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Bovine tuberculosis (*Mycobacterium bovis*) in British farmland wildlife: the importance to agriculture

Fiona Mathews[1,*], David W. Macdonald[1], G. Michael Taylor[2], Merryl Gelling[1], Rachel A. Norman[3], Paul E. Honess[1,†], Rebecca Foster[1], Charlotte M. Gower[4,‡], Susan Varley[4], Audrey Harris[5], Simonette Palmer[6], Glyn Hewinson[6] and Joanne P. Webster[4,‡]

[1]*Wildlife Conservation Research Unit, Department of Zoology, University of Oxford, Tubney House, Abingdon Road, Tubney, Oxon OX13 5QL, UK*
[2]*Centre for Molecular Microbiology and Infection, Flowers Building, Imperial College London, South Kensington Campus, London SW7 2AZ, UK*
[3]*Department of Mathematics and Computing Science, University of Stirling, Stirling FK9 4LA, Scotland, UK*
[4]*Peter Medawar Building for Pathogen Research, University of Oxford, South Parks Road, Oxford OX1 3FY, UK*
[5]*Department of Food Microbiology, Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK*
[6]*Veterinary Laboratories Agency, Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK*

Bovine tuberculosis (bTB) is an important disease of cattle and an emerging infectious disease of humans. Cow- and badger-based control strategies have failed to eradicate bTB from the British cattle herd, and the incidence is rising by about 18% per year. The annual cost to taxpayers in Britain is currently £74 million. Research has focused on the badger as a potential bTB reservoir, with little attention being paid to other mammals common on farmland. We have conducted a systematic survey of wild mammals ($n=4393$ individuals) present on dairy farms to explore the role of species other than badgers in the epidemiology of bTB. Cultures were prepared from 10 397 samples (primarily faeces, urine and tracheal aspirates). One of the 1307 bank voles (*Clethrionomys glareolus*) live-sampled, and three of the 43 badgers (*Meles meles*), yielded positive isolates of *Mycobacterium bovis*. This is the first time the bacterium has been isolated from the bank vole. The strain type was the same as that found in cattle and badgers on the same farm. However, our work indicates that the mean prevalence of infectious individuals among common farmland wildlife is extremely low (the upper 95% confidence interval is ≤ 2.0 for all of the abundant species). Mathematical models illustrate that it is highly unlikely the disease could be maintained at such low levels. Our results suggest that these animals are relatively unimportant as reservoirs of bTB, having insufficient within-species (or within-group) transmission to sustain the infection, though occasional spill-overs from cattle or badgers may occur.

Keywords: bovine tuberculosis; *Mycobacterium bovis*; epidemiology; voles; PCR; *mycobacterium microti*

1. INTRODUCTION

The control of bovine tuberculosis (bTB) has preoccupied agricultural policy in the UK for more than three decades. The current cost to British taxpayers is £74 million per year, with annual expenditure projected to reach £1 billion in seven years' time (Department of Environment, Food and Rural Affairs; DEFRA 2004). The total costs to agriculture are much higher. Bovine tuberculosis is also an important zoonosis. Worldwide, the incidence of TB in humans is rising rapidly, with bTB being responsible for many cases in sub-Saharan Africa, where human–cattle contact is often close (Grange 2001). Even in the UK, spill-over of bTB from cattle to humans has recently been reported (Smith *et al.* 2004). Although the prevalence in cattle has been reduced to low levels in much of England, it remains an intractable problem in the South West (Krebs & The Independent Scientific Review Group 1997). New cases are also emerging in parts of the country previously free of bTB. The number of cases in cattle has risen annually over the past two decades, with the rate of increase now being around 18% per annum (DEFRA 2004). Attention has, therefore, focused on the possibility of a wildlife reservoir.

Badgers in the UK, like possums in New Zealand, are known to be susceptible to bTB and their control has formed an integral, if controversial, part of bTB control strategies for many years (Zuckerman 1980; Krebs & The Independent Scientific Review Group 1997). The incidence of bTB in cattle is highest in areas with greatest badger density, and there is some evidence for a fall in bTB incidence in cattle when effective culls of badgers have

*Author for correspondence (fiona.mathews@zoo.ox.ac.uk).
†Present address: Veterinary Services, Department of Physiology, University of Oxford, Parks Road, Oxford OX1 3PT, UK.
‡Present address: Department of Infectious Disease Epidemiology, Faculty of Medicine, Imperial College (St Mary's Campus), Norfolk Place, London W2 1PG, UK.

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been conducted (Wilesmith *et al.* 1982; Clifton-Hadley *et al.* 1995; Krebs & The Independent Scientific Review Group 1997; Griffin *et al.* 2005). However, the incidence of bTB in cattle has continued to rise in Great Britain despite policies of badger removal. This may be due to difficulties in implementing complete culls in routine practice, and/or because factors other than badgers are important in bTB epidemiology. An experimental cull of badgers is now being conducted (randomized badger culling trial (RBCT); Krebs & The Independent Scientific Review Group 1997) to quantify the impact of culling badgers.

