Applied and Environmental Microbiology, Nov. 2007, p. 7471–7473 Vol. 73, No. 22
0099-2240/07/$08.00+0 doi:10.1128/AEM.00978-07

Environmental Monitoring of *Mycobacterium bovis* in Badger Feces and Badger Sett Soil by Real-Time PCR, as Confirmed by Immunofluorescence, Immunocapture, and Cultivation▽

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Received 1 May 2007/Accepted 16 September 2007

Real-time PCR was used to detect and quantify *Mycobacterium bovis* cells in naturally infected soil and badger feces. 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 postremoval of the known animal reservoirs (16) and that the probability of detection of *M. bovis* in soil and badger feces is correlated with the prevalence of excreting badgers (2). For 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 by using a fluorescent (TaqMan) probe which discriminates *M. bovis* from other *M. tuberculosis* complex members since it hybridizes with both the 5′ and 3′ RD4 deletion flanking sequences, which only occur directly adjacent to each other in *M. bovis* (1).

M. bovis cannot be directly cultured from soil because of the harsh decontamination techniques required to remove competing organisms. This limitation was overcome in a previous study by using immunomagnetic capture (IMC) to extract cells of *M. bovis* from mixed cell communities with a polyclonal antibody to *M. bovis* BCG and thus enabling cultivation of *M. bovis* from soil samples for the first time (13). Greater specificity could be achieved by using a monoclonal antibody, MBS43 (14, 15), which recognizes MPB83, a glycosylated cell wall-associated protein (8), differentiating *M. bovis* from other members of the *M. tuberculosis* complex (6).

We report here the first use of an *M. bovis*-specific real-time PCR to detect and quantify *M. bovis* DNA in environmental samples and confirm the presence of viable cells of *M. bovis* by using IMC, immunofluorescence, and cultivation.

Badgers are an important wildlife reservoir of *M. bovis* in the United Kingdom, and infected badgers can excrete the organism into the environment (4, 13). Social groups of badgers dig underground tunnel systems known as setts, and they defecate into communal "latrines," which are often located on cattle pasture. Soil was collected from seven badger setts, and feces was collected from five badger latrines during September 2006 on two cattle farms in a region of the United Kingdom where bovine tuberculosis (bTB) is endemic. Replicate samples were taken from within 10 m of each other at any one sett or latrine, although the setts and latrines were variable in size. The average distance between the nearest neighbor setts sampled was 195 m (range, 40 to 380 m), and that between the nearest neighbor latrines sampled was 234 m (range, 60 to 400 m). The study farms were not under bTB restriction at the time of sampling but had experienced tuberculin skin test-positive herd breakdowns, as defined by the Department for Environment Food and Rural Affairs, in the past. These sites were chosen because they had previously tested positive for *M. bovis* by conventional PCR (2). Four soil samples were used as negative controls, two from an area where bTB is nonendemic and two from an area where bTB is endemic that had tested negative for *M. bovis* by the MPB70 PCR (16). Total community DNA was extracted from 0.2 g of each sample with a QIAGEN Stool DNA extraction kit (QIAGEN United Kingdom) by following the manufacturer's instructions. Triplicate reactions were carried out for all environmental samples, standards, and no-template controls by real-time PCR. For each reaction, the total reaction volume was 25 μl comprising 12.5 μl of TaqMan universal PCR master mix, 1 μl (20 pmol) of forward RD4 flanking primer (5′ TGTGAATTCATACAAG CCGTAGTCG 3′), 1 μl (20 pmol) of reverse RD4 flanking primer (5′ CCCGTAGCGTTACTGAGAAATTGC 3′), 1 μl (20 pmol) of the probe (5′ 6-carboxyfluorescein–AGCGCAA CACTCTTGGAGTGGCCTAC–tetramethyl-6-carboxyrhoda

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▽ Published ahead of print on 28 September 2007.

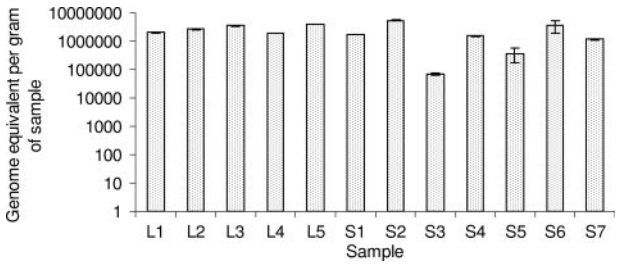

FIG. 1. Mean numbers of *M. bovis* cell copies per gram of environmental sample (L, latrine; S, sett) estimated by TaqMan real-time PCR. Error bars represent the 95% confidence intervals around the mean counts from three replicates per sample.

