Dependent variable	Independent variables	*β* ± s.e.	*F*	df	*p*
a) Field IMS-LFD					
i) 2008-11 ($F_{df=4,420}$= 1.049, p=0.382, AUC=0.591)					
	AIMS-qPCR	0.127 ± 0.341	0.138	1,436	0.711
	AIMS-MGIT culture	0.192 ± 0.392	0.241	1,436	0.624
	Julian date	-0.364 ± 0.243	2.238	1,436	0.135
	bTB in cattle 2008-11	0.130 ± 0.192	0.461	1,436	0.498
ii) 2011 ($F_{df=4,420}$= 1.165, p=0.326, AUC=0.626)					
	AIMS-qPCR	0.066 ± 0.340	0.038	1,436	0.846
	AIMS-MGIT culture	0.091 ± 0.392	0.053	1,436	0.818
	Julian date	-0.516 ± 0.233	4.905	1,436	**0.027***
	bTB in cattle 2011	-0.361 ± 0.205	3.099	1,436	0.079
b) AIMS-qPCR					
i) 2008-11 ($F_{df=4,420}$= 4.841, p=0.001, AUC=0.729)					
	Field IMS-LFD	0.120 ± 0.336	0.127	1,436	0.722
	AIMS-MGIT culture	-0.571 ± 0.365	2.454	1,436	0.118
	Julian date	0.336 ± 0.206	2.649	1,436	0.104
	bTB in cattle 2008-11	0.070 ± 0.180	0.154	1,436	0.695
ii) 2011 ($F_{df=4,420}$= 3.359, p=0.010, AUC=0.737)					
	Field IMS-LFD	0.099 ± 0.338	0.086	1,436	0.769
	AIMS-MGIT culture	-0.573 ± 0.364	2.476	1,436	0.116
	Julian date	0.333 ± 0.204	2.683	1,436	0.102
	bTB in cattle 2011	0.078 ± 0.186	0.173	1,436	0.677
c) AIMS–MGIT culture					
i) 2008-11 ($F_{df=4,420}$= 3.562, p=0.007, AUC=0.633)					
	Field IMS-LFD	-0.058 ± 0.458	0.018	1,420	0.893
	AIMS-qPCR	-0.026 ± 0.443	0.717	1,420	0.398
	Julian date	-0.877 ± 0.261	20.273	1,420	**<0.001*** **
	bTB in cattle 2008-11	0.165 ± 0.231	3.257	1,420	0.072
ii) 2011 ($F_{df=4,420}$= 3.412, p=0.009, AUC=0.633)					
	Field IMS-LFD	-0.020 ± 0.413	0.002	1,436	0.961
	AIMS-qPCR	-0.331 ± 0.404	0.673	1,436	0.413
	Julian date	-1.507 ± 0.339	19.751	1,436	**<0.001*** **
	bTB in cattle 2011	0.383 ± 0.241	2.538	1,436	0.112

Performance of the field IMS-LFD test relative to the laboratory AIMS-qPCR and AIMS-MGIT culture tests for NI badger faeces samples.

2 x 2 contingency tables (Appendix 2) were analysed by Fisher's Exact test to calculate specificity and sensitivity of the field IMS-LFD relative to the two lab-based tests carried out at QUB (AIMS-qPCR and AIMS-MGIT culture). Kappa values were also determined. The field IMS-LFD test has specificity relative to AIMS-qPCR of 0.8206 and to AIMS-MGIT culture of 0.830. However, sensitivity relative to both lab-based tests was low (Se of 0.1714 and 0.219 for AIMS-qPCR and AIMS-MGIT culture, respectively). Kappa values indicated 'poor agreement' between results of field IMS-LFD test and the other two tests.

The ratio of false positives to true positives derived from the Area Under the Curve (AUC value) was approximately 0.5 when testing any one test with any other (Table 2). Thus, the results from any one test were no better than random chance in predicting the results of any other.

Table 2 Area Under the Curve (AUC) values for Receiver Operating Characteristic (ROC) curves testing the binomial result (positive/negative) from each test against the results from the other tests.

	Field IMS-LFD	AIMS-qPCR	AIMS-MGIT culture
Field IMS-LFD	-	0.494	0.521
AIMS-qPCR	0.496	-	0.504
AIMS-MGIT culture	0.524	0.506	-
MEAN	**0.510**	**0.500**	**0.513**

There was a negative relationship (p=0.027) between positive test results with the field IMS-LFD test and Julian date (sampling time) when fitted with the bTB prevalence data for 2011 only, and also for AIMS-MGIT culture regardless of which bTB prevalence data were fitted simultaneously (p<0.001 in both cases, Table 1). No such relationship existed for the AIMS-qPCR test. The frequency of positive results with each the three IMS-based tests performed at QUB relative to sampling date is illustrated in Figure 6. Spatiotemporal bias in sampling throughout the study was ruled out as a potential explanation. The negative relationship between AIMS-MGIT culture result and sampling (Julian) date may be a reflection of the introduction of chemical decontamination prior to culture from sample 304 onwards leading to less positives due to adverse effects of the decontaminant on viability of low numbers of *M. bovis*, rather than more culture positives due to less competition from background microflora. There was no significant relationship between positive results of any QUB test and the prevalence of bTB in cattle herds within a 5 km buffer of the sampling location either from 2008-11 or 2011 only.