The ability of *Mycobacterium bovis* to colonize a wide range of laboratory, domestic and zoo animals host species, in addition to badgers, is well established (Griffith 1937, 1939; Francis 1958). Two groups of animals susceptible in the laboratory—rodents and lagomorphs—are particularly numerous and widespread on farmland. Reviews of bTB control in Britain consistently highlight both the potential importance of wildlife other than badgers in the epidemiology of bTB and the lack of adequate information about them (MAFF 1987; Krebs & The Independent Scientific Review Group 1997; House of Commons Agriculture Committee 2003). It is even unclear which wildlife species are infected. Post-mortem examinations have previously identified *mycobacterium bovis* in ferrets (*Mustela putorius furo*), moles (*Talpa europaea*), rats (*Rattus norvegicus*), foxes (*Vulpes vulpes*), mink (*Mustela vison)*, stoats (*Mustela erminea*), polecat (*Mustela putoris*) and deer (roe, *Capreolus capreolus*; red, *Cervus elaphus*; fallow, *Dama dama*; Muntjac, *Muntiacus reevesi* and sika, *Cervus nippon*) (MAFF 1987; Delahay *et al.* 2002). Of these, only ferrets and deer were thought to be potentially infectious (i.e. likely to be excreting bacilli; Krebs & The Independent Scientific Review Group 1997). Further evidence of the possible importance of deer comes from the USA, where bTB appears to be transmitted from deer to livestock (Schmitt *et al.* 2002; Wilkins *et al.* 2003); and from Spain, where deer and livestock from the same location have been found to share the same spoligotype of *mycobacterium bovis* (Aranaz *et al.* 2004). There has also been a single isolation from a 'vole' in the UK, but this was an archived sample and the species is unknown (Delahay *et al.* 2002). The pathogen has not been isolated from any other wild British mammal (MAFF 1987). However, sample sizes have generally been too low to give a reasonable chance of detecting the disease or to give acceptable confidence intervals around the estimates. We have, therefore, conducted an extensive survey to determine the prevalence of animals infectious for bTB across a range of wildlife species, within a well defined sampling frame in England and Wales.

2. MATERIAL AND METHODS

(a) *Study design*

We undertook a cross sectional survey of wild mammals present on farms with a recent history of bTB in cattle and controls. We primarily used live-sampling in our surveillance, in contrast to previous research which has used lethal sampling (Wilesmith *et al.* 1982; MAFF 1987). The latter is a good means of diagnosing infected individuals since animals not excreting bacilli can be detected. However, live-sampling produces robust estimates of the point prevalence of infectious individuals—a key parameter required for the evaluation of risk to cattle. Extrapolation from post-mortem evidence—which is usually derived from tissue samples rather than excretory products collected post-mortem—is difficult if animals excrete the infectious agent intermittently (there is some evidence for this in badgers (Clifton-Hadley *et al.* 1993) and cattle (Neill *et al.* 2001), and the mechanism could operate in other species). Similarly, the necessary detailed pathological investigations are difficult with small animals, especially if large sample sizes limit examination times. Lethal sampling also precludes the use of systematic or longitudinal surveys of ecosystems, and may have the undesired effect of increasing bTB prevalence (see association between limited badger culling and increased bTB in cattle in randomized badger culling trial Donnelly *et al.* 2003)

(b) *Site selection*

Surveys were conducted on 12 dairy farms. The farms were arranged as geographical 'triplets' comprising an index case farm, a further case farm, and a control farm. A ratio of two cases to one control was used to maximize the power of the study to detect animals infectious for TB if they were truly present on case farms (the issue of greatest interest to DEFRA). Case farms were defined as having ≥ 1 confirmed breakdown since 1997 (confirmation by culture of *M. bovis* from tissues of culled cattle) and ≥ 2 incidents since 1997, where the herd contained positive reactors to the tuberculin skin test (Monaghan *et al.* 1994). Control farms were defined as having no confirmed breakdowns and no reactors since 1994. The index farms were randomly selected from a sampling frame of all 'case' dairy herds in England and Wales with >80 cows which were outside the RBCT areas (on instruction from DEFRA), and which were within 20 km of another case farm. This random selection ensures that the data are free from the unquantifiable biases that could otherwise be introduced through the use of other selection strategies. The second case farm in each triplet was randomly selected from all those within 20 km of the index farm. Cases caused by the importation of bTB-infected cattle from elsewhere were excluded from the study. The cause of the breakdowns on our case farms was attributed, on the DEFRA database, either to 'badgers' or 'unknown' causes (but see Krebs & The Independent Scientific Review Group 1997 for discussion of the difficulties classification of breakdowns as due to badgers). The control farm in each triplet was randomly selected from those present within a 10 km^2 grid centred on the mid-point of the two case farms. The 'triplet' design means that geographical variations in species distribution (for example, yellow-necked mice have a localized distribution) would be expected to affect both case and control farms similarly.

