

Short communication

Avian tuberculosis in naturally infected captive water birds
of the Ardeidae and Threskiornithidae families
studied by serotyping, IS901 RFLP typing,
and virulence for poultry

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Received 15 February 2006; received in revised form 12 September 2006; accepted 15 September 2006

Abstract

Avian tuberculosis was detected in one flock of 38 water birds of the families Ardeidae ($n = 20$) and Threskiornithidae ($n = 18$). *Mycobacterium avium* subsp. *avium* (MAA, serotype 1, genotype IS901+ and IS1245+) was more often ($p = 0.01$) detected in tissue and/or faecal samples in 18 (90.0%) birds from the Ardeidae family: little egret (*Egretta garzetta*), buff-backed heron (*Bubulcus ibis*), great white egret (*Egretta alba*), and bittern (*Botaurus stellaris*) in comparison to two (11.1%) birds from the Threskiornithidae family: sacred ibis (*Threskiornis aethiopicus*). Avian tuberculosis was not diagnosed in spoonbills (*Platalea leucorodia*). Tuberculous lesions were found in nine birds. MAA isolates of IS901 RFLP type F-C3 were present in all of the 20 infected birds and in all environmental isolates. A mixed infection with the MAA isolates of three RFLP types F-C3 (tissue isolate), G-C3, and T-C3 (faecal isolates) was found in one sacred ibis. All 20 tissue isolates of IS901 RFLP type F-C3 from 20 birds and 8 environmental MAA isolates were fully virulent in pullets, whilst the isolates of RFLP types G-C3 and T-C3 were non-virulent in pullets. All of the tested MAA isolates had the same IS1245 RFLP “bird profile”. In 12 of 20 infected birds with MAA *M.a. hominissuis* isolates of serotypes 4, 8, 9 and genotype IS901– and IS1245+ were detected and in 8 other birds mycobacteria not belonging to the *M. avium* complex were found. The presence of MAA in the environment may be a source for further spread of the causal agent of avian tuberculosis among other groups of animals in zoological gardens, farm animals, and also among their keepers.

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Keywords: Avian mycobacteriosis; PCR; Ecology; Captive birds; Zoonosis; Zoological garden

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1. Introduction

Avian tuberculosis caused by *Mycobacterium avium* subsp. *avium* (MAA) of serotypes 1, 2 and 3 and genotype IS901+ and IS1245+ is a serious disease of animals and humans (Mijs et al., 2002; Dvorska et al., 2003; Thegerstrom et al., 2005). Whilst many animal species can be infected, birds are particularly susceptible, often leading to fatal organ tuberculosis. When domestic farm animals are infected, particularly cattle and pigs, tuberculous lesions are commonly found localized to the head and intestinal lymph nodes (Pavlik et al., 2002, 2005; Dvorska et al., 2004; Matlova et al., 2005).

Various methods can be employed to identify particular species and even different isolates of MAA epidemiology, such as biological experiments in pullets (Piening et al., 1972) and serotyping (Wolinsky and Schaefer, 1973). More recently molecular biological methods such as: Accu-Probes (San Diego, California, USA), polymerase chain reaction (PCR), and restriction fragment length polymorphism (RFLP) analysis are being employed (Kunze et al., 1992; Ritacco et al., 1998; Pavlik et al., 2000; Mijs et al., 2002; Dvorska et al., 2003, 2004). The discovery of the specific insertion sequence IS901, led to the development of a rapid method for identification of MAA by PCR. This is due to the presence of 2–13 copies of IS901 in the genome of all three recognized serotypes 1, 2 and 3 of MAA. RFLP analysis can then further discriminate between different isolates, which is particularly useful for epidemiological studies (Kunze et al., 1991; Mijs et al., 2002; Dvorska et al., 2003, 2004; Matlova et al., 2005).

Animals with advanced forms of avian tuberculosis intensively contaminate their surrounding environment and consequently represent a serious risk of spreading the disease. Transmission of disease particularly risky in domestic pigeons (*Columba livia* f. *domestica*), sparrows (*Passer* sp.), turtle-doves (*Streptopelia* sp.), and wild ducks (*Anas* sp.), which are commonly found in large numbers in zoological gardens all the year-round. During winter, crows (Corvidae) and laughing gulls (*Larus ridibundus*) frequently found around zoological gardens could spread the diseases as well (Hejlíček and Tremel, 1994).

