Mycobacterium avium in miniature schnauzer from Argentina: a series of cases

Pablo Borrás , María Jimena Marfil , Matias Tellado , Diego Hernandez , Juan Manuel Osacar , Indiana Piras , Marcela Martinez Vivot , Soledad Barandiaran

PII: S1938-9736(22)00071-X
DOI: https://doi.org/10.1016/j.tcam.2022.100698
Reference: TCAM 100698

To appear in: *Topics in Companion Animal Medicine*

Please cite this article as: Pablo Borrás , María Jimena Marfil , Matias Tellado , Diego Hernandez , Juan Manuel Osacar , Indiana Piras , Marcela Martinez Vivot , Soledad Barandiaran , Mycobacterium avium in miniature schnauzer from Argentina: a series of cases, *Topics in Companion Animal Medicine* (2022), doi: https://doi.org/10.1016/j.tcam.2022.100698

Highlights:

- Some miniature schnauzers are genetically predisposed to *M. avium* infections.
- Confirmation of the *Mycobacterium* species causing infection in pets is essential.
- Combined diagnosis approach is necessary to establish treatment and prognosis.
- Antibiotic therapy should be consider to improve life quality in diseased dogs.

Title: *Mycobacterium avium* in miniature schnauzer from Argentina: a series of cases

Authors:

Borrás, Pablo[1]; Marfil, María Jimena[2]; Tellado, Matias[3]; Hernandez, Diego[4]; Osacar, Juan Manuel[3]; Piras, Indiana[2]; Martinez Vivot, Marcela[2]; Barandiaran, Soledad[2,5].

Affiliations:

1. Servicio de Enfermedades Infecciosas y Parasitarias en Animales de Compañía, Clínica Veterinaria Panda. Ciudad Autónoma de Buenos Aires, Argentina.

2. Universidad de Buenos Aires, Facultad de Ciencias Veterinarias, Cátedra de Enfermedades Infecciosas. Buenos Aires, Argentina.

3. VetOncologia Cancer Clinic, Ciudad Autónoma de Buenos Aires, Argentina.

4. Centro Este Medicina Veterinaria. General Pico, La Pampa, Argentina

5. Instituto de Investigaciones en Producción Animal (INPA), Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad de Buenos Aires, Buenos Aires, Argentina,

Corresponding author: jmarfil@fvet.uba.ar

Summary

Environmental mycobacteria such as those from the *Mycobacterium avium-intacellulare* complex may cause disseminated and severe disease in dogs with genetic predisposition. A series of cases of four miniature schnauzers with nonspecific clinical signs and the diagnostic tests are described. Complementary means of diagnosis including complete blood count, biochemical serum analyses and fine needle aspiration cytology staining were performed. The bacteriological culture followed by PCR amplification of *1245* and *901* insertion sequences, allowed the identification of *Mycobacterium avium* subsp. *hominissuis.* This environmental Mycobacteria normally do not cause severe disease in dogs or other species, but when CARD-9 gene presents mutations, dogs may become extremely susceptible and disease is fast, disseminated and mortal. Antibiotic therapy can be applied under veterinary consideration in specific situations, as treatment is usually applied for a long period of time. Although zoonotic risk is low as the *Mycobacterium* is environmental, contamination of the location may be high, and immunosuppressed animals and humans can develop infection as well. This report may aid clinical veterinarians in the diagnosis, treatment, and prognosis in similar cases of this breed and others with the genetic predisposition.

Keywords: Diagnosis*;* dogs*;* *Mycobacterium avium*; drug therapy.

INTRODUCTION

The *Mycobacterium avium-intracellulare* (MAC) complex is a group of mycobacteria closely related, with different degrees of pathogenicity, differences in their environmental distribution and primary host. Recently, this complex has been re-defined and now includes 12 species: *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium chimaera*, *Mycobacterium colombiense*, *Mycobacterium arosiense*, *Mycobacterium vulneris*, *Mycobacterium bouchedurhonense*, *Mycobacterium timonense*, *Mycobacterium marseillense*, *Mycobacterium yongonense*, *Mycobacterium paraintracellulare* and *Mycobacterium lepraemurium* [1]. Dogs are considered to be naturally resistant to MAC infections, but a CARD9 variant gene may increase the susceptibility of infection in affected dogs leading to a predisposition to the disease. Although this mutation may occur in any breed, it has only been investigated and proven in miniature schnauzers [2]–[6].

