Disseminated *Mycobacterium bovis* Infection in Red Foxes (*Vulpes vulpes*) with Cerebral Involvement Found in Portugal

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Abstract

A total of 49 road-killed red foxes were used for the detection of *Mycobacterium tuberculosis* complex (MTC) in Portugal. MTC infection was detected by PCR in 10 red foxes (20.4%; 95% confidence interval [CI] 8.8–31.2%) and confirmed in three (6.1%; 95% CI 0.0–7.9%) of them by microbiological culture. The complex was detected in 20 tissues out of 441 by PCR techniques (4.5%; 95% CI 16.3–23.7%) and in seven tissues out of 441 (1.6%; 95% CI 4.6–9.4%) by culture. MTC was most frequently detected in the brain (8.2%) and in the mediastinal lymph nodes (8.2%). The seven cultures obtained were positive for *M. bovis* by PCR-based genotyping of the MTC targeting genomic deletions. This study confirms the presence of disseminated *M. bovis* in red foxes in Portugal, and it is the first report in the world of the natural infection in the animals' brains.

Key Words: Epidemiology—*Mycobacterium tuberculosis* complex—Red foxes.

Introduction

Mycobacterium tuberculosis complex (MTC) includes *Mycobacterium tuberculosis*, *M. bovis*, *M. caprae*, *M. africanum*, *M. pinnipedii*, and *M. microti*. These species exhibit similar genetic homology but differ considerably in their host range and pathogenicity; most of them are zoonotic agents (Palgrave et al. 2012). There are few reports of MTC in foxes (Bruning-Fann et al. 2001, Delahay et al. 2007, Millán et al. 2008). In animals, the infection caused by these agents has only been reported in the brain of rabbits, mice, and pigs in an experimental model infection (Hernandez Pando et al. 2010). In humans, tuberculosis infections of the central nervous system, caused by *M. tuberculosis*, are a serious and often fatal disease, predominantly impacting young children (Bartzatt 2011). This is a severe type of extrapulmonary disease that is thought to begin with respiratory infection, followed by hematogenous dissemination and brain infection (Hernandez Pando et al. 2010).

The detection of MTC members has been previously reported in wild canids in reservoirs worldwide, such as in coyote (*Canis latrans*) (Rhyen et al. 1995, VerCauteren et al.

2008), wolf (*Canis lupus*) (Carbyn 1982), gray fox (*Urocyon cinereoargenteus*) (Bruning-Fann et al. 2001), and red fox (*Vulpes vulpes*) (Delahay et al. 2007, Martín-Atance et al. 2005, Millán et al. 2008).

The aim of this study was to screen free-ranging red foxes for MTC infection. This was done in response to concerns about the prevalence of MTC in wild mammals in the central region of Portugal.

Material and Methods

Between 2009 and 2012, post mortem examinations were performed on 49 red foxes (*V. vulpes*) (12 juveniles and 37 adults; 31 males and 18 females) killed on roads in Idanha-a-Nova (39°55'11"N, 7°14'12"W) and Penamacor (40°10'8"N, 7°10'14"W) in Castelo Branco, central-western Portugal. Location, sex, and approximate age data were collected for each animal.

At necropsy, samples of the lungs, tonsils, brain, kidney, liver, spleen, intestine, and retropharyngeal, tracheobronchial, mediastinal, and mesenteric lymph nodes were collected for microbiological culture, molecular studies, and

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histopathology. Samples for culture and direct PCR were frozen before processing. Samples were fixed in 10% neutral buffered formalin and processed routinely stained with Hematoxylin & Eosin and by the Ziehl–Neelsen method for acid-fast bacteria (AFB).

