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The distribution of *Mycobacterium bovis* infection in naturally infected badgers

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ABSTRACT

Populations of Eurasian badgers (*Meles meles*) with tuberculosis (*Mycobacterium bovis* infection) are a significant reservoir of infection for cattle in Ireland and the United Kingdom. In this study the distribution of infection, histological lesions and gross lesions was determined in a sample of 132 culled badgers from naturally-infected wild populations. Badgers were culled when an epidemiological investigation following a tuberculosis breakdown in a cattle herd implicated badgers as the probable source of infection. The definition of tuberculosis infection was based on the isolation of *M. bovis* from tissues or clinical samples. An accurate diagnosis of infection was achieved by culturing a wide range of lymph nodes (LN) and organ tissues (mean 32.1) and clinical samples (faeces and urine) from each badger.

Infection was detected in 57/132 badgers (43.2%). Histological lesions consistent with tuberculosis were seen in 39/57 (68.4%) culture-positive and 7/75 (9.3%) culture-negative animals. Gross lesions were seen in only 30/57 (52.6%) infected badgers, leaving a high proportion (47.4%) of infected animals with latent infection (no grossly visible lesions). The most frequently infected tissues were the lungs and axillary LN, followed by the deep cervical LN, parotid LN and tracheobronchial LN. The data support the hypotheses that in badgers there are only two significant routes of infection, namely, the lower respiratory tract and bite wounds, and that badgers are very susceptible to infection but resistant to the development and progression of the disease. At all levels of disease severity, infection was found in widely dispersed anatomical locations suggesting that there is early dissemination of infection in the period preceding the development of active immunity.

Introduction

Tuberculosis (*Mycobacterium bovis* infection) in Eurasian badgers (*Meles meles*) was first identified in Switzerland in 1951 (Bouvier et al., 1951), in the South West of England in 1971 (Muirhead et al., 1974), and in Ireland in 1974 (Noonan et al., 1975). Infection is now endemic in badgers throughout Ireland and the UK and infected populations are a significant reservoir of infection for cattle (Griffin et al., 2005; O'Mairtin et al., 1998a,b; Woodroffe et al., 2005).

Tuberculosis in badgers is primarily a respiratory disease transmitted by infectious aerosols (Nolan and Wilesmith, 1994). Transmission through bite wounds is also a significant mode of intra-species infection (Gallagher and Clifton-Hadley, 2000). After inhalation of an infectious aerosol and the establishment of pulmonary infection, there is slow progression to overt disease (Gallagher and Clifton-Hadley, 2000). Only a small proportion of badgers develop generalised disease (Dolan, 1993; Gallagher and Clifton-Hadley, 2000; Murphy et al., 2010) and the mortality rate due to tuberculosis is low. Deaths due to tuberculosis do not have a significant effect on the size or structure of badger populations (Wilkinson et al., 2000) and infected badgers may survive for several years (Clifton-Hadley et al., 1993; Little et al., 1982a).

In Ireland badgers are culled when an epidemiological investigation implicates them as the source of a tuberculosis breakdown in cattle (O'Keeffe, 2006). The prevalence of disease in culled badgers, as estimated by routine post-mortem examination for gross lesions, has remained static at 12–13% for many years (O'Boyle et al., 2003). However, it has been established that increasing the resources used in the examination, e.g., the inclusion of bacteriology and increasing the number of tissues examined, improves detection of infection (Corner et al., 2008a; Costello et al., 2006; Crawshaw et al., 2008; Murphy et al., 2010). In one study where the principal diagnostic procedure was bacteriology of pooled samples, the prevalence of infection was found to be significantly higher (36.3%) than that determined from gross lesions (12.1%) (Murphy et al., 2010).

