

First isolation of *Mycobacterium genavense* in a blue headed parrot (*Pionus menstruus*) imported from Surinam (South America) to the Czech Republic: a case report

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ABSTRACT: In 2005 a *Mycobacterium genavense* infection was diagnosed in one blue-headed parrot (*Pionus menstruus*) imported from Surinam (South America), the first such incidence in the Czech Republic. The bird died suddenly. Histopathological examination revealed a diffuse granulomatous inflammatory reaction in the intestinal mucosa. Tissue sections of the intestines stained by Ziehl-Neelsen were microscopically positive for acid-fast bacilli. Culture examinations were made by the conventional solid media, the manual BD-BBL MGIT and the automated MGIT 960 liquid culture systems. We have detected mycobacteria by the automated MGIT 960 liquid culture system in the intestinal tissues after 92 days and on solid Herrold's Egg Yolk Medium without Mycobactin J after 270 days. The manual BD-BBL MGIT liquid culture and the conventional culture system revealed mycobacteria after 150 days of cultivation from three and two tissues samples, respectively. *M. genavense* was identified by HAIN Life Science kits (GmbH, Germany) and was found to be the cause of death for the parrot, which was one of 14 exotic birds kept by its keeper. From the environment *M. a. hominissuis* was isolated only, which was documented by antibody detection of this member of the *M. avium* complex in sera samples of five birds. Three two month old common pet parakeets (*Melopsittacus undulatus*) were for one day in contact with drinking water contaminated by the liver suspension from the infected bird. After 12 months they were euthanized. Subsequent culture was negative for the presence of mycobacteria and histopathological examinations showed no granulomatous inflammatory reaction or any other pathological findings.

Keywords: zoonosis; avian tuberculosis; Psittaciformes; granulomatous inflammation; mycobacteriosis

List of abbreviations:

AFB = acid-fast bacilli; MAA = *Mycobacterium avium* subsp. *avium*; MAC = *Mycobacterium avium* complex; ZN = Ziehl-Neelsen staining

Avian tuberculosis/mycobacteriosis is an important chronic wasting disease in domestic, companion captive, exotic and wild birds worldwide

(Tell et al., 2001; Jones, 2006). Infection is commonly caused by *Mycobacterium avium* complex members (MAC). *M. a. avium* (MAA) is the most

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widely distributed and pathogenic organism causing tuberculous lesions in birds. However, in recent years *M. genavense* has also been reported to cause infections, particularly in captive exotic birds (Hoop et al., 1993; Portaels et al., 1996). Although *M. genavense* was first identified in 1990 in *HIV/AIDS* patients (Bottger et al., 1992), *M. genavense* is currently recognized as the most frequent etiological agent of avian mycobacteriosis in pet birds and particularly in Passeriformes and Psittaciformes bird species (Portaels et al., 1996; Holsboer-Buogo et al., 1997).

M. genavense, while a genetically distinct species, causes a disease with similar clinical and histopathological observations to that of *MAC* members (Antinoff et al., 1996; Portaels et al., 1996; Ramis et al., 1996), although it has unusual fastidious growth requirements and *in vitro* growth is difficult. Epidemiological data on *M. genavense* are so far scarce because of this difficulty in culturing. Apart from this, the origin of infections due to the *M. genavense* in humans and animals remains obscure (Portaels et al., 1996; Lorenzen et al., 2009; Ludwig et al., 2009). However, reports have indicated that pet birds and dogs are considered to be potential sources for the contamination of humans. The mode of transmission among birds is still unclear, in birds and humans an oral route of infection has been suggested (Hoop et al., 1993; Perchere et al., 1995).

M. genavense detection by culture is difficult, though diagnosis is based on identification of acid-fast bacilli (AFB) after Ziehl-Neelsen (ZN) staining combined with culture examination on special media for 6 to 12 weeks (Realini et al., 1999). Detection of *M. genavense* by molecular methods, targeting a fragment of the 16S rRNA gene with specific primers could reveal the characteristic signature sequence (Boddinghaus et al., 1990; Bottger et al., 1993; Portaels et al., 1996; Therese et al., 2009).

