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Is *Mycobacterium bovis* in the environment important for the persistence of bovine tuberculosis?

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***Mycobacterium bovis* is the causative agent of bovine tuberculosis (bTB) in cattle and wildlife. Direct aerosol contact is thought to be the primary route of infection between conspecifics, whereas indirect transmission via an environmental reservoir of *M. bovis* is generally perceived not to be a significant source for infection. Here, we report on the application of molecular technology (PCR) to quantify the prevalence of *M. bovis* in the environment and to explore its epidemiological significance. We show that the detectability of viable *M. bovis* at badger setts and latrines is strongly linked to the frequency of *M. bovis* excretion by infected badgers, and that putative *M. bovis* in the environment is prevalent on a large proportion of endemic cattle farms in Britain. These results raise important questions about the role of an environmental reservoir in bTB persistence.**

Keywords: *Mycobacterium bovis*; bovine tuberculosis; environment; survival; badger; *Meles meles*

1. INTRODUCTION

Bovine tuberculosis (bTB) caused by *Mycobacterium bovis* is a persistent problem among UK cattle herds. Potential obstacles to bTB control are the existence of the badger (*Meles meles*) as a wildlife reservoir (Griffin *et al.* 2005), and the presence of *M. bovis* in the environment where the organism can survive for months (Maddock 1933; Young *et al.* 2005) and may remain infectious (Williams & Hoy 1930; Maddock 1933; Wilesmith *et al.* 1986). Badgers form social groups that use communal underground setts where conditions are likely to facilitate transmission and provide one focus of environmental contamination.

In this study, we apply molecular techniques to quantify the occurrence of mycobacteria belonging to the *Mycobacterium tuberculosis* complex (MTBC) and in particular *M. bovis*, in badger sources on farms in the UK. We also explore relationships between detection of environmental *M. bovis* and badger infection in a naturally exposed badger population.

The electronic supplementary material is available at http://dx.doi.org/10.1098/rsbl.2006.0468 or via http://www.journals.royalsoc.ac.uk.

2. MATERIAL AND METHODS

(a) *Sample collection*

Environmental samples were obtained from 60 cattle farms located in six bTB endemic regions of the UK between February and June 2003 for molecular detection of MTBC. Within these regions, all farms that had at least one main sett and two active setts were selected from a larger sample of 292 farms previously ground surveyed for signs of badgers (Courtenay *et al.* submitted). Replicate soil samples were collected from 2 to 10 badger setts per farm and replicate badger faeces collected from 5 to 20 latrines on nine farms (sample size calculations detailed in electronic supplementary material).

Setts and latrines were also sampled for molecular detection of *M. bovis* from two high-density badger populations in the UK: one bTB endemic population in Woodchester Park, Gloucestershire, sampled in September 2004; the other of unknown bTB status in a region of low cattle breakdown incidence in Wytham Woods, Oxfordshire, sampled in June 2005 (details in electronic supplementary material). From these populations, replicate soil samples were collected from the main sett in 22 and 10 badger social group territories, respectively, and replicate faeces samples were collected from all latrines within 25 m of the main sett (12 and 7 of the territories).

(b) *Molecular detection*

Mycobacterium DNA was detected by a PCR assay targeted at the MPB70 antigen gene specific to the MTBC (Young *et al.* 2005). Positive samples from the Woodchester and Wytham badger populations were subsequently confirmed as *M. bovis* using a second PCR targeting Rv1510 (RD4), present in all members of the complex except *M. bovis* (Huard *et al.* 2003). RT–PCR for 16S rRNA was performed (Young *et al.* 2005) on 22 PCR positive samples to demonstrate cell viability.

(c) *Badger sampling*

In Woodchester, sputum, urine, faeces and bite wound swabs were collected from anaesthetized badgers trapped four times a year between January 2002 and September 2004 in the 22 group territories to detect badgers excreting *M. bovis* (excretors) defined as positive *in vitro* culture and identification of *M. bovis* spoligotypes (Delahay *et al.* 2000).

(d) *Statistical analyses*

Data generated from the badger populations were analysed by maximum-likelihood logistic regression. The outcome variable was the proportion of environmental replicate samples that tested PCR positive per territory. The explanatory variables were (i) the number of excretors and (ii) the number of excreting occasions/the total sampling (trapping) occasions, per social group. McFadden pseudo-R^2 values were computed using log-likelihood values of the null and minimal unrestricted logistic models. Badger demographic variables proved non-significant in all minimal models (detailed in electronic supplementary material).

