Molecular characterization and drug susceptibility profile of a *Mycobacterium avium* subspecies *avium* isolate from a dog with disseminated infection

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*Mycobacterium avium–intracellulare* complex (MAC) infections have been described in many mammalian species, including humans and pets. We isolated and molecularly typed the causative agent of a rare case of disseminated mycobacteriosis in a dog. We identified the pathogen as *M. avium* subspecies *avium* by sequencing the partial genes *gyrB* and *rpsA*. Considering the zoonotic potential of this infection, and in an attempt to ensure the most effective treatment for the animal, we also determined the drug susceptibility profile of the isolate to the most common drugs used to treat MAC disease in humans. The pathogen was tested *in vitro* against the macrolide clarithromycin, as well as against amikacin, ciprofloxacin, rifampicin, ethambutol and linezolid, by the resazurin microdilution assay. It was found to be sensitive to all tested drugs apart from ethambutol. Despite the fact that the pathogen was sensitive to the therapies administered, the dog’s overall clinical status worsened and the animal died shortly after antimicrobial susceptibility results became available. Nucleotide sequencing of the *embB* gene, the target gene most commonly associated with ethambutol resistance, showed new missense mutations when compared to sequences available in public databases. In conclusion, we molecularly identified the MAC pathogen and determined its drug susceptibility profile in a relatively short period of time (7 days). We also characterized new genetic mutations likely to have been involved in the observed ethambutol resistance. Our results confirmed the usefulness of both the *gyrB* and the *rpsA* genes as biomarkers for an accurate identification and differentiation of MAC pathogens.

INTRODUCTION

*Mycobacterium avium–intracellulare* complex (MAC) is the best-studied group of slow-growing non-tuberculous mycobacteria (NTM). MAC includes two species: *M. avium* and *M. intracellulare*. The species *M. avium* is divided into four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *hominissuis*.

Despite their close taxonomic relationship, the subspecies of *M. avium* represent phenotypically diverse organisms with specific pathogenicity and host range characteristics (Rindi & Garzelli, 2014).

MAC infections cause disease in humans and in a wide range of different animal species, including ruminant and non-ruminant wildlife, as well as domestic animals, such as poultry, cattle, swine, sheep, goats and horses (and rarely cats, dogs and ferrets) (Harris & Barletta, 2001; Mentre & Bulliot, 2015; Thorel et al., 1997).

Dogs are reported to be relatively resistant to MAC infections, although certain breeds, such as basset hounds and miniature schnauzers, seem to be at higher risk (Campora et al., 2011; Haist et al., 2008; Horn et al., 2000; Kontos et al., 2014; Miller et al., 1995; Shackelford & Reed, 1989). The most common mode of transmission amongst dogs is...
environmental (Biet et al., 2005). MAC can invade the body through the skin, respiratory or gastrointestinal tracts and usually causes focal disease with skin, respiratory or gastrointestinal lesions. Occasionally, MAC causes disseminated infection, which carries a poor prognosis (Campora et al., 2011; Cucchi et al., 2009; Gow & Gow, 2008; Haist et al., 2008; Horn et al., 2000; Kontos et al., 2014; Naughton et al., 2005; Shackelford & Reed, 1989). In light of the disease’s unfavourable prognosis, its severe clinical signs and the risk it may pose to humans, therapy is not generally prescribed and most dog owners choose to euthanize their pets (Campora et al., 2011; Haist et al., 2008; Horn et al., 2000; Kontos et al., 2014; Shackelford & Reed, 1989).

Humans are commonly and continuously exposed to MAC organisms present in wildlife, domestic animals and natural biotopes (Biet et al., 2005), but only a very small percentage of human/mycobacteria interactions progress to an overt mycobacterial infection. Such progression is much more common in the immunocompromised, notably AIDS patients, in whom MAC organisms in general, and M. avium subsp. avium in particular, exhibit a considerable zoonotic potential (Arastéth et al., 2000).

Traditional antituberculosis antibiotics have not been very successful in the treatment of MAC infections, with in vivo activities as low as 1/100. The introduction of the macrolides azithromycin and clarithromycin has considerably improved clinical outcomes, as these drugs showed good in vivo activity against the pathogens in question (Christianson et al., 2013).