Moreover, direct sequence determination of the PCR-amplified 16S rRNA has also been introduced as an alternative technique for identification of the bacteria (Rogall et al., 1990). As *M. genavense* has been isolated from human patients with *HIV/AIDS* (Bottger et al., 1992; Coyle et al., 1992; Berman et al., 1994; Bottger, 1994), it remains a concern as a potentially important zoonosis. The present report describes the features of a mycobacteriosis in parrot species in which *M. genavense* was identified as the cause of death.

MATERIAL AND METHODS

Anamnestic data

The parrot was imported from Surinam (South America) to the Czech Republic in December 2004

Table 1. *Mycobacterium genavense* detection in the tissues of a naturally infected blue headed parrot (*Pionus menstruus*)

Origin of tissue Sample	Histopathology ¹			Conventional cultures ²			Liquid cultures ³		Identifications ⁴	
	PA	HE	ZN	HEYM	StM	SuM	MGIT I	MGIT II	PCR	HAIN
Stomach (glandular)	0	0	1	0	0	0	0	0	0	0
Stomach (muscular)	0	0	1	0	0	0	0	0	0	0
Intestinal wall	1	1	1	0	0	0	0	0	0	0
Intestinal mucosa	1	1	1	0	0	0	1 (150)*	0 (150)*	0	1
Intestine (whole tissue)	1	1	1	1 (270)	0	0	1 (150)*	1 (92)	0	1
Liver tissue	1	1	1	0	0	0	1 (150)*	0 (150)*	0	1
Total	4	4	6	1	0	0	3	1	0	3

¹Histopathology: PA = gross pathological lesions (edema, swelling and inflammation; non-typical tuberculous lesions) were observed in tissues, HE = granulomatous inflammation after hematoxylin and eosin staining were observed, ZN = acid fast bacilli (AFB) were detected after Ziehl-Neelsen (ZN) staining

²Conventional culture: HEYM = Herrold's Egg Yolk Medium with and without Mycobactin J, StM = Stonebrink Medium, SuM = Sula's Liquid Medium were used (the days at which visible colonies were detected are given in brackets)

³Liquid cultures: MGIT I = Manual BD-BBL MGIT liquid culture system, MGIT II = Automated MGIT 960 liquid culture system were used (in brackets are given the days, when AFB were detected by signal or * AFB were detected after ZN staining and microscopy after the stated days in brackets without observation of the growth signal in the MGIT system)

⁴Identifications of AFR: PCR = IS900, IS901, IS1245 were detected by a method described previously (Bartos et al., 2006), HAIN = According to Genotype Mycobacterium CM/AS HAIN Life Science (GmbH, Germany) mycobacterial isolates were identified as *Mycobacterium genavense*

and kept together with 13 other exotic birds until January 12th, 2005. Then, due to its illness, it was separated from the other birds. The bird was submitted to the clinic on January 26th, 2005 and died on January 29th, 2005. Faeces examined from the clinically suspected bird by ZN staining were negative. X-ray analysis showed dilatation of the proventriculus; a non-specific pathological lesion (thickening) on the intestine wall at the cranial part of the small intestine was also observed.

Intravital diagnosis

Serological testing. For the purpose of epidemiological studies serum was collected from the 13 parrots for the presence of antibodies in agglutination tests against antigens prepared from three members of the MAC: MAA of serotype 1 originally isolated from an infected heron little egret (*Egretta garzetta*) No. 1 (Dvorska et al., 2007), *M. a. hominissuis* of serotype eight isolated from a pig with tuberculous lesions in mesenteric lymph nodes (Pavlik et al., 2007) and *M. intracellulare* serotype strain of serotype 19 from our collection (Bartos et al., 2006). The test aims at the detection of the causative agents of avian tuberculosis/mycobacteriosis caused by MAA, which was expected after the histopathological examination of the dead parrot. The result is evaluated within a minute by holding the glass plate against a dark ground and if a marked agglutination and/or formation of white complex of antigen antibody reaction is observed this is considered as a positive reaction. No such agglutination reaction would constitute a negative result. In addition, 26 faecal samples (twice individual collection from the rest of the 13 living exotic birds) and nine environmental samples from the surroundings of the cage (where the infected parrot was kept) were collected and examined by ZN staining and culture.