Investigation by Hejlíček and Tremel (1995) allowed classification of bird species according to their susceptibility/resistance to avian tuberculosis into the following four groups: (i) highly susceptible: domestic fowl (*Gallus domesticus*), sparrows (*Passer* sp.), ring-necked pheasants (*Phasianus colchicus*), grey partridges (*Perdix perdix*), and laughing gulls, (ii) less susceptible: guinea fowl (*Numida meleagris* f. *domestica*) and domestic turkeys (*Meleagris gallopavo* f. *domestica*), (iii) moderately resistant water birds: the domestic goose (*Anser anser* f. *domestica*), and the domestic duck (*Anas platyrhynchos* f. *domesticus*) and (iv) highly resistant: the domestic pigeon (*C. livia* f. *domestica*), the collared turtle-dove (*Streptopelia decaocto*), and the rook (*Corvus frugilegus*).

Stress factors appear to enhance the development of avian tuberculosis in birds living in captivity. Galliformes, Gruiformes and Anseriformes are the orders that appear to be most susceptible in zoological collections (Saha et al., 1995). The most common route of infection for susceptible birds is via the alimentary tract; however, MAA has also been detected in eggs from an infected flock of domestic birds (Hines et al., 1995; Thorel et al., 1997). Most tuberculous lesions caused by MAA develop in the intestinal tract, liver and spleen but occasionally also in the heart, ovaries or testes. Pulmonary avian tuberculosis is only seen occasionally and the central nervous system is rarely affected (Thorel et al., 1997).

The purpose of this study was to investigate the occurrence of MAA in one naturally infected flock of water birds with six different species from two families (Ardeidae and Threskiornithidae) kept in a zoological garden in the Czech Republic. The MAA infection spread relatively rapidly through the flock and birds were gradually dying. Due to these facts the whole flock was culled. This enabled us to study distribution of MAA in the bodies of respective birds and their faeces by serotyping and IS901 RFLP analysis.

2. Materials and methods

2.1. Examined birds and environmental samples

A total of 38 birds from 6 different species of water birds in 2 families were examined; 20 birds belonged to the Ardeidae family: 13 little egrets (*Egretta garzetta*),

5 buff-backed herons (*Bubulcus ibis*), 1 great white egret (*Egretta alba*), and 1 bittern (*Botaurus stellaris*) and 18 birds belonged to the family Threskiornithidae: 14 sacred ibises (*Threskiornis aethiopicus*) and 4 spoonbills (*Platalea leucorodia*). A total of 417 tissue and 814 faecal samples were examined. A further 491 samples taken from the environment surrounding the aviaries that housed these infected birds (340 and 151 samples from a summer and winter aviary, respectively) were also examined.

After collection, organ samples were transported to the laboratory in insulated boxes with ice packs at 4 °C. Tissue for histological examinations were immersed in a 10% formalin. Organ samples were frozen at –18 °C and kept for up to 3 weeks until processing, whereas the environmental samples were kept at 4–6 °C in a dark room for up to 2 weeks.

2.2. Gross examination and histopathology

Tissue samples from 18 euthanized and 20 naturally dead birds were rapidly thawed at 37 °C and examined for the presence of tuberculous lesions. A total of 417 tissues were formalin fixed, embedded in paraffin blocks and stained by Ziehl-Neelsen (ZN) technique for the presence of acid-fast rods (AFR). Histological samples were observed by light microscopy using a 1000× magnification under oil immersion (Olympus B17, Japan).

2.3. Tissue impressions microscopy and culture examinations

Slides prepared from tissue impressions were stained by the ZN technique and examined by light microscopy for presence of AFR. At least 200 fields of view were examined for each sample. For culture examination, approximately 1 g of tissue or environmental samples were after the homogenisation and decontamination inoculated on solid and liquid media and incubated simultaneously at two temperatures (24 and 37 °C) in quadruplicates for 3 months as described previously (Fischer et al., 2001).