Sampling was conducted on eight 'case' and four 'control' farms and took place from 2001 to 2004, with a gap during the Foot and Mouth epidemic. The farms were located in four regions: Staffordshire/Derbyshire; North Somerset; Carmarthenshire; and Gwent. At one case farm in Staffordshire (farm 2), sampling of small mammals, rabbits and squirrels was incomplete due to the unpredicted start of the RBCT in the area.

(c) *Trapping*

On the first seven farms (five case, two control) we aimed to live-sample a representative cohort of all terrestrial species present on study farms, with the exception of deer which are

extremely difficult to handle within acceptable welfare limits, and which were the subject of a separate study by DEFRA based on lethal sampling. For the remaining farms we focused on sampling small mammals (<30 g), rabbits, squirrels, rats and domestic or feral cats and dogs; other species being again the subject of the separate study based on lethal sampling. Badgers were trapped throughout the study, except during the 'closed' season (Jan–May inclusive, as defined by English Nature and the Countryside Council for Wales). Representative sampling of badgers was, therefore, possible on four case and two control farms (one additional animal was caught during a short trapping period on a further case farm). Detailed ecological surveys to record habitat composition and animal field signs were undertaken on each farm. Longworth traps (Penlon Ltd, Abingdon, UK) to capture small mammals (<30 g) were placed along 100 m sections of field boundary (usually hedgerow, occasionally wall or woodland edge). The trapping sites were randomly selected from all those which bordered fields used for cattle grazing or for forage production in the previous year. Forty traps were used for each section, with the traps being positioned in pairs. The proportion of the available habitat sampled was very similar on case and control farms (18.8% (s.d.=7) on control farms and 19.8% (s.d.=7) on case farms; $t=-0.224$, d.f.=10, $p=0.83$). The total length of hedgerow, and the overall area of the farm, were also very similar on case and control farms.

Traps designed for other species (rats, moles, rabbits, squirrels, stoats and weasels, foxes and badgers) were placed according to field signs, as were additional Longworth traps around farm buildings. All traps were provisioned with food. Bedding was provided in Longworth traps, and bubble wrap provided additional insulation in winter. During extreme weather conditions, traps for larger animals were not set. Traps were set at dusk and checked at dawn. Squirrel traps were also used during daylight. All animals were temporarily marked using both fur clip-marks and hair dye in order to uniquely identify individuals. Decisions to stop trapping at any particular site were based on recapture rates. Conservative estimates of the proportion of the total population of each species sampled at each farm were based on the simple recapture rates and total catch numbers at each trap site (to estimate population size at each trap site), in conjunction with the proportion of suitable habitat trapped (for domestic animals, the entire population was sampled).

(d) *Clinical sampling*

Animals were transported to a mobile sampling station on each farm and, with the exception of polecats, foxes and badgers, were transferred into disinfected polypropylene holding containers. A mesh platform separated the animal, and any voided faeces, from its urine. Sampling was conducted under gaseous anaesthesia with isoflurane (Isoflo, Schering-Plough; Mathews *et al.* 2002). For polecats, foxes and badgers, anaesthesia was induced by injectable agents and maintained with isoflurane (Mathews *et al.* 2002). Tracheal aspirates were taken using gavage tubes (small mammals) (IMS, Congleton) or urinary catheters (Simms Portex, Hythe) of appropriate size for larger species. The aspirate was flushed into a specimen tube using sterile saline solution (0.9% vol.). Where possible, urine samples were obtained using external manual palpation of the bladder. Hartmann's solution (dose appropriate for body weight, 40 ml kg^{-1}; Wolfensohn & Lloyd 1998) was given by subcutaneous injection to counteract overnight dehydration in the trap, and to encourage urination in the holding container during recovery. Where sample volumes were small (<0.1 ml), sterile saline was added to the transport tube to prevent dehydration of the sample. Swabs were taken of any open sores. Faecal samples were obtained by the use of a warm water enema, and were also collected from the holding containers. If insufficient sample was obtained, additional faeces were collected from the animal's trap. Animals were released at the site of capture after recovery.

To minimize the risk of cross-contamination, sampling was conducted on disposable sheets. Work surfaces, holding containers and traps were disinfected between animals using an approved agent for mycobacteria (Trigene, Medichem Int., Sevenoaks) and sampling equipment was either disposable or sterilized.

(e) *Post-mortem examinations*

Carcasses of fresh road casualties, game bags, and accidental trap deaths, were collected. All animals originated within 5 km of each study farm. Post-mortem examinations were also conducted on any animals that died during sampling or recovery ($n=149$). Sampling of shrews under anaesthesia was terminated early in the project because of their high mortality rate (29%, 85/291). The mortality rate for species excluding shrews was 1.6% (64/3890). Post-mortems were conducted according to a standard sampling protocol, with specimens of key lymph nodes (retropharyngeal, bronchial, mediastinal, mesenteric, hepatic, popliteal (for animals >30 g only due to difficulty of locating them in smaller specimens), and prescapular (for animals >30 g)) and organs (lungs, liver, kidney, spleen, heart, bladder) being collected whether or not they showed visible lesions. Wherever possible, the sample types obtained for live animals (i.e. urine, faeces, tracheal aspirate and pus) were also collected. The tissue samples from each animal were pooled for initial culture work.