Culture methodology was performed as described by Juste et al. (1991) and Aduriz et al. (1995). Tissues from different organs of each animal were decontaminated using 0.75% (wt/vol) hexadecylpyridinium chloride (HPC; Sigma-Aldrich, Italy) for 18 h and cultured in duplicate using five specific media, supplemented with a mix of amphotericin B (50 mg/L), penicillin (100,000 U/L), and chloramphenicol (100 mg/L). The media used, in the study, were Löwenstein–Jensen solid medium (LJ; Liofilchem, Italy), LJ medium with sodium pyruvate without glycerol, LJ medium with mycobactin J (Synbiotics Europe, France), Middlebrook 7H11 medium supplemented with oleic acid–albumin–dextrose–catalase (OADC; Becton–Dickinson, USA), and Middlebrook 7H11 medium supplemented with OADC and sodium pyruvate without glycerol. All culture media were incubated at 37°C for 6 months, and checked every week for mycobacterial growth or contamination with undesirable microorganisms.

Total genomic DNA was extracted from samples with a commercial DNA isolation kit (DNeasy Blood and Tissue Kit, Qiagen, Germany) and stored at –20°C until used as the template in PCR assays. DNA from bacteria isolated on tissue culture was extracted by taking a loop-full from a culture and then transferred to a microcentrifuge vial containing 100 µL of 10 mM Tris-HCl/1% Triton X-100/1 mM EDTA and incubated for 20 min at a temperature of 95°C. After centrifugation, the supernatant was stored at –20°C until used.

All of the samples were first tested by a modified 16S rDNA PCR (Moravkova et al. 2008). This assay allows the identification of DNA from bacteria from the genus *Mycobacterium* and the differentiation between *M. avium* and *M. intracellulare*. The PCR amplification reaction was performed in a total volume of 20 mL containing 2 mL of isolated DNA, 1 mL of each primer (10 mM), and 10 mL of Taq PCR Master Mix (Qiagen, Germany). Specific primers for this assay were used, and 2 µL of isolated DNA (from tissues or from bacterial culture) were added to each PCR reaction, except the negative control. The PCR conditions were 94°C for 1 min, 30 cycles of 94°C for 1 min, 62°C for 2 min, 72°C for 1 min, and 72°C for 10 min.

When members of the *Mycobacterium* genus were identified, a second PCR reaction was done to detect MTC (a 372-bp fragment) on the basis of the assay of Cousins and et al. (1991). The PCR amplification reaction was performed in a total volume of 20 µL using the Taq PCR Master Mix Kit and two specific primers. Three microliters of isolated DNA (from tissues or from bacterial culture) were added to each PCR reaction, except the negative control. The PCR conditions were 96°C for 2 min, 35 cycles of 94°C for 30 sec, 60°C for 1 min, 72°C for 2 min, and 72°C for 10 min.

These organisms were identified by multiplex PCR method to differentiate MTC members based on Warren et al. (2006). Primer set 1 included RD1, RD4, RD9, and RD12 primers and primer set 2 included RD1mic and RD2seal primers. PCR amplification reaction for set 1 was performed in a total volume of 40 µL and for set 2 was performed in a total volume of 20 µL using the Taq PCR Master Mix Kit (Qiagen), the specific primers (1 µL of each), and 5 µL of DNA

(set 1) and 3 µL (set 2). The PCR conditions were as described by Warren et al. (2006).

Fifteen microliters of the reaction product was mixed with loading buffer and subjected to electrophoresis on a 3% agarose gel at 100 V for 30 min. Gels were stained with ethidium bromide and photographed on an UV transilluminator.

Results and Discussion

MTC infection was detected by PCR in 10 red foxes (20.4%; 95% confidence interval [CI] 8.8–31.2%) and confirmed in three (6.1%; 95% CI 0.0–7.9%) of them by microbiological culture. Culture is still the gold standard in MTC diagnosis. MTC was recorded in two juvenile males, seven adult males, and one adult female. External examinations of the carcasses indicated that six were in good body condition and four were in fair body condition. All 10 animals showed AFB in different tissues. PCR detected MTC in tissues of seven animals that could not be confirmed by culture isolates. In total, the complex was detected in 20 tissues out of 441 by PCR techniques (4.5%; 95% CI 16.3–23.7%). The complex was detected most frequently in the brain (8.2%; 4 out of 49) and in the mediastinal lymph node (8.2%; 4 out of 49) by molecular methods.

