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SHORT PAPER

Detection and Molecular Characterization of *Mycobacterium celatum* as a Cause of Splenitis in a Domestic Ferret (*Mustela putorius furo*)

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Summary

Mycobacterium celatum is a slow growing non-tuberculous mycobacterium described mainly as occurring in human patients. Only two cases of infection with this pathogen have been reported previously in animals. A 5-year-old, neutered male ferret was presented with progressive weight loss and muscle atrophy. Pale mucous membranes, slight alopecia of the tail and splenomegaly, confirmed by abdominal ultrasound, were observed. Fine-needle aspirations of the spleen revealed extramedullary haematopoiesis and marked macrophage-dominated inflammation associated with mycobacterial infection. Ziehl–Neelsen staining demonstrated sporadic acid-fast bacilli within macrophages. These organisms were identified as *M. celatum* by microbiological and molecular methods. Phylogenetic analysis based on the 16S rDNA gene compared this isolate with previously reported strains and demonstrated close relatedness to the human strains of *M. celatum* types 1 and 3. The ferret was treated with enrofloxacin, rifampicin and azithromycin, resulting in clinical improvement. After 40 days of treatment, the spleen was re-evaluated. Cytological evaluation revealed only extramedullary haematopoiesis without evidence of infection. Discontinuation of therapy was followed by rapid deterioration and death.

Keywords: ferret; *Mycobacterium celatum*; spleen; 16S rDNA gene

Mycobacterium celatum is a slow growing non-tuberculous mycobacterium (NTM) described for the first time in a human patient in 1993 (Butler *et al.*, 1993). Based on genomic sequencing, *M. celatum* has been divided into types 1, 2 and 3 (Butler *et al.*, 1993; Bull *et al.*, 1995). *M. celatum* is pathogenic in man, especially in immunocompromised individuals such as those with acquired immunodeficiency syndrome (AIDS; Tortoli *et al.*, 1995; Gholizadeh *et al.*, 1998), but this infection can lead to serious disease in immunocompetent subjects as well (Tan *et al.*, 2009). *M. celatum* has been considered responsible for disseminated granulomatous infection in a bird (*Trogon viridis*; Bertelsen *et al.*, 2006) and a ferret (Valheim *et al.*, 2001). The present report describes the first detection of *M. celatum* infection as a cause of splenitis in a domestic ferret (Mustela putorius furo) with an initial good response to treatment and the phylogenetic relatedness, on the basis of the 16S ribosomal DNA (rDNA) gene, of this isolate with previously reported human strains.

A 5-year-old neutered male ferret, living at home without other animals and with an up to date vaccination history, was presented to the referring veterinarian with a history of anorexia and progressive weight loss over the previous 2 months. Physical examination revealed poor body condition, muscle atrophy, pale mucous membranes, slight tail alopecia and splenomegaly. Haematological examination revealed a marked macrocytic, hypochromic anaemia (haematocrit 13.6%, reference interval 40.2–46.8%; haemoglobin 4.0 g/dl, reference interval 13.1–15.6 g/dl) with slight anisocytosis, polychromasia and a low number

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of acanthocytes. Moderate leucopenia (1.10×10^9/l, reference interval $4.03-6.87 \times 10^9$/l) with marked lymphopenia (0.022×10^9/l, reference interval $1.23-3.74 \times 10^9$/l) was also observed. Moreover, moderate hypoalbuminaemia (2.1 g/dl; reference interval 3.0–3.6 g/dl) was also present.

Abdominal ultrasound examination revealed a small amount of abdominal fluid, mild hepatomegaly and a markedly enlarged hyperechoic spleen. Fine-needle aspirates of the spleen were taken and slides were stained with a modified Wright's stain (Aerospray slide stainer 7,120, Wescor Delcon®, Arcore, Milan, Italy). Cytological evaluation revealed high cellularity and good preservation of cellular morphology. There were numerous background red blood cells (RBCs) and occasional cellular debris. The main cell population consisted of haematopoietic cell precursors and inflammatory cells (Fig. 1). Megakaryocytes were present in moderate number. The erythroid and myeloid precursors were well represented with complete and orderly maturation. Numerous histiocytic cells, often arranged into epithelioid aggregates, were noted. Some of these contained variable numbers of intracytoplasmic, negative-staining, short- to medium-length, rod-shaped structures (Fig. 2). Occasional multinucleated giant cells were encountered. A moderate number of non-degenerate neutrophils and a low number of small lymphocytes were also observed. Sparse acid-fast bacilli within macrophages were detected by Ziehl–Neelsen staining (Bio-Optica, Milan, Italy)