3. RESULTS

We detected MTBC in badger setts on 47 (78%) of the 60 farms (table 1). An average 43% of setts and 29% of latrines were positive per contaminated farm. 16S rRNA sequences were demonstrated in 3 of 12 positive setts indicating the presence of viable cells.

11.5% of the sampled Woodchester badger population located in 16 of 22 territories were classed as excretors during the 32 months of this study (table 2*a*). The proportion of environmental samples that tested PCR positive per social group territory (table 2*b*) was positively associated with both the absolute number of excretors and the proportion of total sampling occasions on which badgers were detected excreting *M. bovis* (table 3, figure 1). Stratifying these analyses by environmental site (sett or latrine) and excretion route (sputum only, faeces, urine, or all routes combined), consistent associations were observed between environmental detection and

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Table 1. Prevalence of environmental MTBC in soil from badger setts and faeces from badger latrines, on cattle farms in bTB endemic regions of the UK.

	setts		latrines	
sample	*n*	% (binomial 95% CI)	*n*	% (binomial 95% CI)
crude proportion PCR positive	286	40.9 (35.2–46.9)	89	12.4 (6.3–21.0)
proportion PCR positive per farm	286	33.3 (25.0–43.4)	89	15.0 (0–29.9)
proportion PCR positive per positive farm	247	42.9 (33.3–65.0)	47	28.6 (15.0–40.0)
proportion of farms PCR positive	60	78.3 (65.8–87.9)	9	55.6 (21.2–86.3)

frequency of excretion in urine and faeces, but not in sputum (table 3). In six of the social groups that showed evidence of environmental *M. bovis*, no excretors were detected during the study period. Neither *M. bovis* excretion rates nor the prevalence of environmental *M. bovis* were associated with absolute badger social group size or group density.

Mycobacterium bovis was detected at all setts and latrines tested in Woodchester and 16S rRNA was present in one of five setts and two of five latrines tested. All 180 samples positive for MPB70 (135 from setts and 45 from latrines) were confirmed to contain *M. bovis* rather than other members of the MTBC.

In contrast to Woodchester, we did not detect MTBC in any of the 140 replicate environmental samples from the 10 social group territories in the Wytham badger population.

Table 2. (*a*) Prevalence of *M. bovis* excretion events in Woodchester badgers and badger social groups from 2002 to 2004. (*b*) Detection of *M. bovis* in environmental samples collected from the badger social group territories in 2004.

sample	percentage positive (binomial 95% CI)	*n*
(*a*) *badger excretion*		
total badger	11.5 (8.3–15.4)	331
total badger samples	6.1 (4.5–8.0)	803
badger social groups	72.7 (49.8–89.3)	22
(*b*) *environmental contamination*		
total setts	100 (87.3–100)	22
total latrines	100 (77.9–100)	12
total sett replicates	62.8 (56.0–69.3)	215
total latrine replicates	56.3 (44.7–67.3)	80
replicate samples per sett	65.0 (50.0–60.0)	10
replicate samples per latrine	60.0 (40.0–68.9)	5–10

4. DISCUSSION

Evaluation of the environment as a potential reservoir of *M. bovis* is an important step towards understanding its transmission. This study demonstrates wide variation in the prevalence of *M. bovis* in the environment associated with badger setts and latrines, and that the probability of molecular detection is related to the number and prevalence of badgers excreting *M. bovis* within the territories of social groups. The biological integrity of these results is strengthened by identification of a direct relationship between latrine positivity and social group rate of excretion of *M. bovis* in faeces. The failure to detect excreting badgers in six of the social groups that showed evidence of environmental *M. bovis* may be due to the low sensitivity of culture, variation in the probability of capturing a badger while it is excreting (Delahay *et al.* 2000), neighbouring excretors visiting these territories (Rogers *et al.* 1997) and/or *M. bovis* persistence following prior contamination.