Post mortal diagnosis

Sample collection. For the laboratory examination six tissue samples were collected from the dead bird (Table 1).

Sample storage. Tissue samples for culture examination were kept at –20°C for one week until they were processed. Faecal and environmental samples were kept at +4°C for one day and processed for culture on the following day.

Microscopy examination. Tissue impressions were stained according to ZN and examined by light microscopy (Olympus 17, Japan) for the presence of AFB. At least 200 fields of view were examined for each sample.

Histopathological examination. Tissue samples were collected for histopathological examination. Samples were fixed in buffered 10% neutral formalin, dehydrated, embedded in paraffin wax, sectioned on a microtome at a thickness of 4 µm, and stained with hematoxylin and eosin (HE) and ZN for the detection of AFB in tissues.

Conventional culture examination. Approximately 1 g of tissue sample was homogenised by a laboratory blender stomacher (Kleinfeld Labortechnik GmbH, Gehrden, Germany) and the suspension was decontaminated in a 1M HCl solution for 15 min. The suspension was subsequently neutralized by 2M NaOH until the colour changed to light purple. Two to three drops of phenolphthalein (2%) were used as a pH indicator. Faecal and environmental samples were also decontaminated as tissue samples. Eighty µl of the suspension was inoculated with sterile disposable tips and dispensed to each of the solid media and one liquid Sula's medium. Incubation was performed at 37°C and cultures were checked after the first week of incubation and then every two weeks as described previously (Matlova et al., 2003). After observation of the results for up to two months, the incubation period was extended up to nine months.

Culture on BD BBL MGIT liquid culture system. To each 4 ml MGIT test-tube aseptically 0.5 ml of MGIT oleic acid, dextrose, albumin and catalase (ODAC enrichment), 0.1 ml of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) antibiotic mixture and 0.5 ml of centrifuged sample suspension were added. After shaking gently cultures were incubated at 37°C for two months. Monitoring of the growth of mycobacteria was carried out on the next day and then every day using a manual fluorescence reader (BACTEC Micro MGIT from BD, USA with the wave length of 365 nm). Incubation of cultures was carried out for nine months. Positive culture smears were stained using the ZN method and examined for the presence of AFB.

Culture on MGIT 960 Automated liquid culture system. ODAC enrichment and PANTA antibiotic mixture were added to BACTEC MGIT 960 culture tubes containing 7 ml of M7H9 broth base. After inoculation of 0.5 ml of the processed

sample, the tubes were incubated at 37°C in the BACTEC MGIT 960 instrument. The cultures were monitored automatically for an increase in fluorescence. Any sample identified as positive was removed from the instrument and a smear prepared and examined for the presence of AFB. Incubation of cultures in the MGIT tubes was carried out for up to 4 months. Samples with no indication were considered as negative.

Identification of mycobacterial isolates. All AFB positive isolates were examined by PCR for the detection of the *dnaJ* gene specific for genus *Mycobacterium* using primers described by Nagai et al. (1991). For differentiation of subspecies of *M. avium* IS901 detection by primers 5'-GCA ACG GTT GTT GCT TGA AA-3' and 5'-TGA TAC GGC CGG AAT CGC GT-3' (Kunze et al., 1992; Pavlik et al., 2000; Svastova et al., 2002), and IS1245 detection by primers 5'-GCC GCC GAA ACG ATC TAC-3' and 5'-AGG TGG CGT

CGA GGA AGA-3' was carried out (Guerrero et al., 1995; Bartos et al., 2006), and Nested PCR with IS6110 was used for isolation of *MTC*. Identification of isolates was also undertaken according to Genotype *Mycobacterium* CM/AS, HAIN Life Science kits (GmbH, Germany).

RESULTS

Intravital diagnosis

Serological findings. Among the 13 serologically tested parrots birds two (13.3%), and one (6.7%) were found positive for *MAA* and *M. a. hominissuis* antigens, respectively, while three (20%), four (26.7%) and two (13.3%) returned dubious results for *MAA*, *M. a. hominissuis* and *M. intracellulare* antigens, respectively. None were positive for *M. intracellulare* antigens (Table 2).