Mycobacterial susceptibility testing is important for the management of patients with tuberculosis and of those with disease caused by certain slow-growing NTM such as MAC. Currently, however, due to a discrepancy between in vitro and in vivo MAC susceptibility to most antimicrobials, the Clinical and Laboratory Standards Institute (CLSI) recommends antibiotic sensitivity testing of human MAC isolates only for macrolides (CLSI, 2011). Indeed, macrolide monotherapy is the only treatment for which a correlation between in vitro MAC susceptibility and human clinical response has been demonstrated in controlled trials (Chaisson et al., 1994; Tanaka et al., 1999; Wallace et al., 1996). To the best of our knowledge, antimicrobial susceptibility testing of slow-growing NTM isolates from dogs has not been previously described in the literature.

We report the results of investigations performed on the causative agent of a rare case of disseminated mycobacteriosis in a young crossbreed dog. Our aims were (1) to isolate and molecularly type the pathogen, (2) to assess the antibiotic susceptibility of the pathogen in vitro against the most common drugs used to treat MAC disease in humans, and (3) in the case of antibiotic resistance, to molecularly characterize point mutations in the target genes.

**METHODS**

**Clinical examination and sample collection.** According to preliminary enquiries, a mycobacterial infection in an 18-month-old crossbreed female dog was suspected by one of the authors (G.N.). The dog was referred to the Veterinary Clinic San Marco (Padua, Italy). Main complaints were lack of appetite, weight loss and systemic lymphadenomegaly.

A full haematological-biochemical profile was performed. Fine needle aspiration lymph node and bone marrow biopsies were carried out under general anaesthesia. The prescapular and mesenteric lymph nodes were sampled. Fine needle aspiration samples were examined by the May–Gru¨ nwald–Giemsma staining method. Based on cytology results (see Results), DNA was extracted from collected samples and PCR was performed for Mycobacterium spp. according to Roth et al. (2000).

Prescapular lymph node biopsy and bone marrow aspirate samples were sent to the Istituto Superiore di Sanita` (Rome, Italy) for further investigations. Pending pathogen identification, detailed information about the zoonotic potential of the condition was provided to the owner, who explicitly requested to pursue therapy. As the national public health guidelines give no specific indications for antibiotic use in cases of MAC infection in dogs, we prescribed pradofloxacin 3 mg (kg body weight)\(^{-1}\) every 12 h per os and azithromycin 10 mg (kg body weight)\(^{-1}\) every 12 h per os. Rifampicin 5 mg (kg body weight)\(^{-1}\) every 12 h per os was added once antibiotic sensitivity results were available.

**Isolation and molecular typing of the pathogen.** Lymph node cells were isolated by gently crushing the biopsy sample in sterile saline solution. The sample underwent decontamination with 1 vol. 4 % NaOH for 30 min at 37 °C, and was neutralized with 0.067 mol PBS \(\times\) 1 at pH 7.2 and centrifuged for 10 min at 8000 g. The bone marrow aspirate sample was centrifuged for 10 s at 13 000 g and the supernatant discarded. Both pellets were suspended in PBS, inoculated into Middlebrook 7H9 broth (BioLife) with 10 % oleic acid–albumin–glucose complex (OADC; Becton Dickinson), and then incubated in CO\(_2\) for 3 weeks at 37 °C for bacteriological examination.

DNA was then extracted from both cultures using NucliSENS MiniMag (bioMérieux). The multiplex PCR assay described by Kulski et al. (1995), with some modifications, was used to detect and identify members of the genus Mycobacterium, as well as to differentiate between members of the M. tuberculosis complex (MTC), M. avium and M. intracellulare. Reactions were performed in a total volume of 50 µl using MyTaq Red DNA Polymerase (Bioline) following the manufacturer’s instructions. Aliquots of 2 µl of DNA were added. The cycling conditions were: denaturation at 95 °C for 10 min, followed by 35 cycles at 95 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s, and a final extension cycle at 72 °C for 7 min. PCR products were visualized on agarose gels stained with Gel Red Nucleic Acid Stain (Biotium) and photographed. Expected fragment lengths were 1030, 372, 180 and 850 bp for the genus Mycobacterium, MTC, M. avium and M. intracellulare, respectively. Field isolates of Mycobacterium bovis and M. avium were used as controls.