Dogs can get infected through contact with the mycobacteria in the environment, and *M. avium* may enter to the body via different routes: orally, by inhalation or percutaneous inoculation. The infection may be localized or disseminated, depending on the *inoculum*, host's immunity, and the individual susceptibility [7]. Although symptoms are variable, gastrointestinal clinical signs such as vomiting, weight loss, abdominal pain, and diarrhoea, are the most frequent [2]–[5], [8]. Superficial and mesenteric adenomegaly are frequently associated [2]–[4], [6].

The aim of this work is to present a series of cases about *M. avium* infections in miniature schnauzers from Argentina.

MATERIALS AND METHODS

All dogs described in this study were diagnosed following the same diagnostic route. Except for case 4, all cases were referred to the Infectious Diseases and Parasitology service, at Panda Veterinary Clinic, in Buenos Aires, Argentina, in the period between May 2019 and March 2020.

Description of the cases

Case 1

A 2-year-old entire male miniature schnauzer was presented for evaluation due to its lethargy, diarrhoea, and fever lasting 2 weeks. Physical examination revealed pale mucous membranes, splenomegaly, abdominal pain, and enlargement of pre-scapular and popliteal lymph nodes and both tonsils. Complete blood count (CBC) was unremarkable except for a mild normochromic, normocytic, non-regenerative anaemia and leucocytosis. Serum biochemistry abnormalities in alanine transferase (ALT) and alkaline phosphatase (ALP) were detected. A sample was obtained from the pre-scapular lymph nodes through fine needle aspiration (FNA). Giemsa staining was performed and revealed bacilli with negative staining, suggestive of infection by *Mycobacterium* spp. A second sample was referred to the Infectious Diseases Department (FCV UBA) for Ziehl Neelsen (ZN) staining, bacteriological culture, and molecular identification.

Case 2

A 2-year-old intact male miniature schnauzer was referred by VetOncologia Cancer Clinic where he was being examined for a presumptive diagnosis of intestinal Lymphoma due to a significant enlargement of the mesenteric lymph nodes with no involvement of the superficial lymph nodes. The patient showed non-specific gastrointestinal clinical signs, as vomiting, diarrhoea, and loss of body condition. On clinical examination, the patient presented depressed sensorium and significant abdominal pain. In the CBC, hypochromic macrocytic anaemia, leucocytosis associated with neutrophilia, and monocytosis were detected. ALT and ALP's values were above normal parameters. On abdominal palpation, an enlargement of the mesenteric lymph nodes was detected. Material obtained through ultrasound-guided aspiration of the mesenteric lymph nodes was subjected to cytological analyses (Diff Quick staining) and the observation of bacilli with negative staining was suggestive of mycobacteria presence (Figure 1). Intestinal lymphoma was ruled out by the results of the mesenteric lymph node cytology. Material obtained through FNA of the mesenteric lymph nodes was refrigerated and sent to the Infectious Diseases Department (FCV-UBA) for ZN staining, bacteriological culture,

and molecular identification. The patient was referred to the Infectious Diseases and Parasitology service, at Panda Veterinary Clinic for treatment.

Case 3

A 3-year-old neutered male, miniature schnauzer was referred from the San Martín Veterinary Centre to the Infectious Diseases and Parasitology service at Panda Veterinary Clinic. Within his medical clinical history, he had lethargy and gastrointestinal clinical signs that improved with symptomatic treatment. The clinical examination revealed the presence of generalized enlargement of the superficial lymph nodes (mainly the popliteal, submaxillary, axillary and pre-scapular), with firm consistency. The gingival mucosa was slightly pale. No pain was detected on the abdominal palpation. Within the CBC, a mild normochromic, normocytic, non-regenerative anaemia and low plate count were observed, as well as an increase in ALT value. The rest of the CBC parameters were within normal values. Due to the history of ticks and low platelets, a serology for *Ehrlichia canis* was performed and it showed negative results. Among the complementary methods performed, abdominal ultrasound showed clinical alterations in mesenteric lymph nodes and spleen A FNA of the left popliteal lymph node was performed (Figure 2) revealing a granulomatous lymphadenitis with positive ZN-stained bacilli. A refrigerated sample was sent to the Infectious Diseases Department (FCV-UBA) for bacteriological culture and molecular identification.

