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The prevalence and distribution of *Mycobacterium bovis* infection in European badgers (*Meles meles*) as determined by enhanced post mortem examination and bacteriological culture

D. Murphy [a,*], E. Gormley [a], E. Costello [b], D. O'Meara [b], L.A.L. Corner [a]

[a] School of Agriculture, Food Science and Veterinary Medicine, College of Life Sciences, University College Dublin, Belfield, Dublin 4, Ireland
[b] Central Veterinary Research Laboratories, Department of Agriculture, Fisheries and Food, Backweston, Co. Kildare, Ireland

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ABSTRACT

The accurate diagnosis of *Mycobacterium bovis* infection in badgers is key to understanding the epidemiology of tuberculosis in this species and has significant implications for devising strategies to limit spread of the disease. In this study, badgers ($n = 215$) in the Republic of Ireland were examined at post mortem and tissues were collected from a range of anatomical locations and pooled into groups for bacterial culture of *M. bovis*. By assessing confirmed gross visible lesions (VL) alone, infection was detected in 12.1% of badgers. However, by including the results of all culture positive pooled samples, the overall infection prevalence increased significantly to 36.3%. Two-thirds (66.7%) of infected animals had no visible lesions (NVL). While the thoracic cavity (lungs and pulmonary lymph nodes) was found to be the most common site of infection, in a proportion of animals infection was absent from the lungs and draining lymph nodes and was confined to the lymph nodes of the carcase or the head. This may indicate an early extrapulmonary dissemination of infection or alternatively, in the case of the head lymph nodes, a secondary pathogenic pathway involving the lymphoid tissues of the upper respiratory tract (URT).

1. Introduction

The European badger (*Meles meles*) is recognised as the principal wildlife reservoir of *Mycobacterium bovis* (*M. bovis*) infection in Ireland and in the UK (Nolan and Wilesmith, 1994; Eves, 1999) and is implicated in the transmission of infection to cattle. Tuberculosis (TB) – infected badgers were first identified in the Republic of Ireland in 1974 (Noonan et al., 1975) and since then have been found in all of the 26 counties (Dolan, 1993). Although long considered to be a likely source of infection for cattle, the results of the East Offaly and Four Area studies formally demonstrated that the widespread and sustained removal of badgers led to a dramatic and significant reduction in the prevalence of infection in associated cattle populations (O'Mairtin et al., 1998; Griffin et al., 2005). Having established this direct link between transmission of infection from badgers to cattle, a national medium term strategy for the control of TB in cattle was adopted that included removal of badgers. The strategy involved the focal culling of badgers from farms with severe TB breakdowns and where, following an epidemiological investigation, infected badgers were considered as the likely source of the breakdown (O'Keeffe, 2006).

Studies both in Ireland and the UK indicate that tuberculosis in badgers, in common with other species, is essentially a respiratory disease but infection may be detected in other organs (Gallagher et al., 1976; Fagan, 1993; Gallagher, 1998). The precise routes of intra- and inter-species transmission and the relative importance of alternative routes (e.g., aerosol and ingestion) have not been determined. One of the difficulties in establishing the routes of transmission of infection is that diagnosis of the disease has largely focused on the infected animal with gross visible lesions and consequently the contribution of animals with sub-clinical infection may have been underestimated. The bacteriological culture of *M. bovis* from infected tissues collected at post mortem examination offers the most sensitive and specific means of diagnosing tuberculosis in this species. However, it is a time-consuming and labour intensive process and is most often used to confirm infection in lesioned tissue samples rather than to enhance diagnostic sensitivity. Examination for gross lesions alone is likely to provide an underestimate of the true prevalence of infection in targeted populations.

The evidence gathered to date from pathology, histopathology and bacterial culture confirmation indicates that the primary route of transmission among badgers is by aerosol infection of the lungs (Gallagher et al., 1976; Fagan, 1993; Dolan, 1993; Gallagher and Clifton-Hadley, 2000; Gavier-Widen et al., 2001). It has been proposed that further respiratory dissemination of infection may

* Corresponding author. Tel.: +353 1 7166072; fax: +353 1 7166090.
E-mail address: denise.murphy@ucd.ie (D. Murphy).

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occur by spread of endogenous aerosols to other parts of the lungs. In addition, infection at distal anatomical sites may result from migration of bacilli via the lymphatics to other lymph nodes or haematogenously to other organs/tissues in the body (Gallagher and Clifton-Hadley, 2000). However, a significant proportion of infected badgers show no visible lesions (NVL) indicative of disease at post mortem examination (Gallagher et al., 1976; Fagan, 1993) suggesting that these badgers can effectively control the infection.