(f) *Culture*

Culture was conducted in category III containment laboratories. Samples were transported refrigerated, and processed within 24 h of collection wherever possible (samples from two case and one control farm were frozen for up to three months prior to processing due to laboratory relocation). Samples were decontaminated using the *N*-acetyl-L-cystein–NaOH digestion–decontamination procedure (Kent & Kubica 1985; Collins *et al.* 1997). An NaOH concentration of 1.5% was used for urine and swabs, and 3% for faeces. Tracheal and gastric aspirates were not treated. Prepared samples were seeded onto two slopes of modified Middlebrook 7H11 medium (7H11; VLA, Weybridge), two slopes of acidified Lowenstein Jensen (LJp) media modified with pyruvate (MFM, Bridgend), and 1 BBL Mycobacteria Growth Indicator Tube (MGIT 960; Becton-Dickenson, Oxford) containing Middlebrook 7H9 broth plus enrichment supplement. The 7H11 medium contained 'Mitcherson's cocktail' of antibiotics (fungizone, polymixin B, amoxil and trimethoprim), and the MGIT 960 medium contained polymixin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA).

The MGIT 960 tubes were incubated using a BACTEC automated culture system (Becton-Dickenson, Oxford) that monitors the tubes hourly for an increase in fluorescence. Possible isolates were subcultured onto solid media. Solid media were incubated at 37 °C for a minimum of 10 weeks. Initial diagnosis was by morphological means and

Table 1. Details of primers and methods used for PCR.

PCR	primer	sequence	[Mg]	anneal temp (°c)	amplicon size (bp)
IS*1081*	forward	5-CTGCTCTCGACGTTCATCGCCG	1.5	58	135
	reverse	5-GGCACGGGTGTCGAAATCACG			
RD7 flanking	forward 1	5-ATCTTGCGGCCCAATGAATC	2.0	58	211
	forward 2	5-TCGGTCAGCAAGACGTTGAAG			105
	reverse	5-ACTTCAGTGCTGGTTCGTGG			
RD4 flanking	forward 1	5-AATGGTTTGGTCATGACGCCTTC	2.0	58	176
	forward 2	5-TGTGAATTCATACAAGCCGTAGTCG			142
	reverse	5-CCCGTAGCGTTACTGAGAAATTGC			
RD8 flanking	forward	5-GAGTCTATATAGTGTGCTCATGGGGCTAGC	2.0	64	178
	reverse	5-GCTTGCTGGCGATCATTGGTCT			
RD13 flanking	forward	5-ATCGCTCGTTCGTCGGCTTC	2.0	60	136
	reverse	5-GGCAAGACCGGGCCTTTGAC			

Ziehl-Neelsen Staining (Kent & Kubica 1985). Confirmatory testing of presumptive positives was by growth onto selective media by the TB Reference Laboratory, Weybridge. All presumptive positives were also confirmed by PCR.

(g) *DNA extraction and PCR*

DNA was extracted from cultures using NucliSens lysis and DNA isolation kits (Biomérieux UK Ltd, Hants, UK). Three PCR tests were routinely applied to all presumptive cases of *M. bovis*. These were: PCR for IS1081, a multi-copy element generally present in six copies in members of the *Mycobacterium tuberculosis* (MTB) complex (Dziadek *et al.* 2001). Additional PCRs were used to detect and distinguish between *Mycobacterium microti* and classical *M. bovis*. This was achieved by designing primers flanking deletion regions in the *M. bovis* lineage known as RD7 and RD4 primers, respectively, (Gordon *et al.* 1999). PCR products are obtained if these deletion events have occurred, and locate the isolate within the evolutionary model for the MTB complex (Brosch *et al.* 2002). The RD7 method detects some isolates of *Mycobacterium africanum*, all *M. microti* strains and the closely related species *Mycobacterium pinnipedii*, found in seals, as well as all members of the *M. bovis* lineage including the caprine variant (Aranaz *et al.* 1999). The RD4 PCR detects only the 'classical' isolates of *M. bovis* and BCG.

As DNA quality was an issue with some extracts, primers amplifying smaller fragments were additionally used for the RD4 and RD7 methods. The status of deletions RD8 and RD13 was determined in some cases as a quality-control on the allocation of strains to the *M. bovis* lineage. The sequences of these primers and details of the individual methods are shown in table 1. Amplification of templates took place in a final volume of 25 µl using the PCR Excite core kit (BioGene Ltd, Cambs., UK). Routine gel electrophoresis on 3% agarose was used to analyse the PCR products. PCR-based typing (Spacer-OLIGOnucleotide TYPING—'spoligotyping') was conducted to determine strain-types as previously described (Aranaz *et al.* 1996). Authoritative names for spoligotype patterns were obtained from www.Mbovis.org.