Table 2. Antibody detection of *Mycobacterium avium* complex members by rapid agglutination in sera samples from 13 exotic birds from the same breeder

Species	Months of age	Origin	Agglutination with <i>M. avium</i> complex members ¹					
			<i>M. a. avium</i>		<i>M. a. hominissuis</i>		<i>M. intracellulare</i>	
			+	±	+	±	+	±
Blue and yellow Macaw (<i>Ara ararauna</i>)	18	Czech Republic	0	0	0	1	0	0
Blue and yellow Macaw (<i>Ara ararauna</i>)	42	Czech Republic	1	1	1	0	0	0
Sun Parakeet (<i>Arantiga solstitialis</i>)	28	Czech Republic	0	0	0	0	0	0
Sun Parakeet (<i>Arantiga solstitialis</i>)	28	Czech Republic	0	0	0	0	0	0
Sun Parakeet (<i>Arantiga solstitialis</i>)	18	Czech Republic	0	0	0	0	0	0
Sun Parakeet (<i>Arantiga solstitialis</i>)	18	Czech Republic	0	0	0	0	0	0
Blue-headed Parrot (<i>Pionus menstruus</i>)	nk ²	Surinam ²	0	0	0	0	0	0
Blue-headed Parrot (<i>Pionus menstruus</i>)	36	Czech Republic	0	1	0	1	0	0
Blue-headed Parrot (<i>Pionus menstruus</i>)	36	Czech Republic	1	0	0	0	0	0
Cuban Amazona (<i>Amazona leucocephala</i>)	18	Germany	0	0	0	0	0	0
Black-crowned Parrot (<i>Amazona melanocephala</i>)	48	Germany	0	0	0	0	0	0
Black-crowned Parrot (<i>Amazona melanocephala</i>)	23	Czech Republic	0	0	0	1	0	0
White-bellied Parrot (<i>Pionites leucogaster</i>)	9	Germany	0	1	0	1	0	0

¹agglutination result: + positive, ± doubtful, – negative results, antigens were prepared from serotype 1 of *M. a. avium* originally isolated from an infected heron little egret (*Egretta garzetta*) No. 1 (Dvorska et al., 2007), serotype 8 of *M. a. hominissuis* isolated from pig (Pavlik et al., 2007) and serotype 19 of *M. intracellulare* serotype strain (Bartos et al., 2006)

²not known, imported as an adult bird



Figure 1. Multiple whitish miliary nodules in the mucosa of the small intestine (Foto V. Grymova)

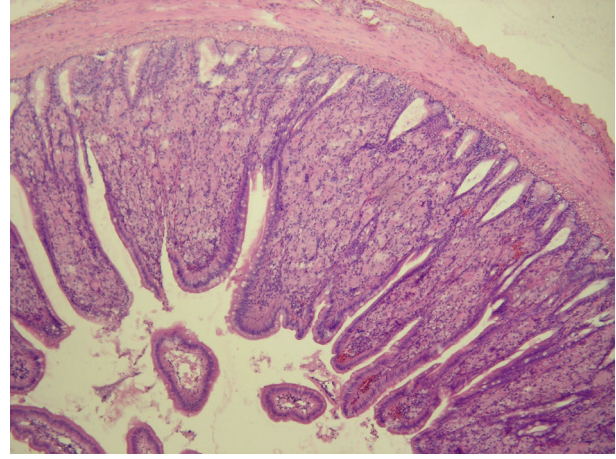


Figure 2. Thickened mucosa of the small intestine due to the presence of granulomatous inflammatory infiltrate, HE, 100× (Foto R. Halouzka)

Post mortal diagnosis

Necropsy and histopathological examination results. At necropsy, a marked dilatation of the proventriculus was observed. In the small intestine (duodenum and jejunum) multiple whitish miliary nodules (with size of 1 mm) were evident (Figure 1). Histopathologically, there was a thickening of the mucosa layer of the small intestine due to the presence of inflammatory infiltrate (Figure 2). A diffuse granulomatous inflammatory reaction was observed with infiltration of mucosa by epithelioid macrophages, lymphocytes and giant multinucleated Langhans cells (Figure 3). There were no signs

of caseation necrosis and mineralization. ZN staining of affected tissues showed the massive presence of rod-shaped acid fast bacilli in the cytoplasm of macrophages (Figure 4).