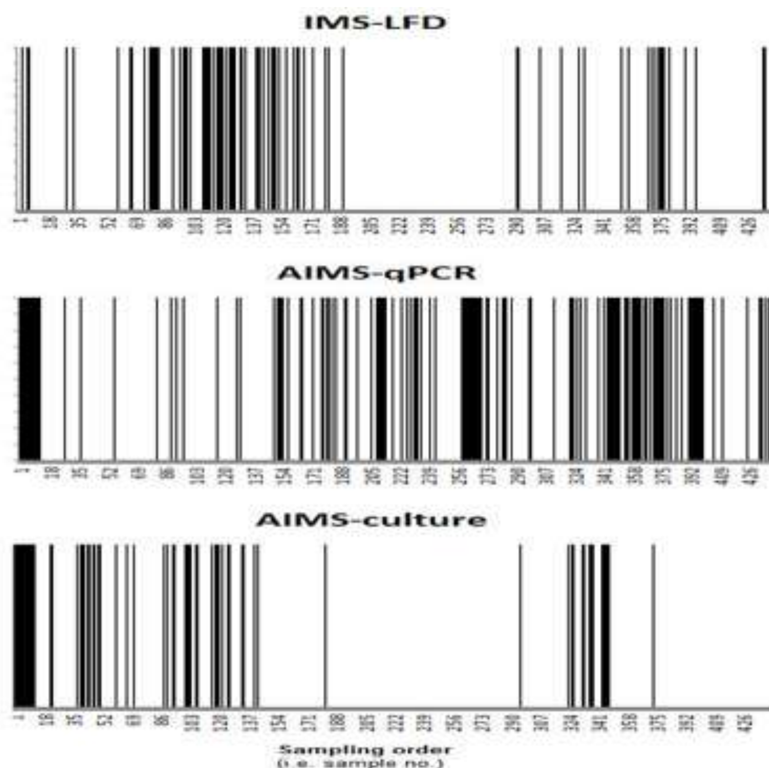

Fig. 6 Frequency of *M. bovis* positive results (black bars) obtained with the three IMS-based tests performed at QUB on NI badger faeces in the order of sample processing (i.e. by sample number) from 3 August - 4 December 2012.

Status of Objective 3: Survey of 441 badger faeces from 110 setts completed, with IMS-LFD test detecting the presence of *M. bovis* in 18% of samples tested.

Objective 4 - To verify that the new IMS-LFD test is applicable to badger faeces samples from England and Wales. (QUB and Sub-contractor (2) FERA)

Milestone 7. Survey of badger faeces from England and Wales (supplied by FERA) completed.

Testing of faeces samples (n=100) from GB badgers of known infection status. FERA supplied faeces samples from badgers in the Woodchester Park study area of known infection status, for which results were

available for StatPak, tissue/faecal culture and Gamma interferon tests. A total of 100 samples were tested using the same protocol as used for the N. Ireland badger faeces samples, i.e. field IMS-LFD, AIMS-qPCR, and AIMS-MGIT culture.

Testing of faeces samples (n=30) from *M. bovis* negative captive GB badgers. At the request of the DPAG committee, following review of the initial findings for the NI badger faeces samples on 10 October 2012, faeces samples from truly *M. bovis* negative badgers were sourced for testing. AHVLA Weybridge kindly supplied 30 faeces samples from captive badgers that had never tested positive for *M. bovis* despite repeated TB testing. These faeces samples were also tested using the same protocol as used for the N. Ireland badger faeces samples, including field IMS-LFD, AIMS-qPCR and AIMS-MGIT culture.

It was noted that almost all of the samples obtained from GB were of a different consistency than the NI badger faeces samples. This difference resulted in a cleaner, less grainy test sample from which the immunomagnetic beads (both the Ademtech and Dynabeads) were more readily and cleanly captured.

For the 100 samples obtained from Woodchester Park that had associated prior *M. bovis* test history, the field IMS-LFD tested positive in 10% of cases whilst AIMS-qPCR and AIMS-MGIT culture tested positive in 56% and 41% of samples, respectively (Figure 7), and 5% of samples tested positive by all three tests. Only two samples tested positive by the IMS-LFD test and not by either or both of the other two IMS-based tests.

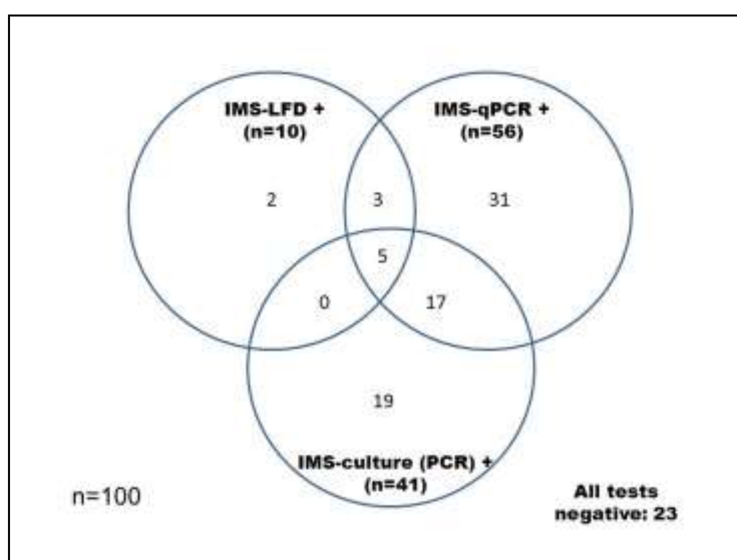

Fig. 7 Venn diagram showing the numbers of Woodchester Park badger faeces samples that tested positive by field IMS-LFD, AIMS-qPCR and AIMS-MGIT culture. Areas of overlap indicate positive test results in common.