(h) *Data analysis*

Prevalences were estimated from the number of animals with at least one positive culture of *M. bovis*. The prevalence of infectious individuals was calculated as the proportion of live-sampled individuals of the species that were *M. bovis* positive. The prevalence of infected individuals was calculated as the proportion of the total number of animals sampled (live and by post-mortem examination) that were *M. bovis* positive. It should be noted that the prevalence estimates for infected (cf. infectious) animals will be underestimates, since complete data could only be obtained by post-mortem examination of all individuals. For simplicity, we made no adjustments for the completeness of sampling on each individual (number of specimens obtained) or for the contamination of culture slopes. Computation of confidence intervals was performed using Wilson's methods for small proportions (Wilson 1927). The structured sampling strategy used in the project facilitates cross-species comparisons of bTB prevalences within farms. Because of the very small number of positive isolates, it was not possible to make sensible estimates of potential positive correlations of bTB events within farms (the intracluster correlation; Fleiss 1981). The true population-level confidence intervals are, therefore, likely to be slightly wider than those we report. However, because of the low disease prevalence, this is unlikely to have a material impact on the interpretation of our results.

(i) *Mathematical models*

The following single host model with free-living infective stages and density dependence in the death rate was used to describe the disease dynamics

$$\frac{dY}{dt} = \beta XW - (b + sH)Y,$$
$$\frac{dH}{dt} = H(r - sH),$$
$$\frac{dW}{dt} = \Lambda Y - \mu W,$$
$$H = X + Y.$$

This is a simple, well-understood model based on the work of Anderson & May (1981) where Y is the density of infected animals, X is the density of susceptible hosts, H it the total population density and W is the density of infectious units, where an infectious unit is defined as sufficient bacilli to cause infection in another host of the same type. The transmission rate between infectious particles and susceptible hosts is given by β, b is the natural host death rate of the host, s is a measure of the density dependent constraints on the host, r is the growth rate of the host in the absence of disease, Λ is the rate of production of infectious units per infected host, and μ is the death/decay rate of infectious units. The carrying capacity of the system, K is given by r/s. The basic reproductive rate of the

Table 2. Numbers of animals trapped and clinical samples obtained by live-sampling. (Notes: *n* refers to numbers in each category. TA is tracheal aspirate. TA column includes 30/41 cats and 20/20 dogs that had throat swabs rather than TA at request of owners. Due to licensing restrictions, badgers were sampled on 6/12 farms only. Polecats and foxes were sampled on the first 8/12 farms. Most species were trapped on all farms. For those which were not (either due to licensing restrictions or failure of capture), the following numbers (%) were obtained on control farms: yellow-necked mouse 40 (15); house mouse 15 (26); pygmy shrew 1 (13); water shrew 5 (45); rabbit 48 (24); polecat 1 (14); fox 3 (33); badger 14 (33).)

		clinical samples (*n* (%))		
species	animals (*n*)	TA	urine	faeces
yellow-necked mouse (*Apodemus flavicollis*)	268	254 (94.8)	216 (80.6)	255 (95.1)
wood mouse (*Apodemus sylvaticus*)	1338	1204 (90.0)	1110 (83.0)	1277 (95.4)
house mouse (*Mus musculus*)	58	51 (87.9)	31 (53.4)	51 (87.9)
bank vole (*Clethrionomys glareolus*)	1307	1131 (86.5)	1130 (86.5)	1221 (93.4)
field vole (*Microtus agrestis*)	330	259 (78.5)	302 (91.5)	317 (96.1)
rat (*Rattus norvegicus*)	76	73 (96.1)	39 (51.3)	68 (89.5)
common shrew (*Sorex araneus*)	272	3 (1.1)	29 (10.7)	208 (76.5)
pygmy shrew (*Sorex minutus*)	8	2 (25.0)	2 (25.0)	6 (75.0)
water shrew (*Neomys fodiens*)	11	0 (0)	7 (63.6)	10 (90.9)
rabbit (*Oryctolagus cuniculus*)	202	196 (97.0)	109 (54.0)	145 (71.8)
grey squirrel (*Sciurus carolinensis*)	189	179 (94.7)	26 (13.8)	95 (50.3)
polecat (*Mustela putorius*)	7	4 (57.1)	3 (42.9)	0 (0.0)
fox (*Vulpes vulpes*)	9	9 (100)	3 (33.0)	8 (88.9)
badger (*Meles meles*)	43	32 (74.4)	25 (58.1)	16 (37.2)
dog (*Cannis cannis*)	21	21 (100)	0 (0.0)	1 (5.0)
cat (*Felis domesticus*)	42	42 (100)	9 (21.4)	5 (11.9)
total	4180	3460	3041	3683