PCR-positive samples for M. avium were examined further for subspecies identification. The following three fragments were amplified: (1) the 644 bp fragment of the hsp65 gene encoding the heat-shock protein (Kim et al., 2005), (2) a 353 bp fragment of the gyrB gene encoding the DNA gyrase B and (3) a 933 bp fragment of the rpsA gene encoding the ribosomal protein S1. We then designed primers in order to amplify the gyrB and rpsA genes. Nucleotide polymorphisms were identified through multiple sequence alignment of gyrB nucleotide sequences from MAC reference strains available in GenBank: M. avium subsp. avium strain DJO-44271 (accession number CP009614), M. avium subsp. avium strain 2285 (accession number CP099493), M. avium subsp. hominisuis strain TH1135 (accession number AP012555) and M. avium subsp. paratuberculosis strain MAP4 (accession number CP005928). The same approach was used to select the region of genetic polymorphisms for rpsA.
Two conserved regions flanking the polymorphic site were chosen to design forward and reverse primers able to amplify a 333 bp fragment of the gyrB gene and a 933 bp fragment of the rpsA gene, as shown in Table 1. The cycling conditions for both PCR amplifications were: denaturation at 94 °C for 10 min, followed by 35 cycles at 94 °C for 20 s, 57 °C for 30 s and 72 °C for 50 s, and a final extension cycle at 72 °C for 7 min.

All PCR products were purified and then sequenced using the same PCR primers. Sequences were analysed using ABI Prism SeqScape software version 2.0 (Applied Biosystems). The consensus sequences generated by matching forward and reverse reads were compared to sequence databases and identified using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Drug susceptibility testing. Susceptibility testing of the isolate was carried out in Middlebrook 7H9 broth with 10 % OADC. The isolate was tested for susceptibility to the macrolide clariithromycin as well as to five additional drugs: amikacin, ciprofloxacin, rifampicin, ethambutol and linezolid. Further details, including the MIC breakpoints used for each antimicrobial agent, are reported in Table 2.

As suggested by the CLSI, drug susceptibility was tested using a broth-based method. We chose the resazurin microtitre assay (REMA), as proposed by Palomino et al. (2002), with slight modifications (Marianelli et al., 2015). The test was carried out in triplicate in 96-well plates. The M. avium subsp. avium isolate, as well as an ethambutol-resistant MAC strain from our collection (positive control) and the drug-sensitive M. bovis ATCC 19210 (negative control), were tested. Briefly, 100 µl Middlebrook 7H9 broth with 10 % OADC enrichment was dispensed in each well. Bacterial suspensions were adjusted to 1.0 McFarland standard, diluted 1:20 in the same medium and inoculated (100 µl). The antituberculosis drugs were added subsequently. A growth control containing no drug and a sterile control without inoculum were also included in the test. Plates were incubated at 37 °C in 5 % CO2 for 7 days. Finally, 30 µl freshly prepared 0.01 % resazurin solution (Acros Organics) was added to each well. The plates were reincubated for an additional 48 h at 37 °C and assessed for colour development. A change from blue to pink indicated resazurin reduction and therefore bacterial growth. All antibiotic susceptibility experiments were performed twice, independently.

Determination of MIC for ethambutol by Etest. As bacterial growth was observed only in the presence of ethambutol (see Results), the MIC of ethambutol was determined by Etest (bioMérieux) following the manufacturer’s instructions. The M. avium subsp. avium isolate and the ethambutol-sensitive M. bovis ATCC 19210 (control) were tested.

