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Environmental monitoring of *Mycobacterium bovis* in badger faeces and badger sett soil using real-time PCR, confirmed by immunofluorescence, immunocapture and cultivation.

Running title: Real-time PCR of environmental *Mycobacterium bovis*

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Abstract

Real-time PCR was used to detect and quantify *Mycobacterium bovis* cells in naturally infected soil and badger faeces. Immunomagnetic capture, immunofluorescence and selective culture confirmed species identification and cell viability. These techniques will prove useful for monitoring *M. bovis* in the environment and for elucidating transmission routes between wildlife and cattle.

Previous studies of *Mycobacterium bovis* shed into the environment by infected hosts using conventional PCR with primers targeting the MPB70 antigen gene (specific to the *M. tuberculosis* complex) provided evidence that the organism is likely to persist in the environment for at least 15 months post removal of the known animal reservoirs (16), and that the probability of detection of *M. bovis* in soil and badger faeces is correlated with the prevalence of excreting badgers (2). For the purpose of epidemiological studies, *M. bovis* detection techniques must be 100% species specific with robust and reliable quantification.

Real time PCR has advantages over conventional PCR because it allows absolute quantification by comparison to a standard curve of known target sequence numbers. The complete genome sequence of *M. bovis* (5) has been used to design primers flanking a region of difference (RD4) between the sequence of *M. bovis* DNA and that of other *M. tuberculosis* complex members (1). The presence of *M. bovis* is confirmed using a fluorescent (TaqMan) probe which discriminates *M. bovis* from other *Mycobacterium tuberculosis* complex members since it hybridises with both the 5'

42 and the 3' RD4 deletion flanking sequences which only occur directly adjacent to
43 each other in *M. bovis* (1).
44
45 *M. bovis* cannot be directly cultured from soil due to the harsh decontamination
46 techniques required to remove competing organisms. This limitation was overcome in
47 a previous study by using immunomagnetic capture (IMC) to extract cells of *M. bovis*
48 from mixed cell communities using a poly-clonal antibody to *M. bovis* BCG, and thus
49 enabling cultivation of *M. bovis* from soil samples for the first time (13). Greater
50 specificity could be achieved using a monoclonal antibody, MBS43 (14, 15), which
51 recognises MPB83, a glycosylated cell wall associated protein (8), differentiating *M.*
52 *bovis* from other members of the *M. tuberculosis* complex (6).
53
54 We report here the first use of an *M. bovis*-specific real-time PCR to detect and
55 quantify *M. bovis* DNA in environmental samples, and confirm the presence of viable
56 cells of *M. bovis* using IMC, immunofluorescence and cultivation.
57
58 Badgers are an important wildlife reservoir of *M. bovis* in the UK, and infected
59 badgers can excrete the organism into the environment (4, 13). Social groups of
60 badgers dig underground tunnel systems known as setts and they defecate into
61 communal "latrines", which are often located on cattle pasture. Soil was collected
62 from 7 badger setts and faeces collected from 5 badger latrines during September
63 2006 on two cattle farms in a region of the UK endemic for bovine tuberculosis (bTB).
64 Replicate samples were taken from within 10m of each other at any one sett or latrine,
65 though the setts and latrines were variable in size. The average distance between
66 nearest neighbour sampled setts was 195m (range 40m-380m), and 234m (range 60m-

400m) between nearest neighbour sampled latrines. The study farms were not under bTB restriction at the time of sampling, but had experienced tuberculin skin test positive heard breakdowns, as defined by Defra, in the past. These sites were chosen as they had previously tested positive for *M. bovis* using conventional PCR (2). Four soil samples were used as negative controls: two from an area non-endemic for bTB and two endemic samples that had tested negative for *M. bovis* using the MPB70 PCR (16). Total community DNA was extracted from 0.2g of each sample using Qiagen Stool DNA extraction kit (Qiagen UK) following the manufactures instructions. Triplicate reactions were carried out for all environmental samples, standards and no template controls using real-time PCR. For each reaction, the total reaction volume was 25 µl comprising 12.5 µl TaqMan universal PCR master mix, 1 µl (20 pmol) forward RD4 flanking primer (5' TGTGAATTCATACAAGCCGTAGTCg 3'), 1 µl (20 pmol) reverse RD4 flanking primer (5' CCCGTAGCGTTACTGAGAAATTGC 3') and 1 µl (20 pmol) of the Probe (5' FAM–AGCGCAACACTCTTGGAGTGGCCTAC 3'- TAMRA), 2.5 µl of a 10 mg/ml BSA solution 6 µl nuclease free sterile water and 1 µl of a 1:10 dilution of the total community DNA.

