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Mycobacteriosis Caused by *Mycobacterium genavense* in Birds Kept in a Zoo: 11-Year Survey

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We report on a disease in 27 birds (1 bird belonging to the order *Coraciiformes*, 3 to *Piciformes*, 4 to *Galliformes*, 7 to *Psittaciformes*, and 12 to *Passeriformes*) caused by fastidious mycobacteria. All birds were caged at the Antwerp Zoo and died suddenly between 1983 and 1994. Seventeen birds had no previous signs of disease, and 10 birds showed emaciation. Gross necropsy findings were generally nonspecific, but all the birds were smear positive for acid-fast bacilli (AFB). Histopathologic evaluation performed on 14 birds revealed predominantly intracellular AFB. Extracellular AFB were more abundant in advanced lesions, especially in necrotic areas. In the intestine the mucosal area was generally heavily infiltrated, suggesting an intestinal origin of the infection. There was extensive invasion of the lungs in most birds. In 11 birds sparse growth was obtained after at least 6 months of incubation on Löwenstein-Jensen medium or on Ogawa medium supplemented with mycobactin. Subculture was unsuccessful in all instances. The 16S rRNA gene sequence of the cultured organisms or tissues from seven birds revealed the characteristic signature sequence for *Mycobacterium genavense*. Direct bird-to-bird transmission in the zoo was unlikely, and the pathogenicity of *M. genavense* in birds seems to be limited. The source of *M. genavense* in nature and the epidemiology of the disease in birds remain obscure. As suspected for human cases of *M. genavense* infection, an oral route of infection has been suggested, and contaminated local water distribution systems may have been the source of the infection. Our study confirms that infections caused by *M. genavense* should be suspected in birds (especially in *Passeriformes* and *Psittaciformes* orders) that die suddenly without previous symptoms and that have AFB in tissues that are difficult to grow on conventional media.

Mycobacterial disease caused by *Mycobacterium avium* is well known in captive birds (11), and avian tuberculosis caused by noncultivable mycobacteria has been reported in the literature (12). Over the last few years, several investigators have reported infections in humans and animals caused by mycobacteria which are difficult to culture (5, 15, 16). In humans, these mycobacteria have been isolated from AIDS patients (2, 3, 7, 9, 14, 19, 25). Sequencing of the 16S rRNA gene indicates that these organisms belong to the new species *Mycobacterium genavense* (6, 16). More recently, a similar mycobacterium closely related to *M. genavense* was isolated from an immunocompetent patient with lymphadenitis (5).

The origin of *M. genavense* infection in humans and animals remains obscure. Investigations on nonhuman sources of *M. genavense* may enhance our knowledge of the epidemiology of the disease in humans.

We report here on a disease in 27 birds caused by mycobacteria that were noncultivable or difficult to grow on conventional media. All of the birds were caged at the Antwerp Zoo and died between 1983 and 1994. For all seven birds whose organisms or tissues were subjected to molecular analysis, *M. genavense* was established as the etiologic agent.

MATERIALS AND METHODS

The birds. From 1983 to 1994, 27 birds (1 bird belonging to the order *Coraciiformes*, 3 to *Piciformes*, 4 to *Galliformes*, 7 to *Psittaciformes*, and 12 to *Passeriformes*) died suddenly in the Antwerp Zoo (Table 1). They were autopsied, and several tissues from each bird were subjected to bacteriologic, histopathologic, and molecular analyses. The birds were kept alone or in groups in the zoo from the 10th month of life for up to 18 years until their death in aviaries with or without outdoors facilities (Table 1) and were fed fruits, finely chopped meat, insects, seeds, or vegetables, separately or mixed.

Bacteriologic and histopathologic analyses. Acid-fast bacilli (AFB) were detected by Ziehl-Neelsen (ZN) staining in smears of touch preparations of various tissues obtained at necropsy (liver, spleen, lung, intestines, kidney, bone marrow). Specimens of some organs from 14 birds were fixed in 10% formalin and were processed routinely for histopathologic examination. Sections were cut to a thickness of 4 μm and were stained with hematoxylin-eosin, ZN, and Fite-Faraco stains for AFB and by the Gomori methenamine-silver method to detect both ZN-stainable AFB and carcasses of ZN-nonstainable AFB.