system, R_0 is the number of secondary infections produced if we add one infectious individual to a completely susceptible population at carrying capacity. It can found via standard stability analysis of the equilibria (Anderson & May 1981) but can also be derived intuitively from the equations as follows. If we assume we are in the disease free state, where $X=H=K$ and we add one infectious individual, they produce infectious particles per unit time and live for $1/(b+sK)=1/(b+r)$. Each infectious particle can infect βK susceptibles per unit time and lives for $1/\mu$ units of time. The number of infections that arise from the initial infection is, therefore, given by the product of theses terms, i.e. $(\Lambda\beta K)/(r+b)\mu$. This model assumes that there is no death due to the disease, since it is unlikely that deaths from bTB have a material impact on population demography. It is also assumed that there is no immunity to, or recovery from, the disease.

We used the model to determine how likely it is that the disease would persist within each of the host species of interest at the prevalences seen in the field, without transmission between species. Values for most of the parameters were estimated for each of the species under investigation using a combination of information in the literature and observations from our study as described below (values in table 4). Then, using the prevalences seen in the field and assuming that the system is at equilibrium, we were able to use the fact that for this model, prevalence $=1-(1/R_0)$ to calculate the basic reproductive number, R_0, for each of the species in question. This result is derived for this model from equilibrium stability analysis. We could then also calculate β, the transmission rate for each species.

Per capita birth and death rate parameters were calculated per day based on average litter sizes and average numbers of litters yr^{-1} and average life expectancies reported in the literature (Macdonald & Barrett 1993; Corbet & Harris 1996; Macdonald *et al.* 1998). For simplicity, these life history parameters were assumed to be the same for yellow-necked mice, wood mice, bank voles and field voles. Therefore, for these rodents, our approach is equivalent to formulating a multi-species model. The density dependence parameter, s, was calculated from the carrying capacity. We did calculations for two different carrying capacities, one of which was based on the upper 95% confidence interval of the number observed per unit area, and one based on the maximum caught per unit area. The latter was included because the mean population estimates are likely to be conservative, since it is not possible to trap the entire population. Although the choice of animal densities influences the transmission parameter, β, it does not affect the basic reproductive rate of the disease, R_0 which is independent of all of the life history parameters.

The rate of production and decay of infectious units was taken to be 1 day^{-1}, this was based on expert opinion of the minimum likely rates. Although the number of bacilli in an infectious unit will vary from host to host, this does not affect our model, since it focuses on within-species transmission (or transmission within similar rodent species). As with the choice of animal densities, inaccuracies in the estimates of infectious unit production and decay rates would influence the transmission parameter, β, but not the basic reproductive rate of the disease, R_0.

3. RESULTS

In total, 4180 animals of 16 species were live sampled (table 2). The numbers of each species trapped were similar on case and control farms (in *t*-tests for differences between farm-types, $p>0.5$ for all species except squirrels, where $t=1.474$, d.f. $=10$, $p=0.171$). Most species were trapped on every farm. For those which were not, the distribution of species occurrence was either similar, or was higher on case farms (table 2). Faecal samples were obtained for 88% ($n=3683$). Excluding the shrews ($n=291$), which we largely did not attempt to sample

Table 3. Prevalence of infectious individuals and confidence intervals. (Notes: raw confidence intervals computed using Wilson's method for small proportions (Wilson 1927).)

species	prevalence infectious animals % (*n*/*n*)	raw 95% confidence intervals
yellow-necked mouse (*Apodemus flavicollis*)	0 (0/268)	0, 1.4
wood mouse (*Apodemus sylvaticus*)	0 (0/1338)	0, 0.3
house mouse (*Mus musculus*)	0 (0/58)	0, 6.2
bank vole (*Clethrionomys glareolus*)	0.1 (1/1307)	0.0, 0.4
field vole (*Microtus agrestis*)	0 (0/330)	0, 1.2
rat (*Rattus norvegicus*)	0 (0/76)	0, 4.8
common shrew (*Sorex araneus*)	0 (0/272)	0, 1.4
pygmy shrew (*Sorex minutus*)	0 (0/8)	0, 32.4
water shrew (*Neomys fodiens*)	0 (0/11)	0, 25.9
rabbit (*Oryctolagus cuniculus*)	0 (0/202)	0, 1.9
grey squirrel (*Sciurus carolinensis*)	0 (0/189)	0, 2.0
polecat (*Mustela putorius*)	0 (0/7)	0, 35.4
fox (*Vulpes vulpes*)	0 (0/9)	0, 30.0
badger (*Meles meles*)	7.0 (3/43)	2.4, 18.6
dog (*Cannis cannis*)	0 (0/21)	0, 15.5
cat (*Felis domesticus*)	0 (0/42)	0, 8.4

under anaesthetic, urine samples were obtained for 77% ($n=3003$), and tracheal or gastric aspirates for 88% ($n=3455$). Across all species, three types of clinical sample were obtained from 65% ($n=2736$), and at least two types from 86% ($n=3577$). The ease with which clinical samples were obtained varied across species (table 2). Sampling was most complete for mice and voles, and these were also the animals trapped most frequently on study farms. Post-mortem samples were obtained for a further 213 animals (5 wood mice, 1 house mouse, 9 bank voles, 1 field vole, 17 rats, 45 common shrews, 1 water shrew, 14 rabbits, 5 squirrels, 1 polecat, 100 foxes, 8 badgers, 1 hedgehog, 3 moles and 2 fallow deer). Post-mortem samples were also obtained from 149 (4%) of the animals previously live sampled.