To date, no study describing the prevalence of infection or the distribution of infection in naturally infected badgers has employed, as the key diagnostic procedure, a comprehensive bacteriological examination of the badgers, i.e. the culture of

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individual samples collected from a wide range of anatomical sites. In the current study each badger was examined using a standardised detailed gross post-mortem examination and the large numbers of tissue samples were collected under strict aseptic conditions. In order to maximise the sensitivity of the isolation of *M. bovis*, each sample was cultured separately using the minimum concentration of decontaminant commensurate with obtaining non-contaminated cultures. The specific objectives of the study were to estimate, with a high level of accuracy, the prevalence of *M. bovis* infection in culled badgers using the isolation of *M. bovis* as the case definition, to describe the anatomical distribution of gross lesions, histopathological lesions and infection, and to compare the sensitivity of the three diagnostic procedures.

Material and methods

Badgers

The badgers used in the study were captured between February 2002 and May 2003 on cattle farms where a severe tuberculosis breakdown had occurred, or were badgers killed in road traffic accidents (RTAs) in areas where culling was being undertaken. Badgers were obtained from six counties across Ireland with a focus on those counties where there was a high incidence of herd breakdowns. Culling was undertaken after an epidemiological investigation deemed badgers to be the most probable source of the infection (O'Keeffe, 2006). Culled badgers were captured in stopped restraints (Murphy et al., 2009). All of the badgers captured at a main sett during the 11-day capturing period were deemed to belong to the same social group (Griffin et al., 2005). The post-mortem examination of the badgers was conducted within 48–72 h of death. The culling was undertaken by the Department of Agriculture, Food and the Marine (DAFM) as part of the Irish National Tuberculosis control program. Culling was conducted under licence from the National Parks and Wildlife Service of the Department of the Environment Heritage and Local Government.

Necropsy

The median time from death to the post-mortem examination was 2 days. The badgers were weighed, and the sex and age recorded. Badgers were placed into three broad age classes (juvenile, adult or old) based on bodyweight and the degree of tooth wear. Prior to dissection, the carcase was washed with water to remove excess dirt, to limit the dispersion of hair and to permit an examination for bite wounds. In this study all penetrating skin wounds or skin abrasions that resulted in exposure of subcutaneous tissues, were classified as 'bite wounds'.

For the post-mortem examination, the carcase was placed in dorsal recumbency on a downdraft table (Astec Microflow). Separate sterile instruments were used to expose lymph nodes, to open the abdominal and thoracic cavities, and to expose and dissect free lymph nodes from surrounding fat and connective tissue, and to collect sections of visceral organs. A systematic, detailed examination was then conducted on all badgers. This involved the visual examination of the skin, buccal cavity and the external surface of the gastro-intestinal tract, along with visual examination and collection of a predetermined set of lymph nodes (LNs) and organs (Table 1), and the collection of any gross tuberculosis-like lesions in other tissues (bite wounds, pleurisy and rectal LN). The sampling of lymph nodes for histopathology and bacteriology was thorough and all of the lymph nodes visible at a site (range 1–8 nodes) were collected.

To minimise the risk of cross contamination, and assuming that the thoracic cavity would be the most severely affected, sites were examined and tissues collected in the following order: lymph nodes of the head, lymph nodes of the body, abdominal lymph nodes and organs, urine and faeces, thoracic lymph nodes and lung lobe, and tonsils.

After being dissected free of surrounding connective tissue and fat, the external surfaces of the intact lymph nodes were examined for gross lesions. All of the nodes collected from each site were cut into two equally representative pieces, one for histopathological analysis and the larger portion for culture. After slicing, the cut surfaces of lymph nodes and organs were examined. Samples for histopathology were preserved in 10% buffered formalin and samples for culture were placed into sterile containers and immediately frozen at −20 °C.

After visual examination of the external surfaces, sections of liver (2–5 g), spleen (2–5 g) and half of each kidney (~4 g) were collected for culture and histopathology. After the lungs were removed from the thoracic cavity, and the mediastinal and tracheobronchial LN removed, each lung lobe was palpated and if lesions were detected they were collected for histopathology and culture. In addition to the lesions and also where no lesions were detected, samples of each lobe (~2 g) were pooled for culture and were also collected for histopathology. The lungs were then fixed in formalin and later examined for gross lesions by serial slicing at 2–3 mm intervals.