In vitro cultures. Tissues from the 27 birds (Table 2) were homogenized individually and were decontaminated with 0.5 N NaOH (final concentration) as described by Petroff (21), by the Zephiran-trisodium phosphate method (26), or with 0.5 N HCl as described by Portaels (22). Before inoculation of the culture media, some samples were mixed with an antibiotic solution to prevent excessive contamination (penicillin, 100 IU/ml; nalidixic acid, 35 μg/ml; natamycin, 50 μg/ml). A few uncontaminated specimens (e.g., bone marrow) were inoculated directly onto culture media. Specimens were inoculated onto slants of both Löwenstein-Jensen and 1% Ogawa egg yolk medium supplemented with mycobactin J (24). All inoculated media were incubated for up to 12 months at 36°C and were observed weekly. All cultures of mycobacteria were subcultured onto the same medium on which the original growth was detected and the subcultures were incubated at the same temperature at which the original cultures were incubated.

Nucleic acid analysis. DNA was extracted from tissue samples as described previously (4). Five microliters of DNA extract or 0.5 μl of the first-run product was used as the template for amplification. PCR was performed on a total volume of 50 μl for the first run (100 μl for the nested PCR); the 50-μl total volume contained 20 pmol of each primer (25 pmol for the nested PCR), 1 U of Ampli Taq DNA Polymerase (Roche Molecular Systems, Branchburg, N.J.), 200 μM (each) deoxynucleotide triphosphate (Boehringer Mannheim, Biochemica), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and 10 mM Tris HCl (pH 8.6) (20°C). For the first run, primer G64R (5′-CGTGCTTAACACATGCAAGT CG) (corresponding to positions 43 to 64 in the 16S rRNA sequence of *Escherichia coli* [8]) and primer rM595R (5′-CCGTGAGATTTCACGAACAACGC;

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TABLE 1. The 27 birds infected by noncultivable or difficult-to-grow mycobacteria

Bird no.	Species	Scientific name	Order	Outdoor access	Mo in zoo
1	Asian golden weaver	*Plocues hypoxanthus*	*Passeriformes*	Yes	ND[a]
2	Cutthroat weaver	*Amadina fasciata*	*Passeriformes*	No	36
3	Cutthroat weaver	*Amadina fasciata*	*Passeriformes*	No	48
4	Eurasian goldfinch	*Carduelis carduelis*	*Passeriformes*	Yes	36
5	Golden-breasted starling	*Cosmopsarus regius*	*Passeriformes*	No	144
6	Gouldian finch	*Chloebia gouldiae*	*Passeriformes*	No	36
7	Java sparrow	*Padda oryzivora*	*Passeriformes*	No	96
8	Red-billed quelea	*Quelea quelea*	*Passeriformes*	Yes	48
9	Red-cheeked cordon-bleu	*Uraeginthus bengalus*	*Passeriformes*	No	ND
10	Silver-eared mesia	*Leiothrix argentauris*	*Passeriformes*	Yes	19
11	White-browed laughing thrush	*Garrulax sannio*	*Passeriformes*	Yes	156
12	Zebra finch	*Poephila guttata castanotis*	*Passeriformes*	No	24
13	Blue and yellow macaw	*Ara ararauna*	*Psittaciformes*	No	144
14	Military macaw	*Ara militaris*	*Psittaciformes*	Yes	10
15	Yellow-collared macaw	*Ara auricollis*	*Psittaciformes*	No	216
16	Yellow-headed amazon	*Amazona ochrocephala oratrix*	*Psittaciformes*	No	84
17	Golden conure	*Aratinga guarouba*	*Psittaciformes*	No	108
18	Grey-cheeked parakeet	*Brotogeris pyrrhopterus*	*Psittaciformes*	Yes	36
19	White cockatoo	*Cacatua alba*	*Psittaciformes*	No	22
20	California quail	*Lophortyx californica*	*Galliformes*	No	72
21	California quail	*Lophortyx californica*	*Galliformes*	No	48
22	Indian blue quail	*Coturnix chinensis*	*Galliformes*	No	72
23	Indian blue quail	*Coturnix chinensis*	*Galliformes*	No	60
24	Saffron toucanet	*Baillonius bailloni*	*Piciformes*	No	96
25	Toco toucan	*Ramphastos toco*	*Piciformes*	Yes	108
26	Toco toucan	*Ramphastos toco*	*Piciformes*	Yes	144
27	Green wood-hoopoe	*Phoeniculus purpureus*	*Coraciiformes*	Yes	144

[a] ND, not determined.