Performance of field IMS-LFD test for testing GB badger faeces samples

2 x 2 contingency tables (Appendix 3) were analysed by Fisher's Exact test to calculate specificity and sensitivity of the field IMS-LFD relative to the two QUB lab-based tests. Kappa values were also determined. The IMS-LFD test has high specificity relative to either AIMS-qPCR (Sp = 0.9545) or AIMS-MGIT culture (Sp=0.915). However, sensitivity relative to both lab-based tests was low (Se of 0.1429 and 0.122 for AIMS-qPCR and AIMS-MGIT culture, respectively). Kappa values indicated 'poor agreement' between results of field IMS-LFD test and the other two tests.

Figure 8 illustrates the relationships between the QUB test results for the 100 Woodchester badger faeces samples and the *M. bovis* test status of the badgers. There was found to be no association between the results for the field IMS-LFD test and any of the contemporaneous or previous tests carried out at either QUB or FERA (Table 3). Indeed, some of the faeces from badgers which had never had a positive *M. bovis* test result at FERA ('None' category in Figure 8) tested positive for *M. bovis* at QUB. However, it must be remembered that the FERA tests were principally blood tests measuring immune response, whereas the QUB tests were carried out on badger faeces and were measuring potential excretion of *M. bovis* cells.

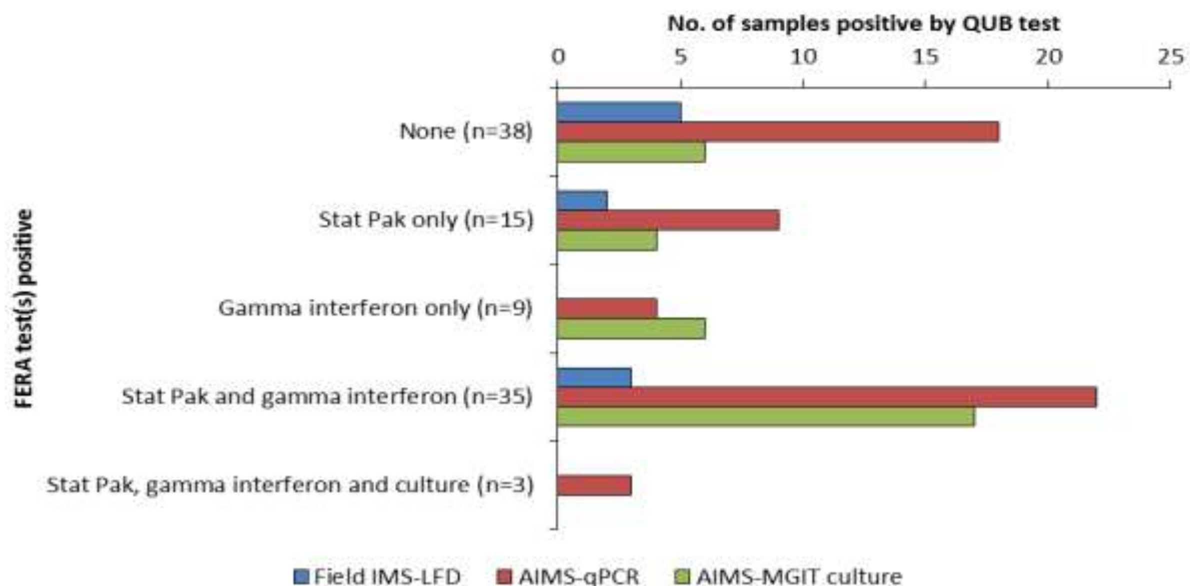

Fig. 8 Relationships between the QUB test results for 100 faeces samples from badgers in the Woodchester Park study area and the *M. bovis* test status of the badgers as indicated by results of contemporaneous or previous StatPak, culture and gamma interferon tests carried out by the Food and Environment Research Agency (FERA) .

Statistical analysis of results using Generalised Linear Mixed Model (GLMM) analysis indicated no significant association between the field IMS-LFD result and the result of any other contemporaneous or previous test, with the possible exception of AIMS-qPCR when P value (0.078) approached significance (Table 3).

Table 3 GLMM analysis of field IMS-LFD results compared to other tests for Woodchester Park badger faeces (n=100).