To confirm the epidemiological significance of an environmental reservoir of *M. bovis* in bTB persistence, further studies to test the infectivity, survival and physiology of environmental *M. bovis* are required. Notwithstanding, our findings strongly indicate that the mycobacterial DNA detected in this study was contained within intact and viable cells: detection of 16S rRNA by RT–PCR is a sensitive indication of active cell metabolism, and extracellular mycobacterial DNA fails to survive for more than a few days in soil (Young *et al.* 2005). Growth of the organism on culture following direct extraction from soil and faeces by MTBC specific immunomagnetic capture (Sweeney *et al.* submitted) is further evidence of its viability. Detection of rRNA in only 6 of 22 PCR positive samples may be due to the poorer stability of RNA and the likely low number of ribosomes within cells in soil. In a study of *M. bovis* persistence in Ireland, little difference was detected in semi-quantitative PCR signal strength in samples from setts tested at 4 and 15 months after badger and

Table 3. Estimated goodness of fit of logistic regression models to test the probability that the detection of *M. bovis* in the environment is related to *M. bovis* excretion rates of badger social groups (*n*). Data are stratified by route of excretion and site of environmental sample for (*a*) numbers of excreting individuals per social group; (*b*) proportion of badger sampling occasions per social group on which a culture positive result was obtained. (Values are McFadden pseudo R^2 estimates. 'Any route' includes cultures of bite wound samples; $p \leq {}^{*}0.05$; ${}^{**}0.01$; ${}^{***}0.001$.)

excretion route	setts only $n=22$	latrines only $n=12$	both $n=22$
(*a*) *badgers*			
sputum only	0.004	0.00009	0.004
faeces	0.21^{*}	0.41	0.30^{**}
urine	0.08	0.24	0.14^{*}
any route	0.27^{*}	0.35	0.34^{**}
(*b*) *samples*			
sputum only	0.03	0.03	0.006
faeces	0.36^{**}	0.44^{*}	0.43^{***}
urine	0.33^{*}	0.10	0.31^{**}
any route	0.43^{**}	0.14	0.42^{***}

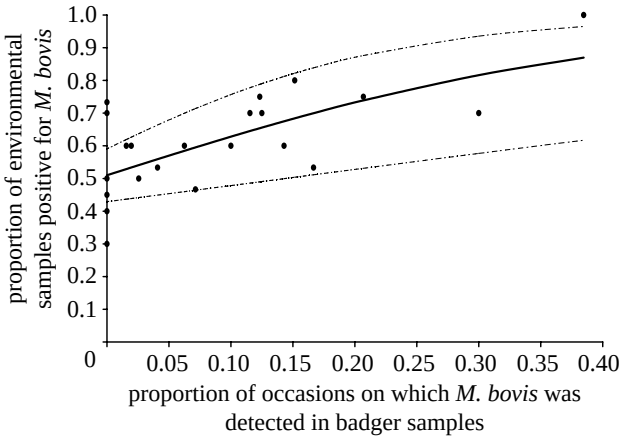

Figure 1. Relationship between the proportion of environmental samples that were PCR positive for *M. bovis* and the proportion of testing occasions from 2002 to 2004 on which badgers were excreting *M. bovis*. Each data point represents a social group territory. Maximum-likelihood model fit (solid line) is shown along with the 95% CI (dashed lines) to the observed data (points). Model fit in logits: $y=4.83x+0.04$. Goodness of fit $R^2=0.42$ (see table 3).

cattle removal (Young *et al.* 2005), highlighting the known ability of the organism to viably persist in soil and faeces for months (Maddock 1933). Critically, historical studies of *M. bovis* showed contaminated soil, badger and cattle faecal inoculates to be infectious in *in vivo* guinea-pig models (Williams & Hoy 1930; Maddock 1933; Wilesmith *et al.* 1986).

These collective results are of interest to agencies involved in bTB control. In particular, this study detected a high prevalence of environmental MTBC on cattle farms in all six bTB endemic UK regions sampled, where 64 (75/117) and 73% (8/11) of the PCR positive setts and latrines, respectively, were accessible to grazing cattle. By targeting the MPB70 region, we confirmed the presence of MTBC mycobacteria. Farm samples were not tested for the specific presence of *M. bovis* using Rv1510 primers; however, *Mycobacterium microti* is the only member of the MTBC likely to be encountered in the environment (apart from *M. bovis*), and all other members are obligate human pathogens. We found no evidence of *M. microti* in this study, nor was *M. microti* detected in 10 397 cultures of 4393 clinically sampled wild animals collected on bTB endemic farms in Britain (Mathews *et al.* 2006). This supports the proposal that the *Mycobacterium* detected on the farms in the current study was predominantly, if not solely, *M. bovis*.