Detailed histopathological examination of infected but NVL badgers has revealed microscopic tuberculous lesions in the lungs and kidneys (Gallagher et al., 1998). This suggests that these NVL badgers may have the potential to excrete *M. bovis* even when gross lesions are not evident. It has also been shown that some NVL animals have minute histological tuberculous lesions with poor staining bacilli that may indicate either a containment phase of the infection or is evidence of a latent state of the disease in badgers, similar to that observed in humans (Parrish et al., 1998). The characteristics of tuberculosis in these NVL animals raise questions as to the role they may play in the epidemiology of the disease in badger populations and the implications for transmission to cattle.

Given the different states of infection that have been found in badgers, we set out to establish the true prevalence and distribution of tuberculosis infection in a population of naturally infected badgers with a view to understanding the pathogenesis of the disease in these badgers, and to gather information on likely transmission pathways. The results clearly showed that the sensitivity of diagnosis of infection was greatly enhanced when *M. bovis* culture results were included in the analysis, with a high proportion of NVL animals identified in the population. This lead to a significantly higher disease prevalence level when compared with the level determined by lesion examination alone. The results also showed that an alternative transmission route, based on the upper respiratory tract (URT), may be important in the epidemiology of the disease, which may have implications for transmission between species.

2. Materials and methods

2.1. Animals

The badgers ($n = 215$) examined in this study were obtained from DAFF culling operations and were sourced from broad geographical areas across 16 counties in the Republic of Ireland. They were removed under license and in accordance with conditions specified by the National Parks and Wildlife Division, Department of Environment, Heritage and Local Government. The study badgers were captured using stopped restraints, anaesthetised with ketamine hydrochloride (0.1 ml/kg) and medetomidine (Domitor®; 0.1 ml/kg) administered by intramuscular injection, and euthanased with an intravenous overdose of pentobarbital sodium (Corner et al., 2007). The badger carcases were kept at ambient temperature for transport and if not examined within 12 h of death were stored at 4 °C.

2.2. Post mortem examination

All badgers were subjected to a detailed necropsy, including an examination for bites and other wounds. Post mortem examinations were generally conducted within 24 h of euthanasia. The age (age classes: young <18 months, adult or old, based on an assessment of tooth wear) and sex of each badger was recorded. Tissue samples for bacteriology were collected using an aseptic technique so that culturing could be carried out with minimal decontamination, and also to minimise contamination with environmental microorganisms and cross-contamination of *M. bovis* between carcases. However, in a modified protocol all lung samples and carcase pool samples were decontaminated prior to culture. The anatomical sites examined for gross lesions and the samples collected and pooled into four groups, (head, thorax, carcase and abdomen pools) for bacteriological examination are listed in Table 1. Suspect gross TB lesions, including bite wounds and abscesses, were collected separately. Only gross lesions confirmed as tuberculosis by bacterial culture were included in the analysis. The lungs were examined visually and by palpation and gross lesions were taken for culture; where lesions were not evident the entire lungs were collected for culture. All samples were stored at −20 °C prior to culture.

2.3. Bacteriology

Samples were thawed at room temperature and macerated in a stomacher with 10 ml phosphate buffered saline (PBS) until a uniform suspension was obtained. The macerated tissue was centrifuged at 2,700*g* for 20 min. The supernatant was poured off and PBS 0.1% Tween added to the pellet. For each sample two plates of modified Middlebrook 7H11 medium (Gallagher and Horwill, 1977), one slope each of Lowenstein–Jensen with pyruvate (LJP) and Stonebrinks media with pyruvate, and one BACTEC MGIT 960 (Becton Dickinson, Sparks, MD) containing 0.8 ml PANTA

Table 1
The pooled samples collected for bacteriology, the tissues examined at post mortem for gross lesions, and the number of visible tuberculous lesions and non-tuberculous lesions confirmed by bacterial culture found in each tissue.