Dependent variable	Independent variables	*β* ± s.e.	*F*	df	*p*
Field IMS-LFD ($F_{df=8,58}$=0.687, p=0.701, AUC=0.719)					
	Julian day	0.004 ± 0.012	0.096	1,58	0.757
	AIMS-qPCR (QUB)	1.410 ± 0.786	3.214	1,58	**0.078**
	AIMS-MGIT culture (QUB)	0.561 ± 0.690	0.660	1,58	0.420
	Current StatPak (FERA)	0.054 ± 1.156	0.002	1,58	0.963
	Current culture any (FERA)	0.693 ± 1.230	0.318	1,58	0.575
	Current gamma interferon (FERA)	0.304 ± 1.187	0.066	1,58	0.799
	Previous StatPak (FERA)	-20.503 ± 43,167	0.000	1,58	1.000
	Previous culture any (FERA)	-1.468 ± 1.187	1.530	1,58	0.221
	Previous gamma interferon (FERA)	0.004 ± 0.012	0.096	1,58	0.757

Information in brackets indicates where test was performed: QUB, Queen's University Belfast; FERA, Food and Environment Research Agency, York.

The AUC values for pairwise comparisons between the field IMS-LFD and other tests were approximately 0.5 (Table 4), suggesting that the results from the field IMS-LFD test were no better than random chance at predicting the outcome of any other test.

Table 4. Area Under the Curve (AUC) values for Receiver Operating Characteristic (ROC) curves testing the binomial result (positive/negative) from the field IMS-LFD performed by Queen's University Belfast (QUB) compared with the results from the contemporaneous and previous tests carried out by QUB or the Food and Environment Research Agency (FERA).

Organisation	Timeframe	Comparator test	QUB Field IMS-LFD
QUB	Contemporaneous	AIMS-qPCR	0.549
		AIMS-MGIT culture	0.519
		Sub-mean	**0.534**
FERA	Contemporaneous	StatPak	0.494
		Culture (any)	- *
		Gamma interferon	0.481
		Sub-mean	**0.488**
	Previous	StatPak	0.494
		Culture (any)	0.448
		Gamma interferon	0.466
		Sub-mean	**0.469**
		Grand mean	**0.493**

* All samples were culture negative upon contemporaneous testing by FERA and thus no AUC value could be generated.

To permit estimation of the bTB diagnostic sensitivity and specificity, and positive and negative predictive values of the field IMS-LFD, AIMS-qPCR and AIMS-MGIT culture tests, the 100 GB badgers from which faeces samples were obtained were classed as '*M. bovis* infected' if a positive result had been obtained for StatPak, culture or gamma interferon tests on any test occasion by FERA, or 'Non-infected' if a negative result had been obtained for StatPak, culture and gamma interferon on all test occasions by FERA. 2 x 2 contingency tables (Appendix 4) were analysed by Fisher's exact test. Test characteristics are summarised in Table 5.

Table 5. Comparison of the test characteristics of field IMS-LFD, AIMS-qPCR and AIMS-MGIT culture. Values highlighted in red indicate the highest value for each characteristic. 95% confidence intervals are in indicated in parentheses.

QUB test	Diagnostic sensitivity, Sp	Diagnostic specificity, Se	Positive predictive value, PPV	Negative predictive value, NPV
Field IMS-LFD	0.0806 (0.0266-0.1781)	0.8684 (0.7195-0.9558)	0.5000 (0.1871-0.8129)	0.3667 (0.2674-0.4754)
AIMS-qPCR	0.6129 (0.4803-0.7341)	0.5263 (0.3581-0.6903)	0.6786 (0.5407-0.7974)	0.4545 (0.3039-0.6118)
AIMS-MGIT culture	0.4355 (0.3102-0.5670)	0.6316 (0.4599-0.7816)	0.6585 (0.4945-0.7991)	0.4068 (0.2809-0.5425)

The AIMS-qPCR test is clearly the best of the three tests in terms of diagnostic sensitivity and positive and negative predictive value, and the field IMS-LFD test has greatest diagnostic specificity but a very low diagnostic sensitivity. However, it appears that not all badgers in the 'None' category in Figure 9 were actually non-infected, or at least non-shedding, given that positive results were obtained with all three QUB tests for varying numbers of faeces from badgers in this category. Misclassification of the infection status of these badgers would have impacted on estimates of diagnostic test characteristics.

QUB test results for the 30 truly *M. bovis* negative GB badger faeces samples provided by AHVLA, Weybridge, indicated that all samples were field IMS-LFD negative and also tested negative via AIMS-qPCR and AIMS-MGIT culture. Hence, no false positives were obtained with the field IMS-LFD test if *M. bovis* infection was not present, which once again confirmed the specificity of the novel field IMS-LFD test.

Milestone 8. Final project report to be submitted to DEFRA by 30 June 2013.

Status of Objective 4: With submission of this final report, objective 4 has been completed successfully and on time. The novel IMS-LFD test was shown to be applicable for the testing of GB badger faeces samples.

General discussion of results and their reliability

We successfully developed a unique prototype lateral flow device (LFD) incorporating two different *M. bovis*-specific antibodies previously generated at QUB. The prototype LFD was optimised in terms of detection sensitivity and specificity for *M. bovis,* and is able to detect only *M. bovis* cells including different spoligotypes. A previously developed IMS procedure for *M. bovis* capture was successfully adapted and optimised for use in conjunction with the LFD to provide a cost-effective, easy to use, rapid IMS-LFD assay with an LOD of 10^5 *M. bovis* per ml in faeces matrix. A standardised protocol was finalised to facilitate use of the novel IMS-LFD assay in the field to detect *M. bovis* in badger faeces samples.

