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Isolation and Phylogenetic Characterization of
Streptococcus halichoeri from a European Badger
(Meles meles) with Pyogranulomatous Pleuropneumonia

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Summary
Clinical and pathological studies in European badgers (Meles meles) are limited. Badgers play a significant role in the epidemiology of bovine tuberculosis (TB) in some countries and an accurate diagnosis is needed for this infection. However, the lesions of bovine TB are similar to those associated with other pathogens, making pathological diagnosis difficult. In the present study, Streptococcus halichoeri was isolated from a European badger with pyogranulomatous pleuropneumonia and suspected of having tuberculosis. TB and other pathogens able to induce similar lesions were ruled out. Comparative 16S rRNA and rpoB gene sequencing studies showed an identity of 99.51% and 98.28%, respectively, with S. halichoeri. This report represents the third description of this bacterium and the first in an animal species other than the grey seal (Halichoerus grypus). It also shows that S. halichoeri can be associated with a pathological process characterized by granulomatous inflammation and resembling tuberculosis.

Keywords: badger; pleuropneumonia; 16S rRNA; Streptococcus halichoeri

Clinical and pathological studies in European badgers (Meles meles) are limited. Only canine distemper virus infection seems to be an important cause of death in this species (Origi et al., 2012). The badger plays a role in the epidemiology of bovine tuberculosis (TB) in Great Britain, with a high percentage of badgers having TB lesions (Corner et al., 2012). Serological studies have shown that badgers have antibodies against several infectious agents typically associated with dogs, cats or man (Sobrino et al., 2008; Millán et al., 2009; Quinn et al., 2012) and a potential involvement in the epidemiology of several domestic animal and human diseases has been suggested.

Pyogranulomatous inflammation can be associated with several pathogens including Mycobacterium spp., Nocardia spp., Actinomyces spp., fungi or the pyogranulomatous form of coronavirus infection (Gallagher and Clifton-Hadley, 2000; Caswell and Williams, 2007; Graham et al., 2012). In the present study, we report the isolation and phylogenetic identification of Streptococcus halichoeri from a badger with pyogranulomatous pleuropneumonia initially diagnosed as TB.

A female European badger, approximately 1 year of age, was found trapped in a concrete pipe and sent to the Wildlife Rehabilitation Centre of La Alfranca, Zaragoza, Spain. Clinical examination revealed good body condition, with a body weight of...
6.605 kg. A superficial abrasion in the back and eroded claws were the only external lesions observed. Antiparasitic treatment was administered and the badger was kept at the Centre for 8 days, showing normal behaviour and an apparent recovery, but it died suddenly without any preceding clinical signs.

A complete necropsy examination was performed at the Rehabilitation Centre and samples of lung with pleura and pericardium, tracheobronchial and mediastinal lymph nodes and spleen were sent for histopathological studies. They were processed routinely and stained with haematoxylin and eosin (HE), Ziehl-Neelsen (ZN) and periodic acid-Schiff (PAS) stains. Pleural samples were sent for microbiological and molecular studies. These were cultured onto MacConkey and sheep blood agar plates (Oxoid, Madrid, Spain) and incubated for 24 h at 37°C, both aerobically and anaerobically. They were also cultured in Sabouraud’s agar for fungal growth for 8 days. Standard biochemical tests and the API 20 Strep system (bioMérieux, Lyon, France) were performed on the colonies.

Molecular studies were also performed on the isolate. For phylogenetic analysis, the 16S rRNA gene and the rpoB gene were amplified as previously described (Drancourt et al., 2004; Vickerman et al., 2007). Sequences were aligned and compared with the known 16S ribosomal RNA sequences from bacteria and Archaea and with the nucleotide collection published in the NCBI database using the BLAST tool (http://www.ncbi.nlm.nih.gov/). Public sequences with identity higher than 95% for 16S rRNA and 85% for rpoB were selected for phylogenetic study. Sequence alignments, maximum likelihood (ML) and the maximum parsimony (MP) phylogenetic trees were constructed following standard procedures (Vela et al., 2010). The reliability of the phylogenetic trees was confirmed using 1,000 bootstrap replications. Direct immunofluorescence for feline coronavirus was performed with a commercial kit (VMRD, Pullman, Washington, USA; CJ-F-FIP-10ML) and a nested PCR for canine distemper virus was performed with a commercial kit (Genekam Biotechnology AG, Duisburg, Germany; Ref. K. 031).

The most significant lesions were found in the thoracic cavity, where there was a very thickened and nodular left pleural surface with underlying purulent exudate and severe pericarditis. The lung was congested and collapsed and the heart was dilated. A congested and slightly enlarged liver and a mottled and mildly enlarged spleen with multifocal white pinpoint foci were also seen. Microscopically, there was severe pyogranulomatous pleuritis and pericarditis (Fig. 1). Numerous neutrophils, macrophages, some small giant cells, fibrin and, in some areas, neo-vascularization were observed (Fig. 2). Mesothelial cells were metaplastic showing a rounded morphology (Fig. 2). The lung showed atelectasis, congestion and haemorrhage, with alveoli occupied by numerous macrophages, some of them with foamy cytoplasm and neutrophils. Mild to moderate proliferative catarhal bronchiolitis was seen (Fig. 3). Some airways and blood vessels were surrounded by a slight infiltration of mononuclear cells (Fig. 3). PAS and ZN stains were both negative. The only other significant lesions were found in the spleen, which showed moderate follicular hyperplasia, and in the lymph nodes, in which there was lymphoid depletion in the follicular areas.