Briefly, mycobacterial broth cultures were adjusted to a turbidity equivalent of 3.0 McFarland standard. A sterile swab was then dipped into the inoculum tube and spread evenly across the surface of the medium [Middlebrook 7H11 agar (Biolife) with 10 % OADC enrichment]. After 24 h incubation at 37 °C in 5 % CO2, Etest strips, with a gradient of ethambutol ranging from 0.016 to 256 g l⁻¹, were applied onto the surface and the plates were re-incubated for 3 weeks, as described above. In this test, incubation renders the inhibition ellipse clearly visible and the MIC values are read at the point where the inhibition ellipse intersects the scale on the Etest strip.

embB gene sequencing. The ethambutol-resistant M. avium subsp. avium isolate underwent embB PCR amplification and subsequent sequencing. The M. avium subsp. avium strain DJO-44271 reference sequence (GenBank accession number CP009614), was used to design primers able to amplify the entire embB gene. The amplification was carried out in 50 µl volumes using MyTaq Red DNA Polymerase following the manufacturer’s instructions. PCR was performed under the following conditions: denaturation at 95 °C for 10 min, followed by 30 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 4 min.

The PCR product (3310 bp in length) was purified by ExoSAP-IT PCR Product Cleanup (Affymetrix) and sequenced. PCR and sequencing primers used are shown in Table 1.

The consensus sequence generated by aligning the resulting sequences using ABI Prism SeqScape Software was compared to the published M. avium subsp. avium reference sequence (GenBank accession number CP009614) and to GenBank sequence databases to detect genetic variations. Mutations were confirmed through re-sequencing. To distinguish silent from missense mutations, amino acid sequences were theoretically deduced.

RESULTS

Identification of the pathogen

Fine needle aspiration cytology showed large mononuclear cells having abundant grey cytoplasm along with a mixed population of small/medium-sized lymphocytes, identified as epithelioid macrophages. Numerous slender, rod-shaped, negative-stained organisms suggestive of Mycobacterium were located inside and outside of macrophages (Fig. 1). A MAC infection was diagnosed.

Bacteria were isolated from lymph node biopsy and bone marrow aspirate samples after 20 days of incubation. Molecular characterization of isolates, performed by the multiplex PCR assay described by Kulski et al. (1995), confirmed the diagnosis of MAC infection: 1030 and 180 bp amplification products indicative of the genus Mycobacterium and MAC, respectively, were obtained from DNA extracted from both cultures. The nucleotide sequence of the 644 bp fragment of the hsp65 gene showed 100 % identity with both M. avium subsp. avium and M. avium subsp. hominisuis strains available in the GenBank databases, and was therefore unable to discriminate between the subspecies. The 335 bp fragment of the gyrB gene as well as the 933 bp fragment of the rpsA gene were, however, 100 % identical only to the M. avium subsp. avium strains registered in the public databases. Our isolate was thus molecularly typed as M. avium subsp. avium.

Table 1. Primers used for PCR and sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward, reverse and sequencing primers</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gyrB</td>
<td>F 5'-GGAGACGCGCAAGGTAATTGTG-3' R 5'-TGAACTGCTTGCTTGATACC-3'</td>
<td>353</td>
</tr>
<tr>
<td>rpsA</td>
<td>F 5'-TTCTCTGAAAATCTCGAGGAC-3' R 5'-CGGCTAGCTGGCTGCAAAGA-3'</td>
<td>933</td>
</tr>
<tr>
<td>embB</td>
<td>F 5'-CGTGCTGACACGGTGTGA-3' R 5'-TTCGCGCGTTATCAGCC-3'</td>
<td>3310</td>
</tr>
</tbody>
</table>

Sequences were analysed using ABI Prism SeqScape Software was compared to the published M. avium subsp. avium reference sequence (GenBank accession number CP009614) and to GenBank sequence databases to detect genetic variations. Mutations were confirmed through re-sequencing. To distinguish silent from missense mutations, amino acid sequences were theoretically deduced.

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<td>F 5'-CGTGCTGACACGGTGTGA-3' R 5'-TTCGCGCGTTATCAGCC-3'</td>
<td>3310</td>
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</table>
Drug susceptibility testing

In order to ensure that the dog received the most appropriate therapy, antimicrobial susceptibility testing was performed by REMA. Results were available after 7 days of incubation and are shown in Fig. 2. The *M. avium* subsp. *avium* isolate was found to be sensitive to clarithromycin, amikacin, ciprofloxacin, rifampicin and linezolid, but was resistant to ethambutol. The resazurin blue dye was reduced to pink at the resistance breakpoint (8 \( \mu \text{g ml}^{-1} \)) (Fig. 2, column 5, rows D–F). Repeated tests confirmed these results.