2.4. Identification of isolates

All the AFR positive isolates were examined by the PCR method for detection of *dnaJ+*, a gene specific

for the *Mycobacterium* genus, using primers (below) according to Nagai et al. (1990): 5'-GGG TGA CGC GAC ATG GCC CA-3' and 5'-CGG GTT TCG TCG TAC TCC TT-3'. DNA of the mycobacterial isolates was first denatured at 94 °C for 1 min, then amplified with 30 cycles of (i) denaturation at 94 °C for 1 min, (ii) primer annealing at 58 °C for 2 min, and (iii) elongation at 72 °C for 1 min, using a thermocycler (model MJ Research PTC200, USA). After the last amplification cycle, the samples were further incubated at 72 °C for 10 min for complete elongation of the final PCR products. Results were confirmed by detection of 1030 bp fragments of 16S rRNA specific for the genus *Mycobacterium* (Wilton and Cousins, 1992). PCR was further employed to detect IS901 (Kunze et al., 1992; Pavlik et al., 2000) and IS1245 to differentiate between *M. avium* subsp. (Guerrero et al., 1995; Bartos et al., 2006). All *M. avium* complex (MAC) isolates were serotyped (Wolinsky and Schaefer, 1973) using the method modified by Süßland and Hrdinova (1976). Mycobacterial isolates not classified as MAC were assessed by biochemical methods (Wayne and Kubica, 1986).

2.5. Virulence testing of *M. a. avium* isolates

Virulence in birds (defined as the ability to produce tuberculous lesions in parenchymatous organs) of 30 selected MAA isolates was studied by gross examination after intramuscular isolate administration to pullets (Pavlik et al., 2000). The isolates selected were from twenty tissue samples (one isolate from each infected bird), two faecal samples from one sacred ibis and eight environmental samples.

2.6. IS901 and IS1245 RFLP method

Ninety two randomly selected MAA isolates (71 tissues and 21 faecal isolates) from all 20 infected birds were analysed using standardized restriction fragment length polymorphism (RFLP) methods with IS901 and IS1245 hybridisation probes (van Soolingen et al., 1993; Dvorska et al., 2003). Mycobacterial DNA was isolated according to the method described by van Soolingen et al. (1993). Approximately, 5 µg of purified mycobacterial DNA was digested with restriction endonucleases *PvuII* and *PstI*. After separation by electrophoresis in agarose gel, DNA fragments were

transferred to a nylon membrane by vacuum-blotting. DNA was fixed and hybridised with a labelled probe according to a previously described method (Dvorska et al., 2003). IS901 RFLP profiles were analysed according to a system described by Dvorska et al. (2003). IS1245 RFLP profiles of MAA isolates were differentiated according to Ritacco et al. (1998), van Soolingen et al. (1998) and Dvorska et al. (2003).

2.7. Statistical evaluation

The Chi²-test (Stat Plus) was applied for the statistical evaluation of the results (Matouskova et al., 1992).

3. Results

3.1. Gross examination and histopathology

From 38 wild water birds of 6 species kept together in one zoological garden, tuberculous lesions of various sizes were detected in 9 (23.7%) examined birds. Tuberculous lesions were found in the liver samples of all except of one bird (little egret L5). The size of tuberculous lesions varied from organ to organ ranging from 1 to 7 mm. Tuberculous lesions between 1 and 5 mm were observed in the spleen of seven birds (all except of little egret L3 and sacred ibis S2). Smaller tuberculous lesions (1–3 mm) were detected

Table 1
Distribution of mycobacteria in 20 naturally infected captive water birds