A pure growth of gram-positive, catalase and oxidase-negative, aerobic coccoid organisms,
compatible with *Streptococcus* spp. was the only significant microbiological finding. No canine distemper virus, coronavirus or other pathogens inducing granulomatous lesions were demonstrated. The *Streptococcus* sp. was tentatively classified as *Streptococcus acidominimus/pluranimalium* by the API 20 Strep system. A continuous sequence of 1,432 base pairs (bp) of the 16S rRNA gene was obtained from the isolate (GenBank accession number KF021280). A BLAST comparison with the 16S rRNA sequences in GenBank revealed a high similarity with *S. halichoeri* strain M512/02/1 (99.51% identity), being the next similar species *Streptococcus dysgalactiae* subsp. *equisimilis* strain CIP 105120 (97.23% identity). The isolate clustered with *S. halichoeri* in both ML and MP phylogenetic trees (Fig. 4), with 100% in both trees. Similarly, a 697 bp sequence was obtained for the *rpoB* gene (GenBank accession number: KF138590). This sequence...
displayed 99.28% similarity with the corresponding gene of *S. halichoeri* culture-collection CCUG:48,342 (JN580236.1) and 99.22% with *S. halichoeri* strain CIP 108195 (DQ232471.1), with the third most similar sequence being that corresponding to *S. dysgalactiae* subsp. *equisimilis* AC-2713 (HE858529.1). As for the 16S rRNA analysis, the new strain clustered together with *S. halichoeri*, displaying 100% bootstrap values for both ML and MP trees (Fig. 4).

In the present case, a gross diagnosis of tuberculosis was made initially, but TB was excluded following microscopical examinations including ZN staining and the absence of Langhans’s type giant cells, which are not typically found in badgers. Other pathogens such as fungi, *Nocardia* spp., *Actinomyces* spp. or coronavirus that can induce similar granulomatous lesions were also ruled out. The only significant microbiological finding was a pure culture of *S. halichoeri* suggesting an aetiological role for this organism. The identification of *S. halichoeri* was only achieved after molecular studies. Sequencing of the 16S rRNA gene has become the new ‘gold standard’ for defining species and genus (Clarridge, 2004). It has been suggested that a minimum of 99% sequence similarity should be present for species identification, with an ideal value >99.5%. Our results met this criterion; however, for some strains from the *Streptococcus* genus, the rpoB gene has been proposed as an alternative to 16S rRNA (Vos et al., 2012). The high similarity observed between the 697 bp rpoB gene fragment sequenced and this gene in two strains of *S. halichoeri*, strongly support the results obtained in the 16S rRNA analysis.

The genus *Streptococcus* includes numerous gram-positive organisms with worldwide distribution (Köhler, 2007). Most species are commensals of the upper respiratory tract and lower urinary tract and may cause opportunistic infections, typically of a suppurative nature. In badgers, the only reference of *Streptococcus* spp. isolation comes from a study of bronchoalveolar lavage fluid in which a light growth of *S. dysgalactiae* subsp. *lactiae* was detected with empyema (Foo and Chan, 2014). The occurrence of *S. halichoeri* in the European badger and the grey seal, two species with a very different way of life, is an interesting finding. One possible explanation for this could be the lack of accurate information about bacterial species in wild carnivores. Additionally, the recent isolation in a human being raises the possibility of a more widespread distribution of *S. halichoeri* and that this *Streptococcus* sp. could be an emerging zoonotic infection (Foo and Chan, 2014). *S. halichoeri* infection might be underdiagnosed due to difficulties in identification by traditional techniques (Foo and Chan, 2014). In the present case sequencing of the 16S rRNA gene permitted identification of this species, although routine techniques failed in the identification.

The origin of the infection in this case remains unclear. Pleuritis and pyothorax are often associated with pulmonary spread from bacteria aspirated from the oropharyngeal microflora. Other routes include haematogenous spread and, less often, bite wounds and perforation of the oesophagus or reticulum (Caswell and Williams, 2007). In the present case, no external injuries suggesting fighting or accidents during trapping were observed and the origin was suspected to be the aspiration of oropharyngeal flora. Further studies are necessary, however, to confirm if *S. halichoeri* is a normal inhabitant of the oral mucosa of badgers. On the other hand, respiratory bacterial infections, as often observed with streptococci, are favoured by stress or previous viral infection (Caswell and Williams, 2007). Canine distemper virus is an important respiratory pathogen in dogs and mustelids, often predisposing to secondary bacterial infections (Barron and Rosenthal, 2012). Although canine distemper was not identified, the possibility of co-infection with other viral pathogens cannot be excluded. In the present case, the stress of being trapped and handled may have precipitated the disease.

This report represents the third isolation of *S. halichoeri* since its first description, the second in a new animal species and demonstrates that it can be associated with respiratory pathology resembling tuberculosis in badgers. Interestingly, the isolation from a man with empyema suggests that this streptococcal species might be associated mainly with supplicative lesions of the respiratory system. It also emphasizes that molecular techniques may be needed for a final identification of some bacterial species and that more studies are needed in wildlife to demonstrate their potential role as reservoirs of both known and unknown pathogens.

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**Conflict of Interest Statement**

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.
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