We then determined the MIC value of ethambutol for our *M. avium* subsp. *avium* isolate by Etest. As can be seen in Fig. 3, the test did not form a clearly visible inhibition ellipse. As complete inhibition was not observed even at the highest concentration, we were unable to define a precise MIC breakpoint for our resistant *M. avium* subsp. *avium* isolate (Fig. 3a). However, our sensitive strain showed a clear inhibition zone at the MIC of 1 \( \text{g l}^{-1} \) (Fig. 3b).

**Table 2.** Antimicrobial agents tested and MIC breakpoints

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC breakpoints (( \mu \text{g ml}^{-1} ))</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Clarithromycin*</td>
<td>( \leq 16 )</td>
<td>32</td>
</tr>
<tr>
<td>Amikacin</td>
<td>( \leq 16 )</td>
<td>32</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>( \leq 1 )</td>
<td>2</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>( \leq 1 )</td>
<td>–</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>( \leq 2 )</td>
<td>4</td>
</tr>
<tr>
<td>Linezolid</td>
<td>( \leq 8 )</td>
<td>16</td>
</tr>
</tbody>
</table>

*Only clarithromycin is routinely reported for MAC isolates (CLSI, 2011). Clarithromycin serves as a class drug for all newer macrolides, especially azithromycin. According to CLSI recommendations, clarithromycin was tested in Middlebrook 7H9 broth at pH 6.8.*

**Fig. 1.** Fine needle aspiration cytology. Epithelioid macrophages containing numerous negatively stained, slender bacterial rods. Negatively stained bacteria were also visible extracellularly in relief against the proteinaceous background. Occasional non-degenerate neutrophils and small lymphocytes are present. The pyogranulomatous inflammatory response and negatively stained bacilli were suggestive of mycobacterial infection. May–Grünewald–Giemsa stain. Bar, 10 \( \mu \text{m} \).

**Fig. 2.** Antimicrobial drug susceptibility by REMA. Rows A–C and D–F: sample triplicates. Rows A–C, columns 1–4: 8.0 (S), 16.0 (S), 32.0 (I) and 64.0 (R) \( \mu \text{g ml}^{-1} \) for clarithromycin; columns 5–7: 16.0 (S), 32.0 (I) and 64.0 (R) \( \mu \text{g ml}^{-1} \) for amikacin; columns 8–10: 1.0 (S), 2.0 (I) and 4.0 (R) \( \mu \text{g ml}^{-1} \) for ciprofloxacin; column 11: positive control containing no drug (+); column 12: negative control containing uninoculated medium (–). Rows D–F, columns 1 and 2: 1.0 (S) and 2.0 (R) \( \mu \text{g ml}^{-1} \) for rifampicin; columns 3–5: 2.0 (S), 4.0 (I) and 8.0 (R) \( \mu \text{g ml}^{-1} \) for ethambutol; columns 6–8: 8.0 (S), 16.0 (I) and 32.0 (R) \( \mu \text{g ml}^{-1} \) for linezolid; column 9: positive control containing no drug (+); column 10: negative control containing uninoculated medium (–). S, susceptible; I, intermediate; R, resistant.
DNA sequencing of the ethambutol target gene

We sequenced the \textit{embB} gene, a gene encoding an enzyme (arabinosyltransferase) targeted by ethambutol, in the hope of gaining insight into the mechanisms underlying ethambutol resistance. The resulting 3214 bp consensus nucleotide sequence was compared to the \textit{M. avium} subsp. \textit{avium} reference strain (GenBank accession number CP009614) and to the genetically closest \textit{M. avium} subsp. \textit{avium} strain found in sequence databases. Results are shown in Table 3. Comparison with the reference strain showed four mutations: three non-synonymous substitutions and one silent mutation. The closest match found in public databases was with the \textit{embRAB} genes of a moderately ethambutol-resistant \textit{M. avium} strain (GenBank accession number U66560) (Belanger \textit{et al.}, 1996) (99 \% identity, 3211/3213 nt). Comparison to the latter strain revealed two new mutations: one synonymous silent mutation and one non-synonymous. The nucleotide sequence of the \textit{embB} gene obtained here was submitted to GenBank (accession number KT363830).