Case 4

A 7-year-old neutered female miniature schnauzer from General Pico city (La Pampa, Argentina), was referred to Centro Este Veterinary with chronic unspecific clinical signs and superficial lymphadenomegaly. She also presented intermittent claudication of the left forelimb. Clinical examination revealed the presence of generalized enlargement of the superficial lymph nodes and abdominal pain. In the CBC, a normochromic, normocytic, non-regenerative anaemia was detected. The serum biochemical parameters were within general reference values. Ultrasound revealed abnormalities in the spleen and liver, as well as mesenteric

lymphadenomegaly. A sample of popliteal lymph nodes was obtained by FNA for cytology. In the Giemsa stain, a population of "foamy" macrophages was observed with negative stained bacilli inside. A ZN staining was also performed and the result was positive, showing acid fast bacilli (AFB). Samples of the obtained material were sent to the Infectious Diseases Department (FCV-UBA) for bacteriological culture and molecular identification.

Table 1. CBC including blood smear examination and biochemical profile from each case on the diagnosis day. The abnormal blood test results are shown in bold letters.

PARAMETERS	CASE 1	CASE 2	CASE 3	CASE 4	REFERENCES INTERVALS
RBC	**4.91**	**4.6**	5.5	**4.8**	5.5-8.5 millions/mm^3
Haematocrit	**34**	**32**	42	**34**	40-60%
Haemoglobin	**10.2**	**10,9**	13.8	**11.3**	12-20 g/dl
WBC	14640	**16500**	11800	11.900	6000-16000/mm^3
Segment neutrophils	**13000**	**13695**	7788	**11186**	3000-10000/mm^3
Bands neutrophils	0	0	0	0	0-3000/mm^3
Lymphocytes	**730**	1155	3894	**119**	1000-4000/mm^3
Monocytes	590	1320	**118**	595	200-1400/mm^3
Eosinophils	290	330	**0**	**0**	100-1300/mm^3
Platelets	171000	170000	**60000**	160000	150000-450000/mm^3
Total Proteins	5.85	7.2	7.3	7.38	5,7-7,7 g/dl
Albumin	**1.7**	2.5	3.4	3.11	2,5-4 g/dl
Aspartate aminotransferase (AST)	**396**	**62**	**79**	58	20-60 UI/l
Alanine aminotransferase (ALT)	**61**	60	**253**	44	20-60 UI/l
Alkaline phosphatase	273	**939**	47	250	30-300 UI/l
Urea	31	28	**67**	34	20-40 mg/dl
Creatinine	0.67	0.67	0.87	0.8	0,5-1,5 mg/dl

Bacteriology and molecular identification

Samples of the different cases were sent refrigerated to the Infectious Diseases Department (FCV-UBA). Samples were first decontaminated using NaOH (0,4%) (Petroff´s method) and then placed in culture media. Bacteriological culture was performed in duplicate, using two egg-yolk based differential growth-media, Stonebrink and Löwenstein Jensen [9]. Cultures were kept in stove at 37 °C until colony development or up to 8 weeks if no growth was detected, with weekly observation. When colony development was observed, ZN staining was performed

to reveal AFB, suggestive of the presence of *Mycobacterium* spp. For molecular analyses, a loopful of colonies were suspended in bi-distilled water and thermal lysis of the colonies was performed to obtain DNA, which was kept frozen (-20 °C) until molecular analyses. Molecular analyses included PCR amplification of the insertion sequence *1245* to detect members of the MAC [10]. Differentiation between species within the complex was achieved through amplification of insertion sequence *901* [11].