IMC was carried out as previously described (13), but with duplicate 0.5g aliquots of the environmental samples blocked with 3% BSA in PBS overnight at 4^{o}C. Dynal magnetic beads (Invitrogen UK) 50µl (50mg) pre-coated with goat anti-mouse antibody were linked to 100 µg MBS43. The reaction was incubated for 3 h with shaking at 4^{o}C. The antibody coated beads were then added to the blocked environmental sample and incubated for 3 h at 4^{o}C with shaking. Cells of *M. bovis* were captured and separated using a magnetic device (Dynal UK), separated cells

were washed three times with PBS containing 0.1% nonident and resuspended in 200µl of PBS. *M. bovis* was cultivated on non-acidified pyruvate LJ media slopes (Media for Mycobacteria Ltd., Cardiff, UK) incubated at 37^{o}C for 4 weeks. Single colonies were transferred to Kirchner medium (Media for Mycobacteria Ltd.), supplemented with sodium pyruvate (4 g / litre) and the following antibiotics, Polymyxin B (200 000 units/ litre), Ticarcillin (100 mg / litre), Trimethoprim (10 mg / litre) and the anti fungal amphotericin B (10mg / litre). A commercially labelled polyclonal antibody to *M. bovis* BCG (DAKO) was coupled to FITC labelled goat anti-rabbit IgG by incubating 50 µg of each at 4^{o}C with shaking. 10 µl was added to 50 µl of the immunocaptured cells. DAPI was also added and the solution left for 1 h at 4^{o}C. Cells were fixed with 4% glutaraldehyde for 2 h before fluorescence microscopy.

TaqMan real-time PCR detected the presence of *M. bovis* in all 12 samples from infected setts and latrines, but no *M. bovis* DNA was amplified from the four negative controls. Gene copies per gram of sample ranged from 6.8×10^4 to 5.4×10^6 (Fig 1), with quantities appearing more variable between sett samples than between latrine samples, although the mean cell count did not differ significantly between sett and latrine samples ($F_{1,10}$=0.77, NS), nor was there a significant difference in the cell count variances between sample types (Bartlett's χ^2=2.01, P=0.156). The product was confirmed as *M. bovis* by its size (142 bp) and sequence.

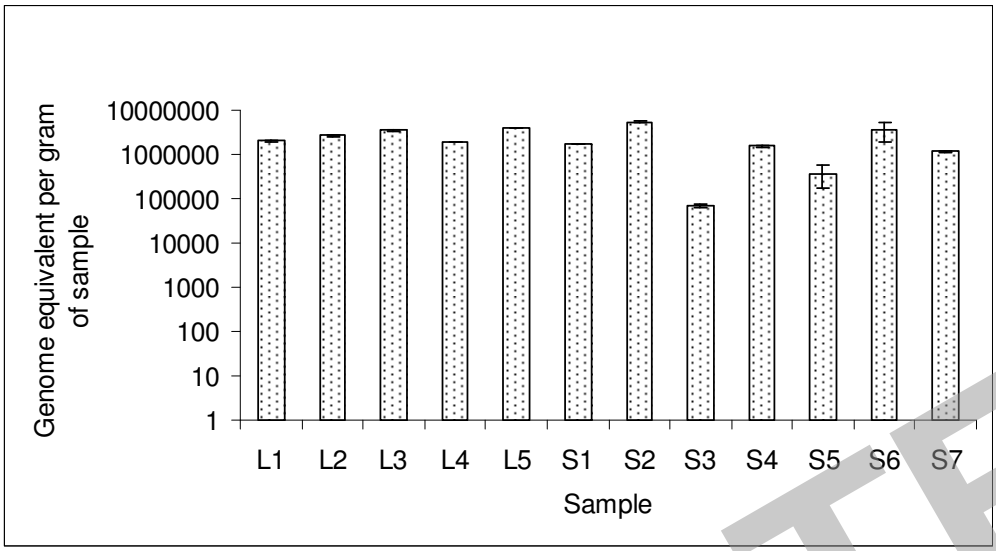

Figure 1. The mean number of *M. bovis* cell copies per gram of environmental sample (L = latrine; S = sett) estimated by Taq Man real-time PCR. Error bars represent the 95% confidence intervals around the mean counts from three replicates per sample.

Immunomagnetic capture was performed on all of the positive samples and in all cases confirmed the presence of *M. bovis* cells by subsequent cultivation. *M. bovis* cells from one sample (L3) captured by the MBS43 coated magnetic beads and stained with FITC coupled *M. bovis* BCG antibody are shown in Fig. 2. DAPI stain detected the captured bacteria and FITC fluorescence was seen to co-localise with the cells.