Culture examination results. The automated MGIT 960 liquid culture system revealed mycobacteria after 92 days in the intestinal tissues, while the manual MGIT (in liver and intestine) and the conventional culture (in intestine) systems detected mycobacteria at 150 and 270 days of incubation, respectively. Culture examination showed ZN positivity in all isolates. Faecal culture was totally negative whilst mycobacteria were detected only in one environmental sample (Table 3).

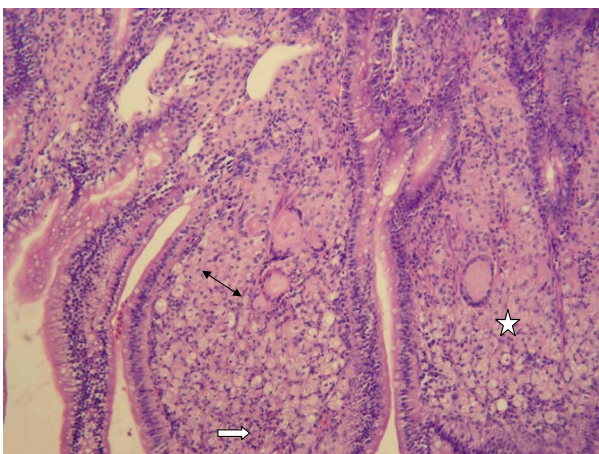


Figure 3. Diffuse granulomatous enteritis with infiltration of mucosa by epithelioid macrophages (double arrow), lymphocytes (arrow) and giant multinucleated cells of Langhans' type (star), HE, 400× (Foto R. Halouzka)

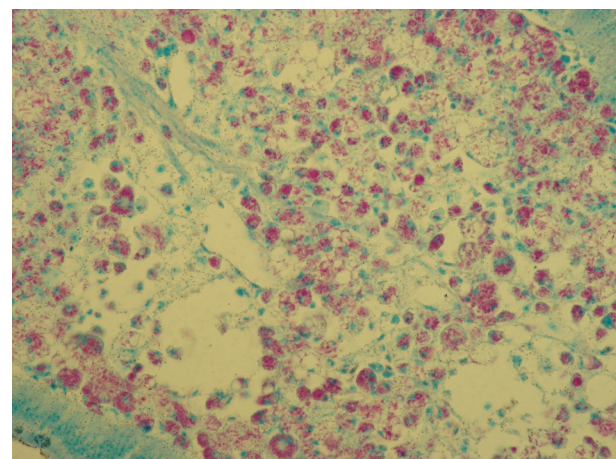


Figure 4. Small intestine, massive presence of *Mycobacterium genavense* in the cytoplasm of macrophages, Ziehl-Neelsen staining, 400× (Foto R. Halouzka)

Table 3. Detection of mycobacteria in the environmental and faecal samples from the surroundings of the aviary

Examined samples		AFB positive ¹		Species identification ²
Origin	No.	ZN	culture	
Piece of tree branches from the roof and floor	3	1	0	
Piece of wood contaminated with mud	2	0	1	
Soil contaminated with faeces	3	0	0	<i>M. a. hominissuis</i>
Dust	1	0	0	
Faeces from surroundings	26	0	0	
Total	35	1	1	<i>M. a. hominissuis</i>
%	100	2.9	2.9	

¹Detection of acid-fast bacilli (AFB): ZN = Ziehl-Neelsen staining, culture = mycobacteria isolation by a conventional method described previously (Fisher et al., 2000)

²Isolates were identified by IS1245 PCR detection specific for *Mycobacterium avium* subsp. *hominissuis* (Bartos et al., 2006)

Identification of mycobacterial species. Organ samples examined by IS901 and IS1245 (MAC) or IS6110 (MTC) were negative. *M. genavense* was isolated from the intestine using Genotype *Mycobacterium* CM/AS, HAIN Life Science kits (GmbH, Germany). A mycobacterial isolate of the *M. a. hominissuis* species was isolated from one environmental sample.

DISCUSSION

The etiology of mycobacteriosis, especially in pet birds, is rarely described. This fact may be due to non-specific post mortem findings or the very small size of any macroscopically observable pathological lesions (Panigrahy et al., 1983; Koppers et al., 1991). On the other hand, it may be down to non-cultivable mycobacteria species (Britt et al., 1980).