Examined tissue	Pathological lesions in parenchymatous organs																			
	Present										Not present									
	L1	L2	L3	L5	L8	L10	L11	H1	S1	L4	L6	L7	L9	L12	H2	H3	H4	E1	B1	S2
Oesophagus	A ^d	nt	nt	nt	nt	nt	nt	A ^a	0	nt	0	0	H	nt	0	nt	0	0	0	0
Stomach-glandular	A ^d	0	0	0	A ^d	A ^d	0	A ^a	H	0	0	0	0	0	0	nt	0	nt	0	0
Stomach-muscular	nt	0	0	0	A ^d	nt	nt	0	0	H	H	0	H	nt	nt	A ^d	0	nt	0	0
Jejunum	A ^d	A ^d	0	A ^d	A ^d	A ^d	A ^d	0	A ^d	H	H	0	A ^d	0	0	0	0	0	0	0
Colon	A ^d	A ^d	A ^d	0	0	H	0	A ^d	A ^d	0	H	0	0	0	0	H	0	0	0	0
Liver	A ^d	A ^d	A ^a	0	A ^d	A ^d	A ^d	A ^d	A ^a	H	H	A ^d	0	0	0	0	0	H	0	H
Spleen	A ^d	A ^d	0	A ^d	A ^d	A ^d	A ^d	A ^a	0	0	A ^d	0	0	0	0	0	0	0	0	0
Heart	A ^d	A ^d	A ^d	0	A ^d	A ^d	nt	A ^a	nt	nt	0	0	A ^d	nt	0	0	A ^d	0	0	nt
Lung	A ^d	A ^d	A ^d	A ^d	A ^d	A ^d	nt	A ^d	0	A ^d	H	H	A ^d	A ^d	0	0	0	A ^d	0	A ^d
Kidney	A ^d	A ^d	A ^d	A ^d	A ^d	nt	A ^d	A ^a	0	A ^d	A ^d	0	0	O	0	A ^d	0	0	0	0
Bone marrow	0	nt	nt	A ^d	A ^d	0	nt	A ^d	0	A ^d	0	0	0	nt	A ^d	0	0	0	0	0
Ovary	nt	A ^d	A ^d	A ^d	nt	nt	nt	nt	nt	0	nt	nt	0	nt	0	0	0	nt	nt	nt
Testes	A ^d	nt	nt	nt	A ^d	nt	nt	A ^a	0	nt	nt	0	nt	nt	nt	nt	nt	0	0	0
No. of tissues	11	10	10	11	11	8	6	12	11	10	11	12	12	7	11	11	11	11	11	11
No. of isolates	10	8	6	6	10	7	4	10	4	6	7	2	5	2	1	3	1	2	0	2
A (<i>M. a. avium</i>)	10	8	6	6	10	6	4	10	3	3	2	1	3	1	1	2	1	1	0	1
H (<i>M. a. hominissuis</i>)	0	0	0	0	0	1	0	0	1	3	5	1	2	0	0	1	0	1	0	1
O (other <i>M. spp.</i>)	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
No. of faecal samples	22	18	30	42	29	35	20	51	80	23	38	32	21	44	48	32	10	50	87	78
No. of faecal isolates	2	0	5	8	5	5	0	1	0	3	7	5	2	3	15	0	0	5	3	9
<i>M. a. avium</i> (A)	0	0	0	6 ^d	1 ^d	3 ^d	0	0	1 ^d	0	0	0	0	1 ^d	1 ^d	0	0	2 ^d	2 ^d	4 ^b
<i>M. a. hominissuis</i> (H)	0	0	0	0	0	2	0	1	0	3	5	2	2	2	0	0	0	3	1	5
Other <i>M. spp.</i> (O) ^c	2	0	5	2	4	0	0	0	0	0	2	3	0	0	14	0	0	0	0	0

L (little egret; *E. garzetta*, Ardeidae); H (buff-backed heron; *B. ibis*, Ardeidae); E (great white egret; *E. alba*, Ardeidae); B (bittern; *Botaurus stellaris*, Ardeidae); S (sacred ibis; *T. aethiopicus*, Threskiornithidae); A (*M. avium* subsp. *avium* of serotype 1 and genotype *dnaJ+*, 1030 bp of 16S rRNA, IS901+ and IS1245+); H (*M. avium* subsp. *hominissuis* of serotypes 4, 8, and 9 and genotype *dnaJ+*, 1030 bp of 16S rRNA, IS901– and IS1245+); O (other mycobacteria not belonging to *M. avium* complex of genotype *dnaJ+*, 1030 bp of 16S rRNA, IS901– and IS1245–).

^a *M. a. avium* IS901+ (RFLP not done).

^b Two isolates of IS901 RFLP type F-C3, one isolate of IS901 RFLP type G-C3, and one isolate IS901 RFLP type T-C3 were detected.

^c *M. terrae*, nt—non tested.

^d *M. a. avium* of IS901 RFLP type F-C3.

in the lung and kidney of eight birds (all except the sacred ibis S2).

Granulomatous inflammation was diagnosed in all tuberculous lesion tissue samples with the detection of numerous AFR. All these samples (except the kidney of little egret L10 and lung of little egret L11: tubes with suspensions were broken during centrifugation) were cultures positive for *MAA*.

3.2. The sensitivity / resistance of captive water birds to avian tuberculosis

MAA infection was found in 18 (90.0%) of the 20 birds from the Ardeideae family; significantly more ($p = 0.01$) than that, found in birds from the Threskiornithidae family, where infection was found in only 2 (11.1%) of the 18 birds. *MAA* isolates from

the 20 (52.6%) infected birds were fully virulent for tested pullets.