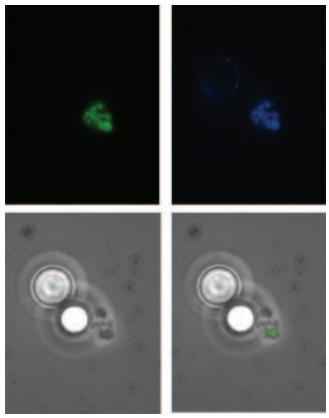

FIG. 2. Immunocaptured *M. bovis* attached to magnetic particles stained with DAPI (blue) and FITC (green). 600× oil immersion.

mine 3′), 2.5 μl of a 10-mg/ml bovine serum albumin (BSA) solution, 6 μl of 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 0.5-g aliquots of the environmental samples blocked with 3% BSA in phosphate-buffered saline (PBS) overnight at 4°C. A 50-μl volume (50 mg) of Dynal magnetic beads (Invitrogen, United Kingdom) precoated with goat anti-mouse antibody was linked to 100 μg of MBS43. The reaction mixture was incubated for 3 h with shaking at 4°C. The antibody-coated beads were then added to the blocked environmental sample and incubated for 3 h at 4°C with shaking. Cells of *M. bovis* were captured and separated with a magnetic device (Dynal United Kingdom), and separated cells were washed three times with PBS containing 0.1% Nonidet and resuspended in 200 μl of PBS. *M. bovis* was cultivated on nonacidified pyruvate LJ medium slopes (Media for Mycobacteria Ltd., Cardiff, United Kingdom) incubated at 37°C for 4 weeks. Single colonies were transferred to Kirchner medium (Media for Mycobacteria Ltd.) supplemented with sodium pyruvate (4 g/liter), polymyxin B (200,000 U/liter), ticarcillin (100 mg/liter), trimethoprim (10 mg/liter), and the antifungal amphotericin B (10 mg/liter). A commercially labeled polyclonal antibody to *M. bovis* BCG (DAKO) was coupled to fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit immunoglobulin G by incubating 50 μg of each at 4°C with shaking. Ten microliters was added to 50 μl of the immunocaptured cells. 4′,6′-Diamidino-2-phenylindole (DAPI) was also added, and the solution was left for 1 h at 4°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. The numbers of 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 ($F_{1,10} = 0.77$ [no statistically significant difference]), nor was there a significant difference in the cell count variances between sample types (Bartlett's $\chi^2 = 2.01$, $P = 0.156$). The product was confirmed to be *M. bovis* by its size (142 bp) and sequence.

IMC 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 colocalize with the cells.

Captured cells from all of the 12 samples were inoculated onto LJ medium slopes (Media for Mycobacteria Ltd., Cardiff, United Kingdom), which gave colonies after 4 weeks at 37°C. These were subcultured into Kirchner medium supplemented with 4 g/liter (wt/vol) Na pyruvate and BSA (Media for Mycobacteria Ltd., Cardiff, United Kingdom).

Many pathogenic bacteria can survive in the environment (7), and several members of the genus *Mycobacterium* 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. bTB is an endemic disease in badgers in Great Britain and Ireland (9); however, the route(s) of transmission to cattle is poorly understood. Cattle are known to be highly susceptible to aerosol transmission (11) but can also become infected through ingestion, although experiments have shown that as many as 10^7 bacilli must be ingested to cause infection by this route (3, 10). Gallagher and Clifton-Hadley (4) estimated by selective cultivation the number of *M. bovis* bacilli that badgers with advanced miliary disease can shed into the environment. They cultivated 200×10^3 and 68 CFU/g from two separate clinical fecal samples and 217×10^3 and 250×10^3 CFU/ml from two separate urine samples (4). The results of this study using real-time PCR show cell densities of 6.8×10^4 to 5.4×10^6 *M. bovis* cells per g of soil at badger setts and of feces at badger latrines, which we assume to be typical in this infected badger population. Previous estimates of cell numbers in similar samples by a different method (MPB70 and Rv1510 primers with PCR product quantified by pixel intensity) were between 2.8×10^5 and 3.2×10^5 (13). In the present study, there was no statistically significant difference between the mean and variance of cell counts at setts versus latrines; however, on visual inspection the quantities detected at setts appeared to be more variable than those at latrines. If this proves to be the case, it

may be due to the greater variability in the distribution of microorganisms in soil compared to feces, and/or differences in the excretory behavior of badgers at setts compared to that at latrines.

In conclusion, we have developed an *M. bovis*-specific molecular detection technique, based on real-time PCR, for monitoring and quantifying cells in environmental samples. This method will be useful for identifying sites of contamination on farms that may constitute an infection risk to cattle and wildlife.

This work was conducted with financial support from the BBSRC (grant BBS/B/08868 awarded to O.C. and E.M.H.W.).

We thank the farmers who granted permission to take samples.

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