Currently, *M. genavense* is considered the most frequent etiological agent of avian mycobacteriosis in pet birds, in Passeriformes and Psittaciformes in particular (Portaels et al., 1996; Holsboer-Buogo et al., 1997). The pathogen has also been detected in birds belonging to the orders Coraciiformes, Piciformes and Galliformes. *M. genavense* infection is most frequently reported in Amazon parrots and has been described in pet birds in Europe, USA and Australia (Hoop et al., 1993; Antinoff et al., 1996; Kiehn et al., 1996; Portaels et al., 1996; Ramis et al., 1996; Holsboer-Buogo et al., 1997; Tell et al., 2001; Manarolla et al., 2007).

In this report from the Czech Republic, we described the clinical, pathomorphological and micro-

biological features of a case of mycobacteriosis in a male parrot (*Pionus menstruus*). An initial gross pathoanatomical examination showed emaciated muscle and dilatation of the proventriculus. Miliary nodules (1 mm in size) were evident in the small intestine, particularly in the duodenum and jejunum (Figure 1). The intestinal mucosa and villi were hypertrophied and thickened due to diffuse granulomatous enteritis (Figures 2 and 3) with the presence of epithelioid macrophages, lymphocytes and giant multinucleated cells of the Langhans' type. We did not observe caseation necrosis and mineralization. These pathological findings are consistent with pathological lesions in mycobacterial infections described in birds (Tell et al., 2001). Ziehl-Neelsen staining confirmed mycobacterial infection with a massive presence of rod-shaped AFB in the cytoplasm of macrophages. In the caudal part of the small intestine granulomatous inflammation was mild and subsequently disappeared along the ileum. No granulomatous inflammatory reactions were histopathologically observed in other organs.

Culture incubated for two months was negative for the growth of mycobacteria and attention was then focused on non-cultivable and/or fastidious mycobacterial species (Hoop et al., 1993; Portaels et al., 1996). Culture was carried out on the manual BD-BBL MGIT and the automated MGIT 960 liquid culture systems. Growth of mycobacteria was detected after 92 days in the automated MGIT 960 culture system from the intestine, which was consistent with previous reports (Hoop et al., 1996).

Mycobacteria were detected after 150 days in the intestine and liver and only in the intestinal

tissues by the manual BD-BBL MGIT and conventional culture systems, respectively. This result, particularly in the solid culture system, may be directly linked with the specific characteristics of *M. genavense*, for which cultivation on whole solid media may often not be suitable (Hoop et al., 1996). *M. genavense*, which is known as a fastidious growing species, was isolated from the intestine using HAIN Mycobacterium Life Science kits.

Some organs were, due to their small size, subjected to complete histopathological examination (spleen, heart, kidneys and lungs) and therefore were not cultured. The difficulty in the culturing of this pathogen resulted in limited information on the epidemiological characteristics of *M. genavense* which remain unclear.

This case report is the first incidence of avian mycobacteriosis caused by *M. genavense* in the Czech Republic. Birds kept in close contact with the infected bird remained uninfected or did not manifest any clinical signs of mycobacteriosis. Faecal and environmental samples collected from the cage were negative for the presence of *M. genavense*. But positive for *M. a. hominissuis* which is common in environment (Matlova et al., 2003; Krizova et al., 2010). Hence, under these circumstances, the source of infection remains unknown. Although the available data are not adequate for the formation of a conclusion, this case study strengthens the suggestion that the specific phenotypic and genomic characteristics of *M. genavense* which give rise to its pathogenicity and virulence may be limited to specific hosts (Hoop et al., 1996).

M. genavense has the zoonotic potential to cause infection in humans, especially in immunocompromised patients (Bottger et al., 1992; Coyle et al., 1992; Berman et al., 1994; Perchere et al., 1995; Hillebrand-Haverkort et al., 1999), and the owners of pet birds in general may be potentially at risk of this pathogenic microorganism. Hence, immunocompromised persons, children and elders, who may be involved in the breeding of exotic birds, should take maximum care during their contact and handling of these birds.

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