The performance of the IMS-LFD in the field was evaluated relative to two laboratory IMS-based tests (IMS-MGIT culture and IMS-qPCR). Whilst the same faecal homogenate was tested by all QUB methods, different volumes of that homogenate were tested in the field and in the laboratory - automated IMS used in the laboratory was restricted to analysing 1 ml amounts of sample whereas the field IMS was carried out on a 6-8 ml volume of homogenate. We have estimated that there was the equivalent of: ~250 mg faeces per 80 µl bead sample applied to the LFD after IMS, ~0.5 mg faeces per AIMS-qPCR reaction, and ~80 mg faeces per AIMS-MGIT culture (assuming no losses during sample processing), so the higher amount of sample applied to the LFD may explain the extra samples testing positive by field IMS-LFD test and not by either AIMS-MGIT culture or AIMS-qPCR. Having said that, we estimate the $LOD_{50\%}$ of AIMS-qPCR to be 1.68×10^4 CFU/ml of faeces homogenate (or 1.68×10^5 CFU/g faeces), indicating that the AIMS-qPCR method should have had greater detection sensitivity than the field IMS-LFD assay ($LOD_{50\%}$ 2.79×10^5 CFU/ml of faeces homogenate or 2.79×10^6 CFU/g faeces) and detected more *M. bovis* positive faeces samples, which it did in the case of both NI and GB samples. A further consideration is that differently sized magnetic beads were used in the field and in the laboratory, because only the smaller coated beads were able to pass along the LFD. Despite being coated with the same binders, we know there is a difference, albeit small, in the capture capability of the 300 nm Ademtech compared with the 1 µm Tosylactivated Dynabeads, which could have impacted results obtained for the field test compared to the laboratory tests.

Despite considerable effort and use of an extensive range of tests in our attempts to isolate and identify *M. bovis* from all of the samples tested (571 badger faeces samples in total), including MGIT liquid culture with and without sample decontamination, solid culture (including LJ slopes and 7H10 agar plates), *M. bovis* specific RT-qPCR and Touchdown PCR (TD-PCR) targeting the *Mycobacterium* complex–specific insertion element IS6110, there is no guarantee that all *M. bovis* infected badger faeces samples were detected during this study. In particular, the AIMS-MGIT culture positive results reported do not represent *M. bovis* isolations confirmed by spoligotyping, but rather PCR positive broth cultures. Spoligotyping was attempted on some of the most strongly PCR positive of these MGIT cultures but no spacer profiles were obtained, possibly due to insufficient *M. bovis* DNA being present, or to *M. bovis* DNA being swamped by larger amounts of other bacterial DNA in mixed cultures. There have not been many previous reports of the successful isolation of *M. bovis* from badger faeces, so we are not alone in being unable to obtain any pure cultures of *M. bovis*.

The person performing the IMS-LFD test on the GB badger faeces samples made the observation that the appearance and consistency of the GB faeces was very different from the NI faeces samples, probably reflecting a difference in the badgers' diets – NI badgers eating lots of worms, grains and berries, GB badgers from a wooded area eating more vegetation. This led to considerable fluctuations in bead retrieval for both automated and field (manual) IMS during processing of the NI samples. The same level of variation was not observed during IMS of the GB samples. More beads were generally retrieved from the GB samples, yet the LFD readings for the IMS-LFD positive NI samples were higher than for the IMS-LFD positive GB samples, as shown in Figure 10. This would be indicative that higher numbers of *M. bovis* were being excreted in faeces of NI badgers compared to GB badgers.

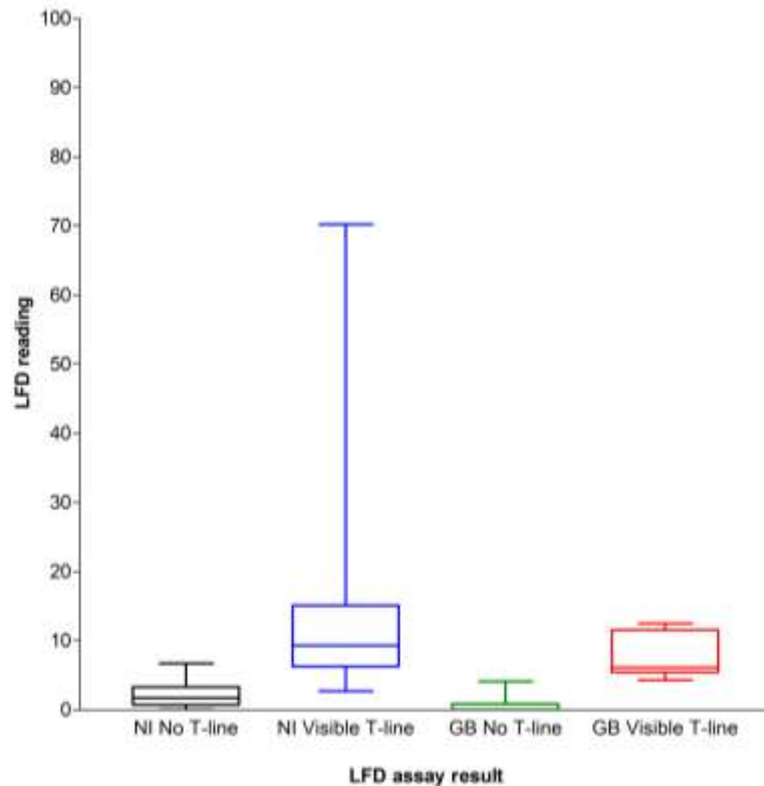

Figure 10. Box plot showing distribution of LFD readings for IMS-LFD test negative (no T line) and IMS-LFD test positive (visible T line) Northern Ireland (NI) and Woodchester Park (GB) badger faeces samples.