Contamination rates for cultures were higher on the egg-based media, and were lower for urine than for faeces or tracheal aspirates (the latter were not decontaminated). The contamination rates (%) for LJp slopes, 7Hll slopes, and MGIT 960 tubes were: urine <1, <1, 13; faeces 24, <1, 20; tracheal aspirates 40; 5; 15. Very few animals ($<1\%$) had contamination on all cultures.

One bank vole and three badgers were found to be infectious for *M. bovis*. The isolates were made on MGIT 960 medium (bank vole), LJp medium ($n=2$ badgers) and 7H11 medium ($n=1$). The specimen types were tracheal aspirates (bank vole and $n=1$ badger) and pus ($n=2$ badgers). Table 3 shows the 95% confidence intervals for the prevalence of infectious individuals for all the species sampled. Inclusion of finite population corrections (Cochran 1977) did not materially alter the 95% confidence intervals with the following exceptions (raw 95% CIs): house mouse (0, 5.0), rat (0, 4.4), rabbit (0, 1.6), grey squirrel (0, 1.8), dog (0, 8.0) and cat (0, 4.0).

One of the eight badgers, but none of the other animals, examined post-mortem was infected with *M. bovis* (spoligotype SB0129). This diagnosis was made on MGIT and LJp media. The prevalences of infection (%) (raw 95% Confidence Intervals) for badgers was 6.7 (2.5, 15.0). Genotyping with a range of PCR methods specific for either the MTB complex or for *M. bovis* provided consistent confirmation of *M. bovis* for all the cases. No isolation of *M. microti* was made using either the live- or post-mortem samples.

M. bovis was isolated from badgers on two of the four case farms, and from no badgers on the two control farms, where representative trapping was possible. The *M. bovis* isolate from the bank vole (1/4) was of the same spoligotype (SB0673) as isolates from cattle and two badgers at the same farm. The spoligotypes of the isolates from badgers were all of the same type as those previously identified in cattle at the same farm (4/4; Fisher's exact test to compare badgers and bank voles $p=0.143$, note power of comparison is constrained by small sample size). The spoligotypes for the badger isolates were SB0129 at farm 1 ($n=2$ badgers, including dead specimen) and SB0673 at farm 2 ($n=2$ live-sampled badgers).

The results of the mathematical modelling are shown in table 4. Since the lower values of the prevalence confidence intervals are all zero we cannot predict exactly what transmission rates and basic reproductive rates will be. We simply know that we must have $R_0<1$ and hence some upper limit on β. The values of the transmission parameter β, were all low. Depending on the prevalence, and the values chosen for the population density parameter, β ranged from 0.002 to 0.007. Even with the upper bound of the confidence intervals for prevalence of infectious individuals, the transmission rates and basic reproductive rates would have to be extremely small to be compatible with the prevalences we observed in the field. By comparison, the R_0 values for badgers ranged from 1.025 to 1.229 (based on the upper and lower confidence limits of the prevalence estimates). This is similar to the estimates of $R_0=1.1$ to 1.2 obtained from other, larger, field studies of bovine TB in the badger (Smith 2001).

4. DISCUSSION

We have identified *M. bovis* for the first time in the bank vole (*Clethrionomys glareolus*). The culture of *M. bovis* from tracheal aspirate suggests that the bank vole was not simply transporting bacilli consumed in food, but had a systemic infection. The strain type we identified in the bank vole was the same as that found in badgers and cattle on the same farm. Bovine tuberculosis is known to have

Table 4. Selected input parameter values and basic reproductive rate, R_0, estimated from the mathematical model. (Notes: $R_0 = 1/1 - \text{prevalence}$, where prevalence is the upper confidence interval for prevalence of individuals infectious for *M. bovis* as given in table 3. The lower confidence intervals for prevalence always gave estimates for R_0 that were <1.0.)