Bacteriology pool	Tissue examined	Gross lesions	
		TB	Non-TB
Head	Right parotid lymph node	3	1
	Left parotid lymph node	3	0
	Right mandibular lymph node	4	1
	Left mandibular lymph node	3	1
	Right retropharyngeal lymph node	2	1
	Left retropharyngeal lymph node	2 (22.4%)[a]	1
Carcase	Right prescapular lymph node	1	1
	Left prescapular lymph node	1	0
	Right axillary lymph node	0	1
	Left axillary lymph node	1	0
	Right inguinal lymph node	2	0
	Left inguinal lymph node	2	0
	Right popliteal lymph node	0	0
	Left popliteal lymph node	2 (11.8%)[a]	0
Abdomen	Spleen	0	0
	Liver	0	0
	Hepatic lymph node	1	0
	Mesenteric lymph node	4 (6.6%)[a]	1
Thorax	Anterior mediastinal lymph node	1	0
	Posterior mediastinal lymph node	4	1
	Right bronchial lymph node	6	1
	Left bronchial lymph node	5 (21.1%)[a]	0
[b]Lungs	Left cranial lobe	4	1
	Left caudal lobe	3	1
	Right cranial lobe	6	1
	Right middle lobe	3	1
	Right caudal lobe	3	1
	Accessory lobe	3 (28.9%)[a]	1
Skin	Bite wound/subcutaneous abscess	7 (9.2%)[a]	3
Total		76	19

[a] Indicates the percentage of visible tuberculous lesions found in each pool.
[b] All lungs were examined and if no lesion was seen or palpated, they were frozen at −20 °C. A sub-sample of the frozen lungs were later examined in detail for lesions and cultured – see text for details.

supplement (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) were inoculated. Where primary cultures were contaminated, a stored portion of the macerated sample was decontaminated with 0.075% w/v cetylpyridinium chloride (CPC) and the media inoculated. All inoculated media were incubated at 37 °C. The Stonebrinks/LJP media were examined weekly for 12 weeks. Microbial growth was detected in the MGIT tubes using a MGIT 960 system with a terminal reading at 3 months. When growth was detected by the MGIT 960 system smears were stained by the Ziehl–Neelsen (ZN) method to demonstrate acid-fast bacteria and cording, a characteristic of *M. bovis*. Modified 7H11 plates were used for colony count estimations and were incubated at 37 °C and examined after 28 days. Preliminary isolate identification was based on colony morphology, growth rate, pigmentation and cording characteristics (Collins et al., 1997) and by the AccuProbe DNA hybridisation probe for the *Mycobacterium tuberculosis* complex (Gen-Probe, Inc., San Diego, Calif). Isolates were confirmed as *M. bovis* by spoligotyping, which was performed according to the method of Kamerbeek et al. (1997).

A sub-sample of badgers' lungs was cultured using a modified protocol. The lungs selected for the modified protocol were from badgers where *M. bovis* had been isolated from at least one of the four pools but where the thorax pool, i.e. the pulmonary lymph nodes, was culture negative. For culture, the lungs were thawed overnight at room temperature. The lobes were then separated from the trachea: each lobe was serially sliced at 2–5 mm intervals using sterile scalpel and forceps and the cut surfaces examined for gross lesions. Where a lesion was identified it was collected for culture, otherwise the entire lobe was macerated in a stomacher and processed as described above except that 5% oxalic acid decontamination was employed and one MGIT tube, one LJP slope, one Stonebrinks slope and one 7H11 plate were inoculated. The inoculated media were incubated as described above.

2.4. Statistical analysis

Differences between groups were analysed using the Chi-square test. All analyses were performed using STATA SE 9 (www.stata.com).

3. Results

3.1. Prevalence and distribution of infection

A detailed post mortem investigation was carried out on each of the 215 badgers. By assessing culture confirmed gross visible lesions alone, infection was detected in 26 of the 215 badgers (12.1%). However, for the purpose of this study, a badger was considered infected when *M. bovis* was isolated by bacterial culture from any sample. Using this standard *M. bovis* was isolated from 78 of the 215 badgers examined, giving a significantly higher prevalence of infection of 36.3%. Generalised disease, which we have defined as infection confirmed by bacterial culture in all four anatomical pools, was uncommon ($n = 5$, Table 2). Those animals that were culture positive in only one pool ($n = 42$) had an even distribution of infection across the head, thorax and carcase pools, however, the abdomen pool was less frequently involved. Among the 78 infected badgers were included the 26 badgers with confirmed gross visible lesions (VL). The individual tissue distribution of culture confirmed VL and non-TB lesions is shown in Table 1. A total of 76 VL were observed in these 26 badgers, including seven tuberculous bite wounds/subcutaneous abscesses. In comparison, 19 non-tuberculous lesions, including three bite wounds or subcutaneous abscesses, were observed in six animals. Most VL (28.9%) were found in the lungs followed by tissues in the head pool, the thorax pool, the carcase pool with the least number of VL found in tissues of the abdomen pool. In 6 of the 7 animals that had VL in the lungs, the thorax pool was also culture positive.

Table 2
Anatomical distribution of *M. bovis* infection in cull badgers.