3.3. Distribution of *M. a. avium* in a host organism

In the birds of the Ardeideae family *MAA* was detected in 17 egrets (Little Egret, Buff-backed Heron, and Great White Egret) in the intestinal tract organs of 9 (52.9%) birds, in the bone marrow of 5 (35.3%) birds, in other organs such as liver, spleen and heart of 8 (47.1%) birds, in lungs of 11 (64.7%) birds, in kidney of 10 (58.8%) birds, and ovary/testes of 6 (35.3%) tested birds. *MAA* was also detected in the faeces of only 6 (35.3%) egrets, and *MAA* (two isolates) was only detected in faeces of the bittern (*B. stellaris*) whilst the tissue culture was negative (Table 1).

Table 2

Mycobacterial contamination of environmental samples from aviaries that housed naturally infected captive water birds

Origin of samples		Examined samples			<i>M. avium</i> complex members		Others <i>M. spp.</i> ^c
Aviary	Biological material collected	No.	Positive	(%)	<i>M. a. avium</i> ^a	<i>M. a. hominissuis</i> (serotype) ^b	
Summer	Water and sediment from lake	17	2	11.8	0	1 (8)	1
	Soil, moss, and leaves	44	2	4.5	0	2 (8/9)	0
	Trunk, bark, and branch of tree	9	0	0	0	0	0
	Web and dust	6	0	0	0	0	0
	Feed	7	1	14.3	0	1 (8)	0
	Regurgitated food	5	2	40.0	2	0	0
	Mixed bird faeces and soil	32	2	6.3	1	1 (agg.)	0
	Faeces of other animals	2	0	0	0	0	0
	Invertebrates	218	1	0.5	0	0	1
	Subtotal		340	10	2.9	3	5
Winter	Dust, plumage, and webs	17	1	5.9	0	1 (9)	0
	Sand	12	3	25.0	0	2 (4)	1
	Water with a sediment	7	0	0	0	0	0
	Mixed sample of bird faeces	65	6	9.2	5	1 (8)	0
	Matter scraped from the wall and decorations	15	0	0	0	0	0
	Soil and faeces	4	3	75.0	0	3 (8)	0
	Wooden branches and bedding	6	0	0	0	0	0
	Invertebrates	25	1	4.0	0	1 (nt)	0
Subtotal		151	14	9.3	5	8	1
Total		491	24	4.9	8	13	3
%			100		33.2	54.2	12.5

^a Isolates were of serotype 1, genotype *dnaJ+*, 1030 bp of 16S rRNA, IS901+ and IS1245+, and IS901 RFLP type F-C3.

^b Isolates were of genotype IS901– and IS1245+ and of different serotypes indicated in round brackets (4, 8, 9: serotypes; 8/9: cross-reactions between serotypes 8 and 9; nt: not tested; agg.: auto-agglutination).

^c Other mycobacteria than members of *M. avium* complex were of genotype *dnaJ+*, 1030 bp of 16S rRNA, IS901– and IS1245–; specifically three different species: one *M. gastri*, one *M. chelonae* and one *M. scrofulaceum* were present.

In the birds of family Threskiornithidae *MAA* isolates were detected in parenchymatous organs, in the intestinal tract and in the faeces of only 2 (14.3%) of 14 sacred ibises but was not detected in the organs or faeces of four spoonbills (Table 1).

3.4. Identification of *M. a. avium* isolates

One hundred *MAA* isolates (genotype *dnaJ*+, 1030 bp of 16S rRNA, IS901+ and IS1245+, serotype 1) were obtained from 20 individual birds (Table 1).

RFLP type F-C3 was detected in 71 (89.9%) randomly selected *MAA* isolates from organs from 19 birds and in 19 faecal isolates from 14 birds. In one bird (sacred ibis S2) two RFLP types G-C3 and T-C3 were found in faeces (Table 1).

A total of 24 (4.9%) mycobacterial isolates were obtained from 491 environmental samples, which were collected from the inside of aviaries that housed these infected birds and the surrounding area (Table 2). Eight (33.2%) isolates were identified as *MAA* of serotype 1 and IS901 RFLP type F-C3 and were virulent by the infection of pullets.

3.5. Identification of *M. a. hominissuis* isolates

M. a. hominissuis (*MAH*) was isolated from 12 (60.0%) of 20 infected birds with *MAA*. Three of 9 birds with *MAH* were found to have tuberculous lesions as 9 of the 11 birds with *MAH* were recorded as not having tuberculous lesions. *MAH* of serotypes 4, 8 and 9 was isolated from 10 faecal samples (50.0%), also from 6 gastrointestinal tract samples (30.0%), from 2 liver and spleen (10.0%), and from the lungs of 2 (10.0%) of 20 infected birds. In any case *MAH* was isolated from bone marrow, testes, and/or ovary glands (Table 1).