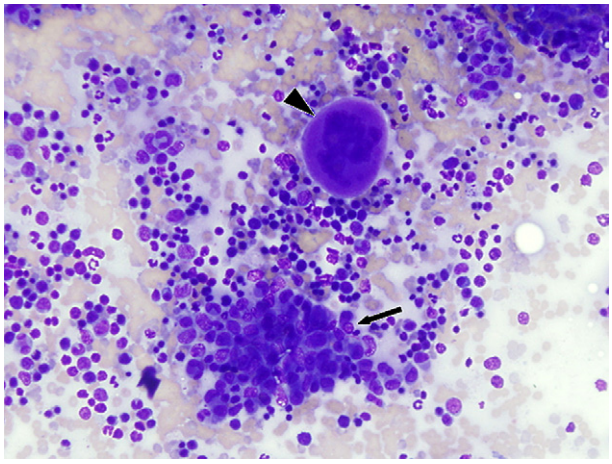

Fig. 1. Fine-needle aspiration of the spleen. Cytological examination reveals a mixed cell population of haematopoietic cell precursors and inflammatory cells. The erythroid and myeloid precursors have complete and orderly maturation. One megakaryocyte (arrowhead) is present. Histiocytic cells, often arranged into epithelioid aggregates are seen (arrow). Lower numbers of small lymphocytes are also encountered. Modified Wright's stain. ×200.

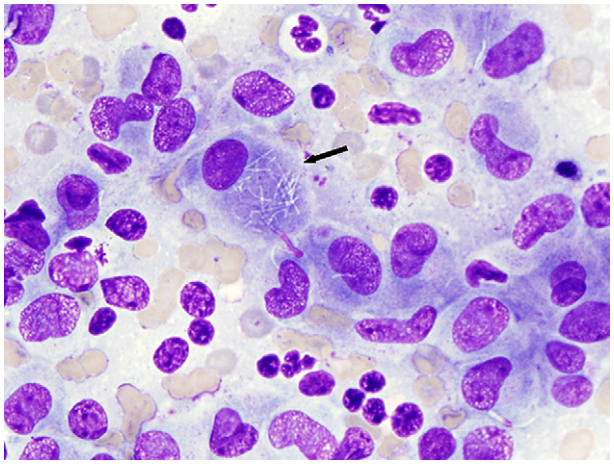

Fig. 2. Fine-needle aspiration of the spleen. Intracytoplasmic, negatively-stained short- to medium-length rods (arrow) in a histiocytic cell. Modified Wright's stain. ×500.

(Fig. 3). The diagnosis of macrophage-dominated inflammation associated with *Mycobacterium* spp. infection and extramedullary haematopoiesis was made. Based on the cytological findings, bacterial culture was performed from the splenic samples. The samples were cultured by standard procedures on Lowenstein–Jensen and Middlebrook 7H10 agars. Small smooth and pale colonies appeared after 8 weeks. Smears from colonies were prepared and stained with Ziehl–Neelsen to confirm that these comprised acid-fast bacilli. Antimicrobial susceptibility was not assessed. No other specific pathogenic bacteria were demonstrated.

DNA was extracted from the mycobacterial cultures after their inactivation by heat at 100°C for 15 min. Polymerase chain reaction (PCR) to amplify a 500 base pair (bp) segment of the 16s rDNA gene

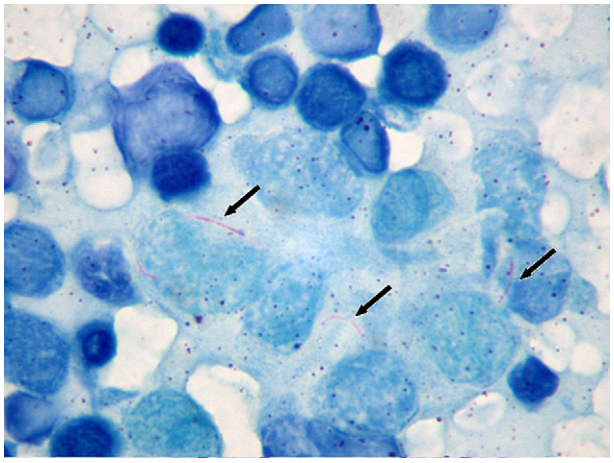

Fig. 3. Fine-needle aspiration of the spleen. Intracytoplasmic acid-fast bacilli (arrow) within histiocytic cells are seen. Ziehl–Neelsen. ×1,000.