	densities (measured in field)			demographic parameters (from literature)		
species	mean	95% confidence interval	maximum	birth rate day^{-1} (*a*)	death rate day^{-1} (*b*)	R_0 (computed from model)
yellow-necked mice/100 m	2.8	1.8, 3.8	22	0.0274	0.001 825	1.0142
Wood mice/100 m	7.2	6.4, 8.0	29	0.0274	0.001 825	1.0030
Bank voles/100 m	6.4	5.7, 7.1	29	0.0274	0.001 825	1.0040
Field voles/100 m	1.4	1.0, 1.7	12	0.0274	0.001 825	1.0122
common shrews/100 m	0.9	0.6, 1.2	12	0.002 16	0.008 120	1.0142
all small rodents/100 m	16.9	15.5, 18.4	49	0.0274	0.001 825	1.0030
rats/0.25 ha	7.5	3.1, 11.9	23	0.035 96	0.002 740	1.0504
rats/0.25 ha	17.8	6.7, 28.8	49	0.0288	0.002 740	1.0194
squirrels/ha	13.0	7.6, 24.4	46	0.0123	0.002 740	1.0204

a wide host range and the epidemiology of multi-host pathogens can be extremely complex (Swinton *et al.* 2002). Generalist pathogens may be endemic to one or more host species, and can occasionally spill over into other host populations, which are incapable of maintaining the infection alone, perhaps because of low host density. Spill-over hosts may or may not be able to transmit the infection to further host populations. The spatial structuring of host populations, as frequently occurs with multi-species host arrays, can offer increased potential for parasite persistence; epidemics can occur asynchronously in the different populations, thereby avoiding the deep global troughs associated with pathogen extinctions (Bolker & Greenfell 1995; Hudson *et al.* 1995). If small mammals were proven to be more than just spill-over hosts, the control of bTB via wildlife management could be even more challenging than previously thought. *M. bovis* was isolated from badgers on two of the six farms, where we were able to conduct representative trapping. The spoligotypes of the badger isolates always (4/4) matched those of cattle isolates from the same farm. However, there were no statistically significant differences between bank voles and badgers in whether their spoligotypes matched those in cows (possibly due to low sample sizes).

Overall, our study has shown that the prevalence of infectious individuals is very low for most non-badger farmland wildlife in the UK. The average numbers of animals trapped, and the distribution of species were similar on case and control farms; there is, therefore, no evidence that there is underestimation of prevalence due to 'over-sampling' of control farms (where prevalences might have been expected to be lower). Estimates of the prevalence of all infected animals will necessarily be slightly higher than estimates of infectious animals, since not all infected individuals will be excreting bacilli at a given time-point. However, it is the prevalence of infectious animals that is the key parameter in the estimation of risk posed to cattle. It is possible that the disease is truly present at higher levels than we have observed using culture—the gold standard method of diagnosis (Kent & Kubica 1985). We suggest that our estimates of the prevalence of infectious individuals should be regarded as minima; we were unable to collect the full range of clinical samples from all individuals, and the numbers of bacilli in the original samples are radically reduced by the decontamination procedures and by adhesion to collection tubes (Chadwick 1981; Kent & Kubica 1985; Collins *et al.* 1997). Contamination of cultures, which is necessarily higher in clinical samples than post-mortems (the method that has been conventionally used for cattle), further reduces the chance of successfully culturing *M. bovis* from an infectious animal. In an attempt to maximize the sensitivity of diagnosis, we employed the MGIT 960 culture system in additional to solid media. The use of both solid and liquid media is recommended to maximize isolation rates (Kent & Kubica 1985; Collins *et al.* 1997) and, at least with clinical samples from humans, the MGIT 960 medium (and its precursor the BACTEC 9000MB system) have been shown to have high sensitivity relative to solid media for the isolation of MTB complex (e.g. Pfyffer *et al.* 1997; Somoskövi & Magyar 1999; Tortoli *et al.* 1999). We are now using direct PCR methods on our stored samples to provide independent estimates of the prevalence of infectious individuals.

Despite the low prevalence of infectious individuals, small mammals, squirrels, rabbits and rats are extremely common on farmland, and might, therefore, be important to bTB control in cattle. We explored the epidemiology of bTB in these species using mathematical models. This work showed that for any of the species to maintain the disease at the levels observed in the field then the basic reproductive number, and consequently the transmission rate, must be extremely low. It is, therefore, unlikely that the disease would persist within single-host systems in the wild: the animals are unable to pass on the infection to their conspecifics at a sufficiently high rate. These findings are robust to the possible under-diagnosis of infection. To have a material impact on the estimates of R_0, such that the maintenance of the disease were likely even in the absence of badgers and cattle, the prevalences of infectious individuals would need to be very substantially higher than those we have observed (for example, for R_0 to equal 1.5, the prevalence would need to be 33%).

If instead of single-host models, multiple-host systems were assumed with the previously estimated within-species transmission rate, then the additional between-species route of transmission would lead to prevalences of infection higher than those we actually observed. Alternatively, the

within-species transmission rate would need to be even lower than that estimated by the single-system model in order to achieve the infection prevalences seen in our surveys. Multi-species transmission of bTB in farmland wildlife communities, therefore, seems unlikely. Although spill overs from established hosts, such as badgers and cattle, may occur, even these must be rare events in order for prevalences to be limited to levels consistent with our field observations. We conclude from our extensive field screening, complemented by modelling work, that common farmland wildlife other than badgers are relatively unimportant to the control of bovine TB in cattle.

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