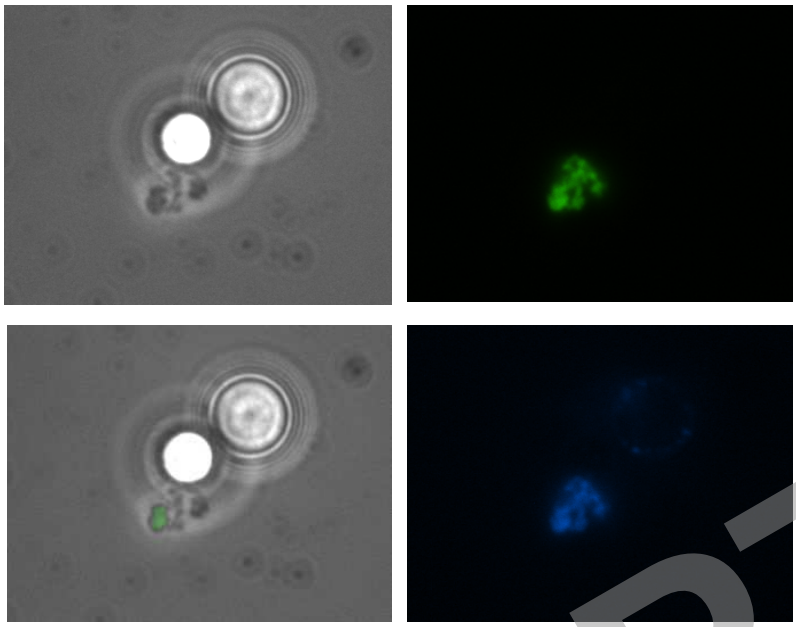

Figure 2. Immunocaptured *M. bovis* attached to magnetic particles stained with DAPI (blue) and FITC (green). 600X oil immersion.

Captured cells from all of the 12 samples were inoculated onto LJ media slopes (Media for Mycobacteria Ltd., Cardiff, UK) which gave colonies after 4 weeks at 37^{o}C. These were sub-cultured into Kirchner medium (supplemented with 4 g/l w/v Na pyruvate and BSA (Media for Mycobacteria Ltd., Cardiff, UK).

Many pathogenic bacteria can survive in the environment (7), and several members of the *Mycobacterium* genus are known to persist even under extremely hostile conditions (12). Several properties that are common to all mycobacteria may help *M. bovis* endure extreme environmental conditions following excretion by an infected host, and a reservoir of the organism in the environment could potentially be a source of infection to cattle and other susceptible species. Bovine tuberculosis is an endemic disease in badgers in Great Britain and Ireland (9), however the route or routes of

143 transmission to cattle are poorly understood. Cattle are known to be highly susceptible
144 to aerosol transmission (11) but can also become infected through ingestion, although
145 experiments have shown that as many as 10^7 bacilli must be ingested to cause
146 infection by this route (3, 10). Gallagher and Clifton-Hadley (4) estimated by
147 selective cultivation the number of *M.bovis* bacilli that badgers with advanced
148 milliary disease can shed into the environment. They cultivated 200 x 10^3 and 68 c.f.u.
149 per g from two separate clinical faecal samples and 217 x 10^3 and 250 x 10^3 c.f.u. per
150 ml from two separate urine samples (4). The results of this study using real time PCR
151 show cell densities of 6.8 x 10^4 to 5.4 x 10^6 *M. bovis* cells per gram of soil at badger
152 setts and of faeces at badger latrines, which we assume to be typical in this infected
153 badger population. Previous estimates of cell numbers in similar samples were
154 between 2.8 x 10^5 and 3.2 x 10^5 using a different method (MPB70 and Rv1510
155 primers with PCR product quantified by pixel intensity) (13). In the current study
156 there was no statistical difference between the mean and variance of cell counts at
157 setts vs. latrines, however, on visual inspection the quantities detected at setts
158 appeared to be more variable than those at latrines. If this proves to be the case, it may
159 be due to the greater variability in the distribution of micro-organisms in soil
160 compared to faeces, and/or differences in the excretory behaviour of badgers at setts
161 compared to at latrines.
162
163 In conclusion, we have developed an *M. bovis* specific molecular detection technique,
164 based on real-time PCR, for monitoring and quantifying cells in environmental
165 samples. This method will be useful for identifying sites of contamination on farms
166 that may constitute an infection risk to cattle and wildlife.
167

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171

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