From a total of 491 examined samples originating from the surroundings of the summer and winter aviaries that housed the infected birds, *MAH* isolates were detected in 13 samples originating from variety of different environmental origins (water, soil, faeces, webs, sand and invertebrates; Table 2).

4. Discussion

The typing of *MAA* by measuring the presence and the genomic distribution of IS901 has been shown to

be a useful rapid indicator of virulence for birds (Pavlik et al., 2000) and epidemiological studies (Dvorska et al., 2003, 2004). Based on previous studies (Dvorska et al., 2003, 2004) IS901 was found to be more plastic than IS1245 and could be associated with host specificity and persistence. From this reason IS901 RFLP analysis confirmed by serotyping was used to study occurrence of *MAA* in one naturally infected flock of water birds with six different species from two families (Ardeidae and Threskiornithidae) kept in a zoological garden in the Czech Republic. All of the birds examined (order Ciconiiformes) in this study share similar habitats and feeding patterns, so the difference in prevalence of *MAA* infection may indicate a genetic difference in susceptibility to this disease. Cromie et al. (1991) published similar results which showed significant differences in susceptibility to infection caused by *MAA* within taxonomic tribes of birds from the order Anseriformes (tribes Cairinini and Mergini) with the percentage of mortality due to avian tuberculosis ranging from 0 to 94% between different species.

All of the *MAA* isolated from the water birds examined in this study were identified as serotype 1; this is the most common serotype of *MAA* found in collections of birds living in captivity (Painter, 1997).

The route of infection of *MAA* in birds is usually through feed or water intake. The source of the outbreak study in our paper is still unknown. *MAA* passes through the intestinal wall and penetrates the intestinal tract and lymph system. As the infection progresses, it spreads to the blood stream and on to the liver via the portal vein (Thorel et al., 1997, 2001). Polycystic livers have been reported to be associated with *MAA* infection in water birds (Roffe, 1989). After colonisation of the liver, mycobacteria are subsequently spread to various organs via the blood stream, and to the intestines through the bile duct. Primary lesions then develop in a number of organs, especially the intestines. Primary lesions can also develop at sites of tissue abrasions, i.e. in the wall of the stomach, oesophagus, pharynx and the bill cavity (Thorel et al., 1997, 2001). This may explain the presence of *MAA* throughout the entire digestive tract as observed in our study (Table 1).

Aerogenic pulmonary infections can also occasionally occur in birds (Thorel et al., 1997, 2001). We have diagnosed single pulmonary infections in three

birds in our study. In the case of the bittern infection no pathological lesions were present in parenchymatous organs (Table 1). The birds may not have been infected with *MAA*; the organism may have been ingested via contaminated food and passed through the digestive tract. The data from the environmental sampling indicates that the birds were exposed to *MAA* (Table 2).

MAH (genotype *dnaJ+*, 1030 bp of 16S rRNA, IS901– and IS1245+, serotypes 4, 8 and 9) was the most prevalent species of other mycobacteria detected in infected birds with *MAA* and was isolated from both parenchymatous organs and the digestive tract. Similar results, published by Morita et al. (1999), detected *MAH* (serotype 9) in the liver, oviduct and intestines of infected painted quail (*Excalfactoria chinensis*).

In our previous study (Dvorska et al., 2003) based on a panel of 173 *MAA* isolates obtained from 27 different hosts and from the environment, RFLP type F-C3 was found only amongst birds and their aviaries (54.3%). The prevalence of *MAA* with the RFLP type F-C3 in all of the birds diagnosed with avian tuberculosis indicates that a single source of infection is probably responsible for this outbreak in the flock of birds. Specific RFLP types of *MAA* rapidly spread among susceptible birds after transmission into a flock. This appears to be the case in this study (Table 1). The consequent dissemination of the pathogen throughout the surrounding environment may lead to the creation of reservoirs for infection. The detection of *MAA* with RFLP type F-C3 in samples taken from the external environment suggests that this has occurred in our case (Table 1).