DISCUSSION

We report a case of fatal disseminated infection in a young crossbreed dog caused by a \textit{M. avium} subsp. \textit{avium} strain. The antimicrobial susceptibility test showed susceptibility to the macrolide clarithromycin and to four additional antituberculosis drugs: amikacin, ciprofloxacin, rifampicin and linezolid. The pathogen was resistant to ethambutol by REMA at the proposed resistance breakpoint. We were unable to determine a precise MIC value by Etest. Nucleotide sequencing of the \textit{embB} gene showed three missense and one silent mutation when compared to the reference \textit{M. avium} subsp. \textit{avium} strain DJ0-44271. A new missense mutation was found when the \textit{embB} gene was compared to a \textit{M. avium} subsp. \textit{avium} strain (GenBank accession number U66560), an ethambutol-resistant strain (Belanger \textit{et al.}, 1996) which was the closest match in the GenBank databases.

MAC infections are considered rare in dogs, presumably due to an innate resistance to these organisms, and cause

<table>
<thead>
<tr>
<th>GenBank accession no.</th>
<th>Synonymous and non-synonymous mutations (amino acid substitutions)</th>
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<tbody>
<tr>
<td>CP009614</td>
<td>Codon 588*</td>
</tr>
<tr>
<td>U66560</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>CTC→CTG</td>
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</table>

*New mutations described in this paper.
severe disease only in immunocompromised individuals. Nevertheless, sporadic cases of disseminated MAC infection involving organs such as the lymph nodes, intestine, spleen, liver, lungs, bone marrow and spinal cord have been described (Campora et al., 2011; Haist et al., 2008; Horn et al., 2000; Kontos et al., 2014; Shackelford & Reed, 1989).

Generally, clinical manifestations of MAC infections tend to be vague or absent, making ante mortem diagnosis difficult. MAC infections are often diagnosed post mortem, on the basis of necropsy, histological and/or cytological examinations (Haist et al., 2008). In the rare cases of in vivo diagnosis, the severity of the clinical picture portends a poor prognosis (Campora et al., 2011; Cucchi et al., 2009; Etienne et al., 2013; Kontos et al., 2014; Miller et al., 1995). In our case study, the diagnosis of MAC infection was made in the live dog based on cytology results and confirmed by PCR. Lymph node and bone marrow samples were then collected for the isolation and molecular characterization of the pathogen, identified as a \textit{M. avium} subsp. \textit{avium} strain. In most canine cases in the literature, the MAC subspecies was not identified (Cucchi et al., 2009; Etienne et al., 2013; Friend et al., 1979; Horn et al., 2000; Kontos et al., 2014; Miller et al., 1995; O’Toole et al., 2005; Shackelford & Reed, 1989; Zeiss et al., 1994); in others, \textit{M. avium} subsp. \textit{hominissuis} (Campora et al., 2011; Haist et al., 2008; Lam et al., 2012) or \textit{M. avium} subsp. \textit{paratuberculosis} (Kukanich et al., 2013) infections were described. To the best of our knowledge, this is the first case of systemic \textit{M. avium} subsp. \textit{avium} infection in a dog to be described in the literature.