The age of the GB samples at time of testing at QUB may have had affected results obtained, although this is a little unlikely as the IMS-LFD test doesn't depend on the presence of viable *M. bovis*, just intact cells. GB samples were received by QUB as one batch of samples at end December 2012, however sampling dates ranged from May-October 2012 and samples had been stored frozen in the interim.

In summary, the pros and cons of the novel IMS-LFD test are:

PROS	CONS
IMS-LF test is specific for *M. bovis* rather than MTB complex	Sample preparation and application of IMS not optimal in field environment
Test detects whole *M. bovis* cells in badger faeces, NOT DNA or antibodies	Capture of *M. bovis* from faeces may have been affected when performed in field in cold weather
Rapid result, in the field – total test time approx. 1.5 h for 6 samples (sample prep 20 min, IMS 45 min, LFD 20 min)	Degree of clean up of sample during IMS and bead recovery influenced by faeces composition (due to badger's diet)
No expensive equipment required – LFD reader can be used to confirm positive T-lines	IMS-LF lacks detection sensitivity compared to AIMS-qPCR ($LOD_{50\%}$ of 2.9×10^5 CFU/ml and 1.7×10^4 CFU/ml of faecal homogenate, respectively)
Larger amount of faeces is tested on LF test (250 mg/80 µl) than is tested per qPCR reaction (0.5 mg/5 µl DNA)	IMS-LF test will detect badgers that are high *M. bovis* shedders not all shedding animals

Main implications of findings

- The novel IMS-LFD test represents a rapid, specific and relatively cheap method of detecting badgers shedding high numbers of *M. bovis* in their faeces. However, the test's lack of detection sensitivity would limit its potential use in the context of diagnosis of TB in badgers because not all *M. bovis* shedding animals would be detected by the IMS-LFD test. AIMS-qPCR would be a more sensitive test in this context.

- Some NI and GB badgers are shedding high numbers of *M. bovis* (>10^5 cells/g) in their faeces and potentially spreading infection in the environment.
- GPS coordinates recorded in the course of badger faeces sampling throughout NI provide new information on the location of setts containing *M. bovis* infected/shedding badgers, which could be of value to the Department of Agriculture for Northern Ireland as, to our knowledge, currently information on bovine tuberculosis in badgers is only derived from road-kill investigations.
- Whilst this study has demonstrated that the IMS-LFD test can be performed in the field, it is our view that optimal sample preparation, IMS capture and LFD performance would only be achieved in the laboratory. Health and safety of the person performing the test in the field would also be an important consideration.

Possible future work

The novel IMS-LFD assay was shown to be capable of detecting *M. bovis* in both NI and GB badger faeces, albeit only in samples containing high numbers of the bacterium (>10^{5-6} CFU/ml faecal homogenate). The test may, therefore, be useful as a surveillance tool to detect/locate badgers which are shedding high numbers of *M. bovis,* to inform future bTB control interventions. However, given the high specificity (i.e. ability to detect only *M. bovis*) but limited detection sensitivity demonstrated by the novel IMS-LFD test (i.e. inability to detect *M. bovis* in faeces of badgers shedding low numbers of the bacterium) compared to AIMS-qPCR, the novel test is likely to be more suited for application in a context where high numbers of *M. bovis* are frequently encountered. Further research funding has been secured by QUB researchers from Invest NI POC programme for a 1 year project to permit the evaluation of the IMS-LFD test in the bovine TB laboratory setting. The LFD has the potential to be applied either in conjunction with immunomagnetic separation (IMS-LFD test) to directly test tissue samples taken at slaughter or as a standalone LFD test later in the culture process to quickly confirm if *M. bovis* has been isolated in acid-fast positive MGIT cultures before spoligotyping.

The binding reagents used for IMS (an IgM and a phage-display derived peptide) and on the LFD (a polyclonal IgG and a monoclonal IgG) have been shown to be specific for *M. bovis*. They represent binders to unique *M. bovis* cell surface epitopes present during infection, however the identity of these epitopes is currently not known. They may represent crucial factors for *M. bovis* pathogenicity and/or appropriate markers for immunodiagnosis or targets for vaccine-induced immunity. Future work should include identification and characterisation of the binding epitopes of these specific affinity ligands on the *M. bovis* cell surface. Identification of the mimetic sequences of the true epitopes of the antibody could lead to a possible target for vaccine production or help in the elucidation of the pathogenicity of *M. bovis*. These targets could subsequently be useful for development of diagnostic or therapeutic reagents.