was performed as described by Hall *et al.* (2003). PCR products were purified using the Qiaquick™ purification Kit (Qiagen, Hilden, Germany). Forward and reverse sequencing reactions were performed using the MicroSeq 500 16S rDNA sequencing Kit (Applied Biosystems, Foster City, California). After purification with the Dye Ex Spin Kit™ (Qiagen) sequencing reactions were run on the 3,130 Genetic Analyzer (Applied Biosystems). The sequence file was assembled, edited and compared with those in the MicroSeq 500 bacterial database (version 1.4.2, February 2002; Applied Biosystems), revealing an identity of 99.7% with a *M. celatum* sequence. The sequence obtained was also compared with those deposited in GenBank®, demonstrating a 98% identity with two *M. celatum* strains isolated from human patients (Bull *et al.*, 1995; Hall *et al.*, 2003). Moreover, the nucleotide sequence had 98%, 97% and 95% similarities to those of *M. celatum* types 3, 1 and 2, respectively. The sequence was deposited into the GenBank® database (accession number GQ503037). For the phylogenetic analysis, the 16S rDNA sequence of *M. celatum* from the ferret was aligned with 10 references of *M. celatum* sequences retrieved from GenBank®, using the Molecular Evolutionary Genetics Analysis (MEGA) 4.1 programs with bootstrap values based on 1,000 replicates. The phylogenetic tree was rooted with *Nocardia* spp. (EU404381) as an outgroup. To better characterize the phylogenetic position of the *M. celatum* isolate from the Italian ferret, other closely related *Mycobacterium* spp., such as *M. xenopi*, *M. branderi* and *M. kyorinense*, were used to construct the phylogenetic tree. The phylogenetic analysis revealed the formation of two clades. The major clade, which included the ferret sequence, showed close homology of the new isolate with *M. celatum* types 3 and 1, while *M. celatum* type 2 and *M. kyorinense* formed a separate smaller clade (Fig. 4).

The ferret was treated with enrofloxacin (5 mg/kg q24 h per os [PO]), rifampicin (20 mg/kg q12 h PO) and azithromycin (10 mg/kg q12 h PO). Treatment was recommended for at least 6 months. The ferret improved clinically after 40 days on treatment. Abdominal ultrasound demonstrated a reduction of splenic volume and cytological investigation of the spleen revealed only extramedullary haematopoiesis without evidence of macrophage inflammation and mycobacterial infection. The therapy was sustained for 2 more months, with continuous improvement of the clinical condition. However, the owner discontinued the treatment due to difficulties in drug administration and thereafter the clinical condition of the ferret rapidly deteriorated and it died at home. Necropsy examination was not performed.

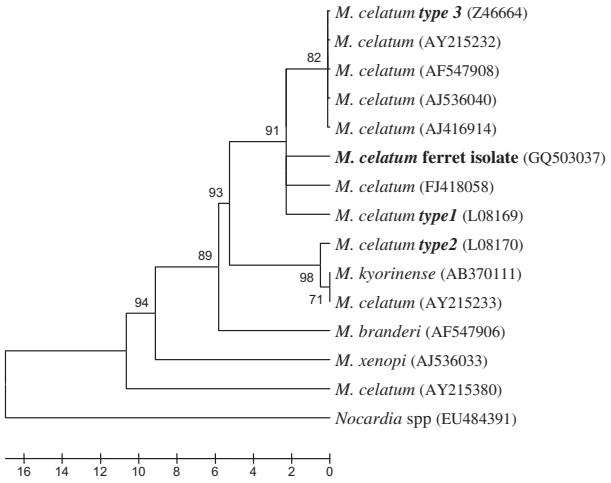

Fig. 4. Phylogenetic tree showing the *M. celatum* isolate from the ferret (GQ503037) and type strains of other closely related mycobacterial species and *M. celatum* sequences available from GenBank®, based on the partial 16S rDNA gene. The GenBank® accession number of each sequence is given in brackets.

Ferrets are susceptible to infection with various mycobacteria and the most common species involved are *M. bovis* and *M. avium* (de Lisle *et al.*, 2008). Many other mycobacterial species, such as *M. microti*, *M triplex*, *M. fortuitum*, *M. florentinum*, *M. interjectum* and *M. intracellulare*, have also been isolated from ferrets (Xavier *et al.*, 2007; de Lisle *et al.*, 2008). *M. celatum* has been reported previously in only one ferret (Valheim *et al.*, 2001). *M. celatum* is reported to cause disseminated infection in human AIDS patients (Piersimoni *et al.*, 1997; Gholizadeh *et al.*, 1998) and an infection limited to the lungs in some cases (Gholizadeh *et al.*, 1998; Shahdad *et al.*, 2005). An exclusively extrapulmonary involvement has been described in one case of penile infection (Dahl *et al.*, 1996). In contrast, in patients that do not have human immunodeficiency virus (HIV) infection, *M. celatum* is reported to be responsible for localized infections, mainly in the lung and lymph nodes (Tan *et al.*, 2009). The first report of *M. celatum* infectionin in a sick ferret described a disseminated granulomatous disease (Valheim *et al.*, 2001). In the present case, due to the investigation being limited to the spleen, dissemination of infection was not evaluated.