The common technique for the molecular identification of mycobacteria is the sequencing of the entire 16S rRNA gene or parts of it. This method is often inconclusive for a number of closely related species, such as members of the MTC and MAC. Fast and accurate molecular approaches for the identification and differentiation of MAC members are therefore needed in order to assess the pathogenicity of MAC subspecies and to provide routine diagnostic tools for the clinician. The partial \textit{hsp65} gene has been proposed by Kim et al. (2005) as a useful target molecule for the identification and phylogenetic analysis of \textit{Mycobacterium} species. In the case of our isolate, however, the nucleotide sequence of the \textit{hsp65} gene perfectly matched both the \textit{M. avium} subsp. \textit{avium} and \textit{M. avium} subsp. \textit{hominissuis} strains available in GenBank databases, thus excluding the possibility of an accurate identification of the species. A new molecular target was therefore required to differentiate these closely related pathogens. Marsh & Whittington (2007), searching for genomic polymorphisms between the sheep and cattle strains of \textit{M. avium} subsp. \textit{paratuberculosis} and \textit{M. avium} subsp. \textit{avium}, identified 11 single nucleotide polymorphisms in eight genes. Amongst those genes, \textit{gyrB} showed the highest number of nucleotide polymorphisms. We selected this gene to be evaluated as a potentially useful target molecule for subspecies differentiation of MAC organisms. We also evaluated the \textit{rpsA} gene, recently suggested by Duan et al. (2015) as an additional potential molecular target for MAC differentiation, but selected a larger region than that proposed by Duan et al. (2015), so as to include all polymorphic sites potentially useful for discrimination. We were able to identify our isolate as \textit{M. avium} subsp. \textit{avium} by sequencing either the \textit{gyrB} or \textit{rpsA} amplified fragments. Our results suggest that both the \textit{gyrB} and \textit{rpsA} genes may be used as biomarkers for the identification and differentiation of MAC members.

Therapy with azithromycin and pradofloxacin was initiated immediately after diagnosis. In order to ensure the most appropriate antimicrobial therapy for the dog, especially in light of the infection’s zoonotic potential, we proceeded to test the \textit{M. avium} subsp. \textit{avium} isolate for antimicrobial susceptibility. Its sensitivity to clarithromycin was tested, as well as to the five drugs generally used to treat MAC disease in humans. MAC infections in humans are generally treated with a combination of antimicrobials, including a macrolide (either clarithromycin or azithromycin), ethambutol and a rifamycin (rifampicin or rifabutin) (Griffith et al., 2007). Aminoglycosides, such as amikacin or streptomycin, may be added in severe cases (Kasperbauer & Daley, 2008).

Due to the unavailability of standard laboratory guidelines for in vitro susceptibility testing for MAC (with the exception of clarithromycin and linezolid), we used tentative breakpoints from the literature (Brown-Elliott et al., 2013; Heifets, 1988; Inderlied, 1997) and the CSLI (CLSI, 2011). The pathogen was sensitive to all tested drugs, apart from ethambutol. Rifampicin was promptly added to the treatment regimen.

Despite the \textit{M. avium} subsp. \textit{avium} isolate being sensitive to the administered therapies, the dog’s overall clinical status worsened and it died 4 days after rifampicin was added to the regimen. The treatment of disseminated MAC infections in dogs has not been very successful. To the best of our knowledge, none of the cases reported in the literature were cured. Infections resulted in either death (O’Toole et al., 2005) or euthanasia of the animals (Campora et al., 2011; Haist et al., 2008; Horn et al., 2000; Kontos et al., 2014; Shackelford & Reed, 1989).

The \textit{M. avium} subsp. \textit{avium} isolate was resistant to ethambutol in vitro by REMA. Resistance to this drug is mainly ascribed to mutations in the \textit{embAB} genes that encode an arabinosyltransferase involved in cell wall arabinan biosynthesis (Belanger et al., 1996). For ethambutol-resistant \textit{M. tuberculosis}, several mutations of the \textit{embB} gene have been described (Alcaide et al., 1997; Ramaswamy et al., 2000; Sreevatsan et al., 1997). Data on ethambutol-resistant MAC are limited, however.

Comparing our sequence to those available from the GenBank databases, the closest match found was to the \textit{embrAB} region of a resistant \textit{M. avium} strain (accession number U66560): a new missense mutation at codon 589 (G589R). We believe that the mutated \textit{embB} gene of our \textit{M. avium} subsp. \textit{avium} isolate resulted in it being resistant to ethambutol.
In conclusion, our case study resulted in a contribution to the development of valid methods for the molecular identification and drug susceptibility profile assessment of MAC isolates, hopefully increasing the chances of curing affected animals and humans. Nevertheless, further research is necessary to enhance the correlation between in vitro drug activity and in vivo therapy outcomes.

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