Action arising from the research (e.g. IP, Knowledge Exchange)

- The novel LFD has been shared with former research collaborators (on Defra project SE3262) at Veterinary Science Division, Agri-Food and Biosciences Institute for Northern Ireland, to allow personnel there to independently verify the specificity of the assay with *M. bovis* and *M. tuberculosis* cultures. AFBI-VSD will be collaborators on, and facilitators of, the forthcoming InvestNI funded project. AFBI-VSD are part owners, with QUB, of the cell lines of the antibodies used for IMS and LFD.
- No IP exists in relation to the novel *M. bovis* IMS-LFD assay that could be exploited at this stage.

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Mycobacterium bovis. PLoS One 6(11), e27369.
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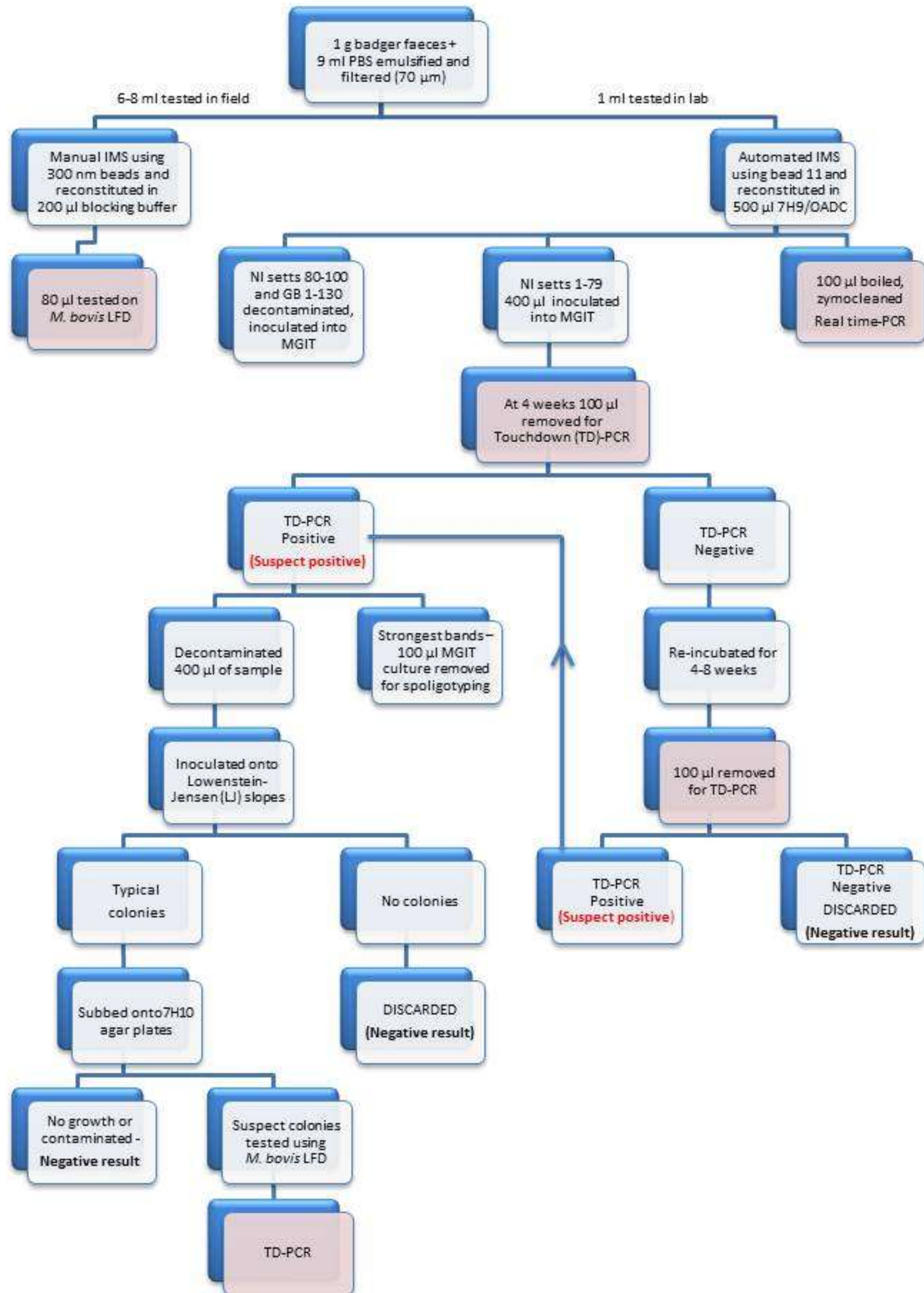
Appendix 1:

Schematic of AIMS-MGIT culture protocol demonstrating the exhaustive nature of steps taken at QUB to isolate *M. bovis* from badger faeces.



Appendix 2

Analysis of 2 x 2 contingency tables comparing field IMS-LFD results with AIMS-qPCR and AIMS-MGIT culture results for NI badger faeces samples.