Histopathology

Samples were embedded in paraffin and 3 μm sections were stained with haematoxylin and eosin, and by the Ziehl–Neelsen method for acid-fast bacteria (AFB). Sections were examined for tuberculous granulomas, defined as those where AFB with the characteristics of *M. bovis* were observed (Gallagher et al., 1976; Nolan and Wilesmith, 1994; Gavier-Widen et al., 2001). The presence of nematode larvae or adult worms in alveolar spaces or airways was recorded, and the degree of accumulation of crystalline material in the lungs was scored subjectively on a scale of 0 (absent) to 3 (extensive accumulations).

Bacteriological procedures

For culture, samples were thawed overnight at 4–6 °C. Tissue samples were cut into small pieces with sterile instruments and macerated in sterile phosphate buffered saline (PBS) in a Colworth Stomacher 80 (Steward Laboratory). Faecal samples were broken up and suspended in PBS. The macerated tissue was decontaminated with 0.075% cetylpyridinium chloride (CPC) for 10–20 min at room temperature and faecal samples with 0.75% CPC overnight at room temperature. After centrifugation, the supernatant was discarded and the pellet re-suspended in 1 mL of PBS. The following media were inoculated with 0.1 mL of the suspension: two slopes of Lowenstein-Jensen medium with pyruvate, two slopes of Stonebrink's medium with pyruvate and one bottle of Middlebrook 7H9 medium (BACTEC 12B). The unused macerated tissue and faeces were stored at −20 °C. If the inoculated media were contaminated, the frozen macerated tissue or faeces were thawed, decontaminated with 0.75% CPC and media inoculated as described above. All inoculated media were incubated at 37 °C for 12 weeks.

Preliminary isolate identification was based on colony morphology, growth rate, pigmentation and cording (Collins et al., 1997). Spoligotyping of isolates was performed according to the method of Kamerbeek et al. (1997). Isolates with the absence of spacers 39–43′ on spoligotyping were identified as *M. bovis*.

Statistical analysis

Only those gross lesions where there was confirmation by bacteriology were used in the description of the distribution of gross lesions except for one bite wound which was histologically positive but no fresh tissue was available for culture. For the purpose of analysis two different definitions of the tuberculous status of animals have been used. For most of the analyses and descriptions 'infection status' is used and defined as the isolation of *M. bovis*: a very specific definition. A less specific histopathological definition was used to try and more accurately estimate the true prevalence by identifying badgers that were false-negative on culture. These were culture-negative badgers with histological lesions that contained AFB.

Differences in the infection rate between males and females was analysed using the χ^2 statistic and Fisher's exact statistic, and differences between the sexes in bodyweight and differences in the number of sites of infection in badgers with and without bite wounds were analysed using the *t* test. Analysis was done using Prism version 4 (GraphPad Software) and EpiCalc 2000 version 1.02.[1]

Results

Badgers

Of the 132 badgers examined, 127 were captured during culling and 5 were killed in RTAs in areas undergoing culling: all were from rural areas. *M. bovis* was isolated from 57 (43.2%) (Table 2), histological lesions consistent with tuberculosis were observed in 46 (34.8% of the sampled badgers) and gross tuberculous lesions were observed in 30 (22.7% of the sampled badgers). The anatomical distribution of infected tissues, tissues containing histological lesions and the location of gross lesions are shown in Table 1. A greater number of females ($n = 77$) than males ($n = 55$) were examined, also there were more adult badgers ($n = 73$) than old ($n = 31$) or juveniles ($n = 28$). The age-class, gender, and mean bodyweight of each class are shown in Table 3. Although the mean weight of males (11.3 kg) was greater than for females (10.7 kg) the differences were not significant (*t* test; $P = 0.08$).

[1] See: http://www.vetschools.co.uk/EpiVetNet.