positions 617 to 595) were used. The nested PCR was performed with primer G64R and 5′-biotin-labeled primer rM592B (5′-TTTCACGAACAACGCGAC; positions 609 to 592). The primer combination resulted in genus-specific amplification of any mycobacterial 16S rRNA gene, irrespective of the species affiliation. PCR mixtures were overlaid with mineral oil and were placed in an automatic thermal cycler (Thermolyne Temp Tronic, Barnstead, Iowa). After an initial denaturation step (5 min at 94°C), the samples were submitted to 35 and 30 (first and second runs, respectively) thermal cycles (denaturation, 45 s at 94°C; annealing, 1 min at 56°C; extension, 45 s at 72°C). The last cycle was followed by a final extension step (72°C, 10 min).

Forty microliters of the second-run PCR products was used for sequencing. The biotinylated single-stranded DNA of the amplified gene fragment was separated from its complementary strand by using streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin; Dynal A.S., Oslo, Norway) and a Dynal MPC-6 instrument (Dynal A.S.) according to the manufacturer's instructions. The single-stranded DNA attached to the beads was resuspended in 10 μl of H_2O, and the complementary strand in the supernatant was recovered in the same volume after the pH was adjusted to 7.6. Each strand of DNA served as a template for direct sequencing reactions with the T7 sequencing kit (Pharmacia Biotech), which were performed according to the manufacturer's instructions. The primers used were M82 (5′-CATGCAAGTCGAACGGAAAGG; positions 54 to 82) and rM587 (5′-ACGAACAACGCGACAAACC; positions 605 to 587). Labeling was performed with [^{35}S]dATP. Three microliters of each reaction mixture was heated at 80°C for 5 min before loading onto a 6% polyacrylamide gel. After electrophoresis, the gels were fixed in 10% acetic acid–10% methanol and dried. The gels were exposed to X-ray film for 48 h.

RESULTS

Seventeen of the 27 birds had no previous signs of disease, and 10 birds (37%) showed emaciation. At necropsy, gross lesions typical of granulomatous inflammation in tuberculosis were noted in only one bird (bird 23). Other necropsy findings were generally nonspecific: congestion of intestines and lungs, enlarged spleen, and swollen liver. In all birds, smears from one or more organs showed variable numbers of AFB (Table 2) (1+ to 4+ according to the scale of the American Thoracic Society [1]).

Tissues from 14 birds (birds 1, 4, 10, 11, 13 to 18, 20, and 24 to 26, as listed in Table 2) were available for histopathologic evaluation; the numbers and kinds of specimens studied are given in Table 3, which shows the numbers of AFB, the amount of cellular exudates, and necrosis on a scale of 0 to ++++. The intestine was involved in all specimens; this was followed, in descending order of frequency, by the lung, liver, spleen, and kidney. While AFB were predominately intracellular, in more advanced lesions some AFB were extracellular, especially in necrotic areas. AFB usually stained equally well by the ZN and Fite-Faraco techniques. In most tissues the Gomori methenamine-silver stain revealed much larger numbers of bacillary bodies than did the acid-fast stains, suggesting that many residual degenerating or dead bacilli were not being cleared by the tissue reaction. Most AFB were granular or irregularly stained and measured 1.5 to 4 μm in length. In no instance were AFB found in the absence of cellular exudates, and, conversely, except in one kidney, AFB were found in all specimens containing cellular exudates. Organized tuberculoid types of granulomas were never seen. In the intestine the mucosal area was regularly and most heavily infiltrated, suggesting an intestinal origin of infection. Macrophages predominated in all lesions, but they were intermixed with various numbers of lymphocytes. Most advanced lesions showed necrosis. All lung specimens contained focal, small to large infiltrations of macrophages with few lymphocytes and various amounts of necrosis. The liver was infiltrated in 8 of 11 birds, with extensive lesions in three liver specimens. Most lesions were composed of small foci of macrophages with few lymphocytes. In three specimens these foci tended to fuse into large lesions, with central necrosis in two of the three specimens. Three of four spleens contained small infiltrations of macrophages, and only one spleen showed necrosis. All infiltrations in the kidney were slight, and hearts from two birds were free of infection.