Treatment

Each case was treated different depending on the clinical case and the animal´s welfare. All treatments will be presented on the following paragraphs.

In case 1, the patient was treated with an antibiotic combination of ethambutol (10mg/kg PO q24h) + ciprofloxacin (10mg/kg PO q12h) + clarithromycin (10mg/kg PO q12h) from June 2019 to August 2020. The patient began to improve clinically within the first two weeks of starting treatment. During the 15 months of treatment, the patient showed clinical improvement along with a decrease in lymphadenomegaly. However, he had major gastrointestinal clinical signs of relapse in the last month of treatment that he could not resolve. The owners decided to euthanize their dog and the necropsy of the patient could not be performed.

For patient of the case 2, hospitalization was indicated for supportive treatment as well as the establishment of empirical treatment for *M. avium*, using clarithromycin (7.5mg/kg PO q12h) + ethambutol (10mg/kg PO q24h) + ciprofloxacin (10mg/kg PO q12h) [3]. The patient died 10 days after starting the treatment and did not improve during the administration of the specific's antibiotics. The necropsy could not be performed.

In case 3, the treatment included clarithromycin (7.5mg/kg PO q12h) + ethambutol (10mg/kg PO q24h) + ciprofloxacin (10mg/kg PO q12h). The period of treatment was from June 2019 to November 2020 with significant improvement in the first week of initiating the specific treatment and a decrease in the size of the mesenteric lymph nodes in the first month. The patient evolved favourably for 16 months until the euthanasia was decided due to worsening of

the digestive and systemic symptoms with a lack of the response to treatment. The necropsy could not be performed.

In case 4, an antibiotic therapy protocol with clarithromycin (7.5mg/kg PO q12h) + azithromycin (10mg/kg PO q24h) + ciprofloxacin (10mg/kg PO q12h) was initiated. Azithromycin was replaced after one month by ethambutol (10mg/kg PO q24h) and a decreasement in the size of the superficial lymph nodes was observed, as well as an important clinical improvement in the first two weeks. Analgesics (firocoxib 5mg/kg PO q24h) were added to treat claudication of the forelimb, since the patient was being treated with anti-arthrosis medication without any improvement. Six months after starting treatment, her oestrous cycle began, which was probably the cause of a clinical relapse. The digestive clinical signs, with partial anorexia, were treated with ranitidine (2mg/kg q12hs) and maropitant (1mg/kg q24hs) with a good clinical evolution within three days. The treatment period was from March 2020 to March 2021, up to when the patient's euthanasia was decided due to a worsening of the clinical signs with lack of response to treatment. The necropsy could not be performed.

RESULTS AND DISCUSSION

This is the first presentation of a series of cases of *M. avium* subsp. *hominissuis* in miniature schnauzers´ from Argentina, in which treatment and prognosis are included.

All FNA samples cultured demonstrated bacteriological development in Löwenstein Jensen medium within the second and the third week of culture. AFB were observed after ZN staining (Figure 3). None of the Stonebrink medium culture showed bacteriological development. PCR results were positive for IS*1245* and negative for IS*901*, identifying all isolates as *M. avium* subsp. *hominissuis*.

M. avium subsp. *hominissuis* is an opportunistic environmental pathogen and is clinically relevant for both humans and animals [12]. Soil and water are the natural reservoirs of the organisms. As for many other non-tuberculous Mycobacteria, drinking water and tap aerosols

are thought to be the main sources of *M. avium* subsp. *hominissuis* infections in humans and this source may be possible for dogs as well [13].

Although MAC infection in dogs is uncommon, there is an association between some miniature schnauzer lineages and a predisposition to clinical *M. avium* infection [2], [6], [14]. This predisposition is associated with an alteration in *CARD9* signalling protein expressed in those miniature schnauzer homozygous for the *CARD9* variant gene [14]. Therefore, genetic testing of the breeders will be essential to avoid the perpetuation of susceptible miniature schnauzer to *M. avium* infections. DNA from all dog were sent for screening for the presence of this variant gene to Laboklin laboratories (Germany). Out of the 4 cases, 3 of the dogs were homozygous and carried the predisposition to be more susceptible to MAC infections and explains the severity of the symptoms shown. One dog´s DNA did not yield any genotyping results.