No. pools positive	Head	Carcase	Abdomen	Thorax	No. of animals infected
1	14	11	3	14	42
2	12	13	7	12	22
3	5	7	6	9	9
4	5	5	5	5	5
Total	36	36	21	40	78

Bites wounds/abscesses accounted for 9.2% of VL. In total, 10 overt bite wounds or subcutaneous abscesses possibly arising from bites, were observed in 9 badgers (4.2% of the total examined) but, whereas *M. bovis* was cultured from all 9 badgers, only 7 of the bite wounds/abscesses were culture positive for *M. bovis*. The confirmed tuberculous bite wounds/abscesses were found on the cheek and around the mouth ($n = 4$), the axilla ($n = 1$), the groin ($n = 1$) and the back of the knee ($n = 1$).

3.2. Culture of M. bovis in lung samples

Analysis of the data revealed that there were 37 infected badgers in the study population where the thorax pool (pulmonary lymph nodes) was culture negative and there were no VL in the lungs. In addition, there was one badger where the thorax pool was culture negative but with a VL in the lungs. In order to gain a more complete understanding of the profile of disease in the 37 animals, sections of the lungs were processed for culture. *M. bovis* was isolated from the lungs of seven of the badgers (mean no. lobes infected 3.4, range 1–6). When combined with the data generated from culture of the thorax pool, *M. bovis* was isolated from the thoracic cavity (pulmonary lymph nodes and/or lungs) of 48 of the 78 infected badgers (61.5%). The distribution of infection in the remaining 30 badgers from which *M. bovis* was not isolated from the thoracic cavity was similar across the head ($n = 17$) and the carcase pool ($n = 16$) but the abdomen pool ($n = 4$) was less frequently infected. Visible lesions were detected in 5 of these 30 badgers (16.7%), 2 animals with head lesions, one of which had a bite wound on the right cheek, 2 carcase lesions and one mesenteric lymph node lesion.

3.3. Demographic factors analysis

The age, sex and infection status of the badgers examined is shown in Table 3. While more females than males were examined, the difference in the proportion of males (37.7%, $n = 34$) and females (35.2%, $n = 44$) infected was not significant ($P = 0.70$). Although a higher percentage of old badgers (47%, $n = 17$) were infected than young (36%, $n = 10$) or adult (34%, $n = 51$) badgers the

Table 3
The age, sex and infection status of 215 cull badgers examined for infection with *M. bovis*.

	Old		Adult		Young		Total	
	I	NI	I	NI	I	NI	I	NI
Male	3	3	24	43	7	10	34	56
Female	14	16	27	57	3	8	44	81
Subtotal	17	19	51	100	10	18	78	137
Total	36		151		28		215	
Age specific prevalence (%)	47		34		36		36	

I = infected.
NI = non-infected.

difference in disease prevalence between the age categories was not significant ($P = 0.32$). The difference in the proportion of infected animals with NVL or VL was not significant across either age group ($P = 0.48$) or sex ($P = 0.87$). The 215 badgers were caught at 133 setts (data not shown). Social group sizes ranged from 1–7 with a mean of 1.62 and a median of 1. There was no significant difference in infection prevalence between social groups of different sizes ($P = 0.25$, data not shown). The analysis showed that there was no association between the demographic factors analysed and the prevalence of infection.

4. Discussion

In this study we showed that by incorporating a comprehensive bacteriological culture examination into the diagnostic protocol, *M. bovis* infection was identified in 36.3% of the badgers compared with 12.1% based on confirmed gross visible lesion detection. This latter estimate is similar to the reported annual prevalence level (range 12.1–13.2%) in culled badgers in Ireland in the years 1998–2003 (O'Boyle et al., 2006). This annual prevalence data is based on confirmed gross lesions with bacterial culture of a limited number of pooled tissues from *c.* 20% of animals with no gross lesions at post mortem examination. The results from the current study suggest that the annual prevalence levels may have been significantly underestimated. The increased diagnostic sensitivity that a bacteriological examination can achieve was also noted in the analysis of the post mortem examination carried out in badgers removed as part of the Four Area Study (Griffin et al., 2005). Although only a limited range of tissues (bronchial, mediastinal, retropharyngeal and mesenteric lymph nodes, 1–2 g of each kidney and of lung tissue) were collected and cultured from each badger in that study, the overall estimated prevalence of infection was 19% (Corner et al., 2008). A UK study compared a standard post mortem examination protocol used in the randomised badger culling trial (RBCT) with a more detailed protocol that included enhanced post mortem examination for lesions, additional culture and histological examination (Crawshaw et al., 2008). The standard protocol was found to have a sensitivity of 54.6% relative to the detailed protocol. It appears therefore that more accurate figures for prevalence of infection can be determined by the use of sensitive bacteriological methods that increase the probability of detecting infection, and also culturing from a wide range of tissues irrespective of the presence of gross lesions.