TABLE 2. Microscopic and culture findings for 27 birds with mycobacteriosis caused by noncultivable or difficult-to-grow mycobacteria

Bird no.	Score by direct smear examination[a]:					Organ(s) cultured	Decontamination[b]	Growth on[c]:	
	Intestines	Lung	Liver	Spleen	Others			LJ	OM
1		2+	1+			Liver, lung	NaOH	−	
2	3+	2+	2+		Kidney, 1+; feces, 4+; femur, −	Kidney, lung, intestines, feces	NaOH	−	−
3	−	3+	1+		Kidney, 1+	Lung	HCl	−	−
4	2+	3+	3+	3+		Liver, lung	Z-TSP	+	
5	−	3+	3+		Airsac, −	Liver	Z-TSP		−
6	−	3+	4+			Liver, lung	HCl		−
7		3+	−			Lung	HCl	C	−
8		3+	3+			Liver, lung	Z-TSP	+	+
9		1+	1+		Kidney, 1+	Liver, lung	NaOH	−	−
10		2+	1+	2+	Kidney, 1+	Lung	NaOH	−	
11	−	3+	1+	1+		Lung	Z-TSP	+	+
12	4+	4+	1+		Kidney, 1+	Intestines, lung	HCl		+
13	4+	−	1+	3+	Ascites, 3+	Spleen	Z-TSP		+
14	4+	3+	3+	3+		Liver	NaOH	−	
15		3+	3+			Liver	Z-TSP		−
16		4+	−	−		Lung	Z-TSP		+
17	4+	2+			Kidney, 2+	Lung, kidney	Z-TSP	+	+
18	4+	−	3+	4+		Liver	Z-TSP	+	
19	−	4+	−	3+	Kidney, 1+	Lung	Z-TSP	+	+
20	4+	3+	3+	3+	Marrow, 4+	Marrow	None		−
21	4+		2+		Crop, 3+	Liver	NaOH		C
22	−	3+	3+	3+	Marrow, 3+	Lung, liver, spleen	Z-TSP		−
						Marrow	None		−
23	−	2+	2+		Marrow, 2+	Marrow	None		−
24	4+	4+	4+	4+	Kidney, 4+	Liver	Z-TSP	+	
25	1+	3+	3+			Liver	NaOH	C	
26	1+	4+	3+			Liver	Z-TSP	C	
27		2+	−	−	Kidney, −	Lung	Z-TSP	+	+

[a] Results according to the scale of the American Thoracic Society (1); blank spaces, no data.
[b] NaOH, 0.5 N NaOH (final concentration); HCl, 0.5 N HCl (final concentration); Z-TSP, Zephiran-trisodium phosphate method (26).
[c] LJ, Löwenstein-Jensen; OM, Ogawa plus mycobactin; blank spaces, no data; C, contaminated.

For the 11 birds whose specimens yielded a positive primary culture after 6 months of incubation on Löwenstein-Jensen slants or on Ogawa-mycobactin slants (Table 2), growth was sparse (a few small greyish colonies). No positive culture was obtained after specimen decontamination with 0.5% NaOH (final concentration). All attempts at subculture were unsuccessful. Two primary cultures of the lungs of birds 11 and 17 were lyophilized.

DNA was extracted from the two lyophilized strains as well as from the tissues of the last five birds received: the intestine and the feces of bird 2, the lungs of birds 3, 6, and 7, and finally, the intestine and lung of bird 12. Direct sequencing of PCR-amplified DNA preparations revealed in all samples tested the characteristic signature sequence for *M. genavense* (7).

DISCUSSION

M. genavense has been found in patients with advanced human immunodeficiency virus infections, most often associated with chronic illness characterized by fever, diarrhea, and marked loss of weight (7). This mycobacterium was first reported by Hirschel et al. (15). Since 1990, more than 50 cases of disseminated infection caused by *M. genavense* (7, 18, 20, 25) have been described. Hoop et al. (16) demonstrated *M.*

TABLE 3. Histopathologic findings in 14 animals[a]

Organ	Total no. of specimens	No. of specimens													
		AFB					Cellular exudate					Necrosis[b]			
		0	+	++	+++	++++	0	+	++	+++	++++	0	+	++	+++
Intestine	7			3	3	1				4	3	2	1	3	1
Lung	13		4	6	3			4	4	5		7	5	1	
Liver	11	3	3	4	1		3	3	2	3		9	2		
Spleen	4	1	1	2			1	1	2			3	1		
Kidney	6	4	2				3	3				6			
Heart	2	2					2					2			

[a] The scales for AFB, cellular exudate, and necrosis are entirely subjective and unrelated to each other in any quantitative way.
[b] No specimens scored ++++.

genavense in six pet birds (four of the order *Psittaciformes* and two of the order *Passeriformes*) by PCR amplification and sequencing of BACTEC primary cultures. Recently, *M. genavense* DNA has been amplified by PCR from intestinal tissues of human immunodeficiency virus-uninfected individuals (13).