In total, seven tissues out of 441 (1.6%; 95% CI 4.6–9.4%) were positive by culture. MTC bacteria were grown from tissues from three animals, all PCR positive. Culture positive samples came from five different types of tissues, and PCR-positive samples came from eight different types of tissues (Table 1). From the four brain samples belonging to four different animals that exhibited positive amplification of MTC, three were confirmed by culture isolates. In one of them, infection was also confirmed in the lungs.

Deletion analysis using multiplex PCR targeting RD1, RD4, RD9, and RD12 (Warren et al. 2006) further confirmed the seven isolates as *M. bovis* owing to the amplification of DNA products with band sizes of 146 bp, 268 bp, 108 bp, and 306 bp, respectively.

Histological lesions consistent with a diagnosis of *M. bovis* infection were not detected in any animals observed. Samples from four animals were in advanced autolysis and unsuitable for histopathology. However, two animals that rendered positive results in the PCR of the lungs had parasitic pneumonia (probably

TABLE 1. DISTRIBUTION OF POSITIVE RESULTS IN 441 TISSUES OF 49 RED FOXES BY DIAGNOSTIC METHOD

Tissue examined	Culture positive no. (%)	PCR positive no. (%)	Ziehl–Neelsen positive no. (%)
Mediastinal lymph nodes	0 (0.0%)	4 (0.9%)	13 (2.9%)
Retropharyngeal lymph nodes	0 (0.0%)	2 (0.5%)	10 (2.3%)
Ileocecal valve	1 (0.2%)	2 (0.5%)	8 (1.8%)
Ileum	1 (0.2%)	0 (0.0%)	5 (1.1%)
Tonsils	0 (0.0%)	3 (0.7%)	11 (2.5%)
Lung	1 (0.2%)	3 (0.7%)	9 (2.0%)
Liver	0 (0.0%)	1 (0.2%)	4 (0.9%)
Kidney	1 (0.2%)	1 (0.2%)	12 (2.7%)
Brain	3 (0.7%)	4 (0.9%)	9 (2.0%)
Total	7 (1.6%)	20 (4.5%)	81 (18%)

from *Crenosoma vulpis* or *Angiostrongylus vasorum*), which was accompanied by vascular medial hypertrophy and thrombosis in one of the cases. Parasitic pneumonia was characterized by granulomatous lesions, with the parasites located at the center of the lesion, surrounded by inflammatory cells, namely lymphocytes, plasma cells, eosinophils, and macrophages.

From the animals that also rendered positive results in culture and/or PCR, mesenteric lymph node reactivity was observed in four animals, one of which also presented discrete macrophagic lymphadenitis and a discrete macrophage infiltration in the tonsils. Three other animals showed interstitial chronic nephritis, with abundant lymphocytes and plasma cells, but scarce macrophages. In two other cases, enlarged retropharyngeal lymph nodes, due to lymph node reactivity, were observed. No AFB were associated with any of the lesions described.

Reports of mycobacterial infection in free-living carnivore species are rare. From our literature review, this is the first report of *M. bovis* infection in red foxes in Portugal and the first report of natural infection in animals with brain involvement. The extent to which infected red foxes may be involved in the epidemiology of *M. bovis* in other wild mammals remains unknown. Foxes most probably become infected after scavenging of infected wild ungulate carcasses (Millán et al. 2008), because bovine tuberculosis was previously reported in wild ungulates in the studied area (Santos, et al. 2009).

These findings provide new information on the prevalence of *M. bovis* in wild mammals in Portugal. The results suggest that *M. bovis* circulates widely in the studied area, which is a serious concern for wildlife. Our findings have also revealed a public health concern because veterinarians and other professional workers, as well as hunters who come into contact with these species, have a high risk of being infected when handling infected samples and tissues (Millán et al. 2008).

On the basis of our results, we hypothesize that *M. bovis* may contribute to the presence and transmission of disease between wild mammals. The potential role of this species in the epidemiology of tuberculosis will require further investigation.

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