The clinical presentation was mainly of unspecific gastrointestinal signs, such as anorexia, vomiting, diarrhoea, and weight loss. Three of the four patients also had superficial lymphadenomegaly. This is in accordance with what is described in the literature on the clinical presentations of MAC in dogs [2]–[5], [8]. None of them showed respiratory, neurological and / or skin compromise. Veterinarians should consider *M. avium* infection in miniature schnauzer with chronic gastrointestinal signs as a differential diagnosis.

When a mycobacterial infection is suspected, bacteriological culture or ZN staining should be attempted [7]. Molecular confirmation of the isolates obtained through PCR must be performed, and the species and subspecies identification are fundamental to assess a correct treatment and transmission risk to owners or other pets.

Treatment of MAC infections in dogs are a true challenge. As a primary drug, the use of macrolides, such as clarithromycin or azithromycin, is essential. Initial therapy should include at least two antibiotics and, in some cases, up to three to avoid antibiotic resistance [5], [7], [15]–[17]. There are no consensus guidelines on the treatment of MAC in the veterinary literature and no completely effective treatment for widespread infections caused by MAC is described. One

patients died while on treatment (Case 2). In the other cases, the owners decided to euthanize their dogs (Case 1, 3 and 4). Non-tuberculous Mycobacteria resistance to commonly used anti-mycobacterial drugs has been described previously in human medicine [18], but *in vitro* susceptibility testing in not a common practice in isolates obtained from dogs, being only a few reports where this test was attempted [19], [20]. Also, some parameters for susceptibility detection of some drugs are not described for this agent, and tentative breakpoints for them have been used for testing *M. avium* subsp. *hominissuis* susceptibility. Therefore, standardization of this test is necessary in order to obtain reliable results. No *in vitro* susceptibility testing was performed for any isolates obtained in these cases. Nevertheless, drug susceptibility testing should always be considered by veterinarians and laboratory technicians once isolates are obtained, this will be a useful tool when deciding the drug therapy for each patient.

Although none of the dogs´ owners allowed the necropsy, veterinarians should always suggest it, as the information obtained will help understand better the disease and treatment results. However, early diagnosis, regular veterinary check-ups as well as the use of combined antibiotic therapy may extend life expectancy in miniature schnauzers with the CARD9 genetic mutation.

Ethambutol is used within antibiotic protocols for MAC infections in humans [18]. Recently, in isolates of *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis* from dogs, a natural resistance to this antibiotic was detected by antibiogram [19], [20]. In the two patients with a survival of more than 15 months, the treatment used was ethambutol within the specific therapy to treat *M. avium* infections. Routine antimicrobial sensitivity testing is laborious and overall not useful, and it is best to follow guidelines such as those of Clinical and Laboratory Standards Institute (CLSI) to better determine the antibiotic to use [21]. Investigation regarding the antimicrobial sensitivity of the isolated agents with the aim of improving the design of treatment protocols may be beneficial.

Three of the four patients had a survival greater than 10 months once the antibiotic protocol was established. There are no guidelines on the duration of the treatment and therefore it is advisable

that it should be prolonged and sustained in time. It should include clarithromycin, as well as the association of two more antibiotics [7], [16], [17], [22].

Some authors advise euthanasia, either because of the potential risk as a source of infection for owners [23], [24] or because of these dogs' susceptibility and the probability of developing severe symptoms due to *M. avium* [14]. Nevertheless, there are many references in the international bibliography about the use of antibiotic protocols with the aim to eradicate the infection or improve patients' quality of life [16], [17], [19], [20], [25] even in miniature schnauzers [3]. Any treatment must be previously agreed with owners after carefully evaluating dogs' life entire context. People are continuously exposed to Mycobacteria of the MAC present in the environment, pets, and wild animals, but only a small percentage of the human - Mycobacteria interaction, progresses to a clinical infection [20]. Non-tuberculous Mycobacteria are considered opportunistic, and the risk of infection of owners through close contact with sick dogs is low, but environmental contamination through feces can be high and risk may increase, especially in those with immunosuppressive diseases [18], [24].