Most HIV-negative adult human patients with *M. celatum* infection have had one or more underlying diseases, such as previous pulmonary tuberculosis, chronic obstructive pulmonary disease, lung transplantation or diabetes mellitus (Tan *et al.*, 2009). Human cases have rarely not been associated with concomitant diseases or immunodeficiency disorders (Christiansen *et al.*, 2004; Tan *et al.*, 2009). In the previous (Valheim *et al.*, 2001) and present cases,

the ferrets did not appear to have an underlying disease or immunodeficiency disorder.

A cough is the most common symptom of *M. celatum* infection in man, followed by fever, weight loss and occasional local lymphadenopathy (Piersimoni *et al.*, 1997; Gholizadeh *et al.*, 1998; Shahdad *et al.*, 2005; Tan *et al.*, 2009). Cough was also described as the predominant clinical sign in the previous case of *M. celatum* infection in a ferret, where the organism was isolated from trachea, lungs, liver, stomach and lymph nodes (Valheim *et al.*, 2001). In contrast, in the present case, only weight loss, anorexia and splenomegaly were noted as previously reported in experimental studies of mice infected with *M. celatum* (Ehlers and Richter, 2001).

Based on the symptoms in human patients and due to false-positive nucleic acid probe test results, *M. celatum* has been misidentified as *M. tuberculosis* (Dahl *et al.*, 1996; Piersimoni *et al.*, 1997; Christiansen *et al.*, 2004). Cross-reactivity with the AccuProbe assay (Gen-Probe Inc., San Diego, California) for the *M. tuberculosis* complex (MTB) was observed by probing types 1 and 3, but not type 2 (Butler *et al.*, 1994; Bull *et al.*, 1995; Somoskövi *et al.*, 2000). Definitive identification of *M. celatum* is provided by mycolic acid high-performance liquid chromatography (Butler *et al.*, 1993; Tortoli *et al.*, 1995) or nucleic acid sequencing (Butler *et al.*, 1993; Bull *et al.*, 1995). In the present case, the cytological examination and special stain helped to diagnose a *Mycobacterium* spp. infection. Bacterial culture of the splenic specimens allowed confirmation of the cytological diagnosis; however, molecular analysis was essential to identify the mycobacterial species.

The sequencing of 16S rDNA by MicroSeq 500® is a rapid and specific method to identify *M. celatum* as previously described (Hall *et al.*, 2003). Different levels of homology were observed with *M. celatum* sequences available in GenBank®. The nucleotide sequence was closer to *M. celatum* type 3, consistent with a previous report of an isolate from a ferret (Valheim *et al.*, 2001). The phylogenetic analysis also confirmed a close relationship between *M. kyorinense* and *M. celatum* strains as recently described (Okazaki *et al.*, 2009). The other closely related species included *M. branderi* and *M. xenopi*, which formed a branch that diverged early in the evolution of the slowly growing species (Tortoli, 2003).

Correct identification of the infecting *Mycobacterium* spp. is of importance, since *M. celatum* is known to be poorly susceptible *in vitro* to many antituberculous drugs (Butler *et al.*, 1993; Fattorini *et al.*, 2000; Tortoli, 2003). In man, the treatment of *M. celatum* infection is usually based on a combination of three to four anti-*M. avium* complex drugs, including clarithromycin, azithromycin and rifabutin, administered for several months (Bull *et al.*, 1995; Tortoli *et al.*, 1995; Piersimoni *et al.*, 1997; Gholizadeh *et al.*, 1998). *M. celatum* is known to be resistant to rifampicin, while macrolides have been shown to be able to significantly reduce the colony forming units in experimentally-infected animals (Butler *et al.*, 1993; Fattorini *et al.*, 2000). Antimicrobial susceptibility in this case was unknown, but a combination of anti-*M. avium* complex drugs resulted in considerable clinical improvement as confirmed by negative results of cytology. A special consideration of the potential risk of inducing resistance to antibiotics is needed when managing mycobacterial infection in animals.

The source of infection in this case was unknown. Based on the fact that *M. celatum* has been isolated from aquarium water (Beran *et al.*, 2006), the environment remains the most probable source of infection. Domestic ferrets kept as pets live close to man and thus there is a possibility for transmission of infectious agents between man and ferrets and *vice versa*, especially to immunocompromised subjects. In conclusion, further studies are needed to determine the distribution, transmission, risk factors, pathogenesis and required treatment for *M. celatum* infection in the domestic ferret.

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