While reliance on gross pathology alone underestimated the true disease prevalence level, our results are consistent with previous reports (O'Boyle et al., 2006) where diagnosis based solely on the visible appearance of lesions resulted in an overestimate of the proportion of badgers with gross tuberculosis. Of all the gross tuberculosis-like lesions detected in this study, 19 were culture negative for *M. bovis*. These non-tuberculous lesions were found in six badgers although in three of these badgers *M. bovis* was isolated from another lesion or tissue.

The majority of infected badgers in this study were NVL and this is likely due to the highly sensitive post mortem examination and bacterial culture techniques employed in this study. While these methodologies are not pragmatic for routine surveillance, the increased disease prevalence revealed by comprehensive bacteriology has important implications for routine surveillance methodology and interpretation. A significant proportion of infected badgers with NVL have been a consistent finding of previous badger necropsy studies (Fagan, 1993; Gallagher, 1998). However, in this study three times as many badgers were actually infected compared to what examination for gross lesions would suggest. The high proportion of these NVL infected badgers indicates that they are important in the epidemiology of the disease and this may have implications for transmission to cattle.

Infection in the thoracic cavity, in lungs and thoracic lymph nodes, was detected in 61.5% of infected badgers, confirming the findings of other studies that tuberculosis in the badger is primarily a respiratory disease resulting from aerosol infection (Fagan, 1993; Gallagher and Clifton-Hadley, 2000). However, this may also be an underestimation of the true proportion of infected animals with pulmonary involvement. Culture of the entire lungs was carried out in only 20% of badgers and these samples were biased towards those animals with extra-thoracic infection. Secondly, culture of the lung tissue for *M. bovis* required decontamination of the sample and both CPC and oxalic acid are known to have some degree of toxicity for *M. bovis* (Corner et al., 1995), thus effectively decreasing the sensitivity of the culture method. When each lung lobe from the sub-set of badgers was examined in detail for gross lesions and each lobe was cultured separately, the culture protocol was likely to have been less sensitive due to decontamination with 5% oxalic acid, which has been shown to be more toxic to *M. bovis* than 0.075% CPC (Corner et al., 1995).

In 38.5% of infected badgers, infection was restricted to extra-thoracic sites. This pattern of disease appears to be inconsistent with commonly accepted pathogenesis of infection following aerosol transmission. We cannot rule out that these badgers had an undetected primary lung infection or that this distribution of infection was associated with transmission of infection by bite wounds (Fagan, 1993; Gallagher and Clifton-Hadley, 2000). However, allowing for the fact that some healed bite wounds may have gone undetected and considering the low incidence of tuberculous bite wounds (3.3%) recorded, we can speculate on alternative explanations for some of the extra-thoracic infections.

Following infection, a cell mediated immune (CMI) response develops in the host that is characterized by the emergence of antigen specific T cells that mediate the recruitment and activation of macrophages to form granulomas and kill intracellular bacteria. During the time it takes for the host to generate this response, it is possible that the *M. bovis* may disseminate throughout the body via the lymphatic system. Until the CMI response is sufficiently developed the organism will not be immobilised in a lymph node. The delay may vary with challenge dose and the individual animal response (Rodgers et al., 2007), and it is possible that the infection could become established in any lymph node in the body distal to the primary site of infection.

In this study, *M. bovis* was isolated from the head pool only in 15% of badgers. This might be indicative of an alternative route of infection via the mucosa of the pharyngeal or upper respiratory tract (URT). Lesions confined to the head lymph nodes in 32% and 23% of infected cattle have been reported (Neill et al., 1994; Corner, 1994). The tonsils have been identified as a site of *M. bovis* infection in white-tailed deer, red deer and cattle and the lymphoid tissue of the URT and tonsils is believed to play a role in the pathogenesis of tuberculosis in these species (Lugton et al., 1998; Cassidy et al., 1999; Palmer et al., 2002). The tonsils have not been found to be a significant site of *M. bovis* isolation in badgers in Ireland (Corner, unpublished). However, if infection in badgers can become established through the mucosa of the URT then *M. bovis* contamination of the sett environment could play an important role for transmission of infection among badgers. By increasing the levels of detail in the necropsy and bacterial culture of tissues, such as described in this study, the contribution of alternative transmission route to the epidemiology of the disease may become more apparent and appropriate control measures can be devised to limit the spread of infection.

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