In AIDS patients, *M. genavense* is usually found in the blood, feces, bone marrow, colon, duodenum, mesenteric lymph node, liver, and spleen (7, 18, 19, 25). The lung is rarely or only slightly affected, even in patients with progressive disease (18, 19). Böttger et al. (7) described a patient whose bronchoalveolar lavage revealed pulmonary toxoplasmosis but no mycobacteria, even though two blood cultures were positive for *M. genavense*. Because of the striking gastrointestinal symptoms (7, 18), the gut has been suggested as the primary site of infection and the reservoir from which *M. genavense* invades other human tissues.

In birds, at necropsy AFB are usually most abundant in the intestines, suggesting an oral route of infection. The lung, liver, and spleen are the other organs most commonly invaded by *M. genavense*. In the present series there was extensive invasion of the lungs in all birds except birds 9, 13, and 18 (Table 2).

The origin of *M. genavense* infection in birds remains obscure. The birds were kept in aviaries with or without access to outdoors facilities (Table 1). The aviaries with outdoor facilities give free access to wild birds which could have soiled these areas with their droppings. Therefore, 10 of the 27 birds in the present series (Table 1) could have been contaminated by wild birds. Five of 12 *Passeriformes* birds (42%) were exhibited in a dark gallery where they were kept in open cages but could occasionally intermingle with each other. Most of the birds in the present series were kept in groups (the same or mixed species); eight birds (30%) were kept in isolated cages. The 19 birds kept in constant contact with larger groups of birds were the only birds of their respective cages which were affected, while the other birds in the respective cages remained healthy. This argues against direct bird-to-bird transmission and suggests that the pathogenicity of these fastidious mycobacteria is limited, as mentioned by Hoop et al. (16). On the contrary, *M. avium* infection is readily transmitted in the feces of birds (10). Unfortunately, it was not possible to perform a systematic examination of all asymptomatic birds. During 1983 until 1994, 18 other birds belonging to the same orders (e.g., *Passeriformes* and *Psittaciformes*) died under similar circumstances. Although there were no nodular lesions at necropsy, ZN staining of tissues from these birds showed numerous AFB. No cultures were performed and no material was kept for further investigation; however, these birds are suspected of having been infected with the same species of mycobacteria.

All birds were fed a commercial seed mixture, insects, fruits, vegetables, and chopped meat. Zookeepers prepared the food in a central kitchen, and small bowls filled with fresh tap water were provided each day. White sand was used as litter in the indoor cages. The sand was replaced every year, or earlier in the event of the diagnosis of an infectious disease among the birds. Potential contamination of food or of the environment by wild birds was not obvious. However, as suspected for human cases (9), contaminated local water distribution systems may be the source of infection.

The epidemiologic significance of the prevalence of *M. genavense* infection in such varied hosts as birds (16), human immunodeficiency virus-uninfected individuals (13), and AIDS patients (2, 3, 7, 9, 14, 19, 25) should be ascertained. In all three of these hosts, an oral route of infection has been suggested (7, 13, 16, 18). *M. genavense*, like many other atypical mycobacteria (23), may be an ubiquitous environmental organism that infects only a small proportion of exposed humans or animals. Additional studies are in progress to detect *M. genavense* in the environment. In animals, as in humans, certain risk factors related to the state of immunity may promote the dissemination of *M. genavense*. Subclinical disease may be more common than suspected. Further research should be aimed in this direction.

We believe it is significant that the identification of *M. genavense* in the present study and that of Dumonceau et al. (13) is based primarily on the characteristic signature sequence in the 16S rRNA gene (7). It is known that mycobacteria with identical 16S rRNA gene sequences could be responsible for different types of clinical infections. This is the case for *M. avium*, which has a 16S rRNA gene sequence undistinguishable from that of *Mycobacterium paratuberculosis* (17).

In conclusion, infection caused by *M. genavense* should be suspected in birds (especially in birds belonging to the orders *Passeriformes* and *Psittaciformes*) that die suddenly without a significant clinical history or gross specific postmortem findings and that have in their tissues AFB that are noncultivable or difficult to grow on conventional media. The present study also demonstrates the importance of the currently available biomolecular technology that permits the identification of such microorganisms.

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