Field IMS-LFD relative to AIMS-qPCR

	AIMS-qPCR +	AIMS-qPCR -	Total
Field IMS-LFD +	24	54	78
Field IMS-LFD -	116	247	363
Total	140	301	441

Test sensitivity = 0.1714 (95% CI: 0.1128-0.2438)
Test specificity = 0.8206 (95% CI: 0.772-0.8623)
Kappa value = -0.009 (P=0.8939), 'worse agreement than expected by chance alone'

Field IMS-LFD relative to AIMS-MGIT culture

	IMS-culture +	IMS-culture -	Total
Field IMS-LFD +	14	64	78
Field IMS-LFD -	50	313	363
Total	64	377	441

Test sensitivity = 0.219 (95% CI: 0.125-0.3397)
Test specificity = 0.830 (95% CI: 0.7883-0.8666)
Kappa value = 0.045 (P=0.3754), 'poor agreement'

AIMS-qPCR relative to AIMS-MGIT culture

	IMS-culture +	IMS-culture -	Total
IMS-qPCR +	21	119	140
IMS-qPCR -	43	258	301
Total	64	377	441

Test sensitivity = 0.328 (95% CI: 0.2162-0.4569)
Test specificity = 0.6844 (95% CI: 0.6345-0.7306)
Kappa value = 0.008 (P=0.8848), 'poor agreement'

Appendix 3

Analysis of 2 x 2 contingency tables comparing IMS-LFD results with AIMS-qPCR and AIMS-MGIT culture results for GB badger faeces samples from Woodchester Park.

Field IMS-LFD relative to AIMS-qPCR

	AIMS-qPCR +	AIMS-qPCR -	Total
Field IMS-LFD +	8	2	10
Field IMS-LFD -	48	42	90
Total	56	44	100

Test sensitivity = 0.1429 (95% CI: 0.063-0.262)
Test specificity = 0.9545 (95% CI: 0.845-0.994)
Kappa value = 0.088 (P=0.1785), 'poor agreement'

Field IMS-LFD relative to AIMS-MGIT culture

	AIMS-MGIT culture +	AIMS-MGIT culture -	Total
Field IMS-LFD +	5	5	10
Field IMS-LFD -	36	54	90
Total	41	59	100

Test sensitivity = 0.122 (95% CI: 0.041-0.262)
Test specificity = 0.915 (95% CI: 0.813-0.972)
Kappa value = 0.042 (P=0.7364), 'poor agreement'

AIMS-qPCR relative to AIMS-MGIT culture

	AIMS-MGIT culture +	AIMS-MGIT culture -	Total
AIMS-qPCR +	22	34	56
AIMS-qPCR -	19	25	44
Total	41	59	100

Test sensitivity = 0.54 (95% CI: 0.37-0.69)
Test specificity = 0.424 (95% CI: 0.296-0.559)
Kappa value = -0.038 (P=0.8379), 'agreement worse than expected by chance'

Appendix 4

Analysis of 2 x 2 contingency tables comparing QUB test results with badger infection status (as indicated by results of FERA tests).

(1) Field IMS-LFD relative to badger *M. bovis* infection status (as indicated by results of FERA tests)

	M. bovis infected	Non-infected	Total
Field IMS-LFD +	5	5	10
Field IMS-LFD -	57	33	90
Total	62	38	100

Diagnostic sensitivity = 0.0806 (95% CI: 0.0266-0.1781)
Diagnostic specificity = 0.8684 (95% CI: 0.7195-0.9558)
Positive predictive value (PPV) = 0.5000 (95% CI: 0.1871-0.8129)
Negative predictive value (NPV) = 0.3667 (95% CI: 0.2674-0.4754)

Kappa value = -0.040 (P=0.4982), 'worse agreement than expected by chance'

(2) AIMS-qPCR relative to badger *M. bovis* infection status (as indicated by results of FERA tests)

	M. bovis infected	Non-infected	Total
AIMS-qPCR +	38	18	56
AIMS-qPCR -	24	20	44
Total	62	38	100

Diagnostic sensitivity = 0.6129 (95% CI: 0.4803-0.7341)
Diagnostic specificity = 0.5263 (95% CI: 0.3581-0.6903)
Positive predictive value (PPV) = 0.6786 (95% CI: 0.5407-0.7974)
Negative predictive value (NPV) = 0.4545 (95% CI: 0.3039-0.6118)

Kappa value = 0.135 (P=0.2146), 'poor agreement'

(3) AIMS-MGIT culture relative to badger *M. bovis* infection status (as indicated by results of FERA tests)

	M. bovis infected*	Non-infected**	Total
AIMS-MGIT culture +	27	14	41
AIMS-MGIT culture -	35	24	59
Total	62	38	100

Diagnostic sensitivity = 0.4355 (95% CI: 0.3102-0.5670)
Diagnostic specificity = 0.6316 (95% CI: 0.4599-0.7816)
Positive predictive value (PPV) = 0.6585 (95% CI: 0.4945-0.7991)
Negative predictive value (NPV) = 0.4068 (95% CI: 0.2809-0.5425)

Kappa value = 0.061 (P=0.5370), 'poor agreement'

References to published material

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

The findings of this research project were orally presented by Dr Irene Grant at an Expert Workshop on Mycobacterial Detection Methods for Badgers which took place at AHVLA Weybridge, Surrey, on 19 April 2013. A summary of the main messages arising from this workshop is to be published on Defra website.

A scientific paper will be prepared in due course for submission to Journal of Clinical Microbiology (or other appropriate high impact journal), the provisional title of which is: Field application of a novel immunomagnetic separation-lateral flow immunoassay as a non-invasive test for detection of *Mycobacterium bovis* cells in badger faeces.