The epidemiological significance of widespread occurrence of *M. bovis* in the farm environment is necessarily speculative at present. Apart from being potentially infectious to cattle, either by ingestion or more likely by creation of an aerosol during olfactory investigation, there is a possibility that the presence of environmental *M. bovis* could compromise test-and-slaughter programmes for bTB control and help explain bTB persistence. Sensitization of cattle to other species of environmental mycobacteria can compromise cattle immune responses to subsequent vaccination with BCG (Buddle *et al.* 2002), and can cause cattle to remain skin-test negative even though they are potentially infectious (Hope *et al.* 2005).

One novel line of bTB research would be to evaluate the potential of molecular technology in non-invasive diagnostic screening. Appropriately, such technology could be developed to identify *all* likely animal sources and hotspot contamination points on farms.

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Buddle, B. M., Wards, B. J., Aldwell, F. E., Collins, D. M. & deLisle, G. W. 2002 Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine* **20**, 1126–1133. (doi:10.1016/S0264-410X(01)00436-4)

Delahay, R. J., Langton, S., Smith, G. C., Clifton-Hadley, R. S. & Cheeseman, C. L. 2000 The spatio-temporal distribution of *Mycobacterium bovis* (bovine tuberculosis) infection in a high-density badger population. *J. Anim. Ecol.* **69**, 428–441. (doi:10.1046/j.1365-2656.2000.00406.x)

Griffin, J. M., Williams, D. H., Kelly, G. E., Clegg, T. A., O'Boyle, I., Collins, J. D. & More, S. J. 2005 The impact of badger removal on the control of tuberculosis in cattle herds in Ireland. *Prev. Vet. Med.* **67**, 237–266. (doi:10.1016/j.prevetmed.2004.10.009)

Hope, J. C., Thom, M. L., Villarreal-Ramos, B., Vordermeier, H. M., Hewinson, R. G. & Howard, C. J. 2005 Exposure to *Mycobacterium avium* induces low-level protection from *Mycobacterium bovis* infection but compromises diagnosis of disease in cattle. *Clin. Exp. Immunol.* **141**, 432–439. (doi:10.1111/j.1365-2249.2005.02882.x)

Huard, R. C., de Oliveira Lazzarini, L. C., Butler, W. R., van Soolingen, D. & Ho, J. L. 2003 PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis* complex on the basis of genomic deletions. *J. Clin. Microbiol.* **41**, 1637–1650. (doi:10.1128/JCM.41.4.1637-1650.2003)

Maddock, E. C. G. 1933 Studies on the survival time of the bovine tubercle bacillus in soil, soil and dung, in dung and on grass, with experiments on the preliminary treatment of infected organic matter and the cultivation of the organism. *J. Hyg.* **33**, 103–117.

Mathews, F. *et al.* 2006 Bovine tuberculosis (*Mycobacterium bovis*) in British farmland wildlife: the importance to agriculture. *Proc. R. Soc. B* **273**, 357–365. (doi:10.1098/rspb.2005.3298)

Rogers, L. M., Cheeseman, C. L., Mallinson, P. J. & Clifton-Hadley, R. 1997 The demography of a high-density badger (*Meles meles*) population in the west of England. *J. Zool.* **242**, 705–728.

Wilesmith, J. W., Sayers, P. E., Bode, R., Pritchard, D. G., Stuart, F. A., Brewer, J. I. & Hillman, G. D. B. 1986 Tuberculosis in East Sussex II. Aspects of badger ecology and surveillance for tuberculosis in badger populations (1976–1984). *J. Hyg.* **97**, 11–26.

Williams, R. S. & Hoy, W. A. 1930 The viability of *B. tuberculosis* (*Bovinus*) on pasture land, in stored faeces and in liquid manure. *J. Hyg.* **30**, 413–419.

Young, J. S., Gormley, E. & Wellington, E. M. H. 2005 Molecular detection of *Mycobacterium bovis* and *Mycobacterium bovis* BCG (Pasteur) in soil. *Appl. Environ. Microbiol.* **71**, 1946–1952. (doi:10.1128/AEM.71.4.1946-1952.2005)