This disease must be included in the differential diagnoses of patients with chronic and unspecific gastrointestinal symptoms and superficial lymphadenomegaly, especially in miniature schnauzer. To accomplish an accurate diagnosis, a precise sampling, along with differential and confirmatory tests are essential.

DECLARATIONS

Ethics Statement:

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as samples were obtained by veterinarians for diagnosis purposes.

Acknowledgements:

Dr. Federico Fernández and Dr. Cecilia DiSarli (Laboratorio Panda), Dr. Susana Lloveras & Dr. José Carranza (Servicio de Zoopatología – Hospital "Francisco J. Muñiz"), Dr. Ricardo Iachini (Instituto de Zoonosis Luis Pasteur), Dr. Cristina Flores & Dr. Laura Telesca (Panda Veterinary Clinic). We thank Dr. Urs Giger and his team for discovering and characterizing the genetic predisposition in Miniature Schnauzers and for his offer to screen the affected dogs for their identified *CARD9* mutation by Laboklin, Bad Kissinger Germany.

Conflict of Interest statements

Authors´ declare not to have conflict of interest.

Author contributions:

Pablo Borrás: Conceptualization; Data curation; Formal analysis; Investigation; Roles/Writing - original draft; Writing - review & editing.

María Jimena Marfil: Formal analysis; Methodology; Investigation; Roles/Writing - original draft; Writing - review & editing.

Matias Tellado: Methodology; Writing - review & editing.

Diego Hernandez: Methodology; Writing - review & editing.

Juan Manuel Osacar: Methodology; Writing - review & editing.

Indiana Piras: Methodology.

Marcela Martinez Vivot: Methodology; Writing - review & editing.

Soledad Barandiaran: Formal analysis; Methodology; Investigation; Roles/Writing - original draft; Writing - review & editing.

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Figure Captions:

Figure 1: Diff-Quick stained mesenteric lymph node cytology displaying a large number of macrophages that replace the lymphoid cell component. There are numerous typical non-staining "ghost" bacilli with morphology compatible with myocbacteria.

Figure 2. Presence of lymphadenomegaly. A: pre-scapular lymphadenomegaly. B: Obtaining material from the popliteal lymph node by Fine Needle Aspiration.

Fig 3. Staining Z-N from material of a culture *Mycobacterium* positive.

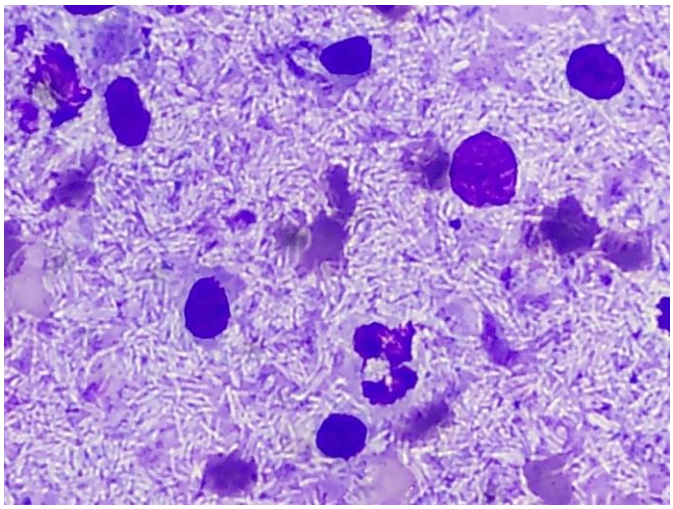

Fig. 1

Fig. 2

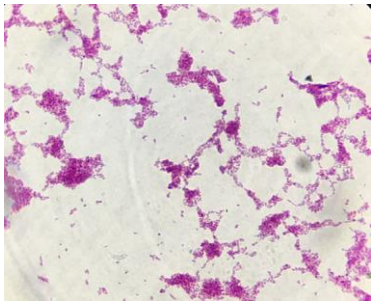

Fig. 3