Water birds exhibit a relatively high resistance to avian tuberculosis; however, it is possible that they may act as hosts of *MAA* over a long period of time without presenting with any of the symptoms of infection. In our study the heron H2 with an infection only in bone marrow could be an example of such animal which excreted *MAA* of same RFLP type F-C3 in faeces. The predominance of the IS901 RFLP type F-C3 in isolates taken from various organs and tissues of the infected 18 birds indicates a monoclonal infection by a primary isolate of this RFLP type from a single source. A similar pattern was seen in another similar case published by Bono et al. (1995).

Only in the case of sacred ibis S2, three different RFLP types F-C3, G-C3, and T-C3 of *MAA* were detected; the following three hypotheses may explain their presence:

- (i) *Hypothesis of polyclonal infection*: The common RFLP type F-C3 was isolated from faeces in the initial stage of infection (Table 1). Isolates of RFLP types G-C3 and T-C3 were isolated after the detection of isolates of RFLP type F-C3. This was likely a case of a mixed/polyclonal infection.
- (ii) *Hypothesis of a single isolate*: G-C3 and T-C3 types were isolated from the faeces of the sacred ibis S2 and were not found in infected tissues, in contrast to RFLP type F-C3. These could be environmental contaminants passing through the gut, particularly as these isolates were not shown to be virulent for pullets. It is much more likely that the ibis was infected by a single isolate, the F-C3 isolate, which was isolated both from tissues and faeces.
- (iii) *Hypothesis of transposition or recombination*: It could be assumed that mycobacteria were evolved as a result of a transposition or recombination of the original F-C3 RFLP type due to some sort of selection pressure on this RFLP type within the host. A further possibility relates to the speed of mutations such as transposition, recombination and crossing-overs, which increases during sudden temperature changes such as from 42 °C (bird body temperature) to 15 °C of the environment.

MAA isolates of RFLP type F-C3 were fully virulent whilst the *MAA* isolates of RFLP types G-C3 and T-C3 were non-virulent to pullets. We have previously shown that *MAA* obtained from atypical hosts (e.g. humans) or kept in collections (e.g. serotype strains) for extended periods of time exhibits a loss of virulence to birds (Pavlik et al., 2000; Dvorska et al., 2003). Birds from the family Threskiornithidae appear to be much more resistant to infection by *MAA* and are possibly following the pattern of “atypical hosts”, which leads to changes in the genome of the original *MAA* isolate, resulting in a change of the RFLP type. It may be caused by mutations that lead to suppression of gene(s)

responsible for the production of functional protein involved in the development of infection in its host.

Despite the examination of many environmental samples from the surroundings of summer and winter aviaries, the source of the avian tuberculosis infection could not be found. It is unlikely that wild birds were the source of infection because the aviaries were well protected by nets that prevented the intrusion of the aviaries by wild birds. We have shown that *MAA* was excreted through the faeces of infected animals and this excrement may be a source of infection for other birds. Bird feedstuff within the aviaries may also come into contact with infected faeces which could be further spread by vertebrates. Previously we have shown that small vertebrates can participate in the transmission of avian tuberculosis (Fischer et al., 2000), where *MAA* (IS901+, serotype 1) was isolated from the organs of the yellow-necked mouse (*Apodemus flavicollis*) and the common shrew (*Sorex araneus*).

MAA is able to survive for a long time in fresh faecal samples (400 days) as well as in dried faeces (308 days) (Hejlícek and Tremel, 1995). From an epidemiological perspective, dried faecal particles and the environmental surfaces that it comes into contact with, in and around aviaries, represent an everyday threat of *MAA* infection to personnel caring for birds in zoological gardens.

MAH has been obtained by culture of sawdust, potted plants, house dust, bedding material and invertebrates; therefore almost all of the material that comes from an aviary that houses birds infected with members of *MAC* could potentially cause infection (Matlova et al., 2004, 2005). Certain atypical mycobacteria (specifically *M. gastri*, *M. chelonae* and *M. scrofulaceum*) were isolated from environmental samples taken in the aviary. These species are usually found in water and soil as well as drinking water and food, but are not recognized as sources of mycobacterial contamination (Marco et al., 2000).

Acknowledgements

Ing. Z. Rozsypalova and Mrs. M. Fisakova are kindly acknowledged for competent technical assistance. The authors wish to thank Ing. L. Faldikova for help with translation, Maria Vass (Swinburne

University of Technology, Victoria, Australia) for critical grammatical correction of the manuscript. This study was supported by the Ministry of Agriculture of the Czech Republic (grants nos. 1B53009 and MZE 0002716201) and grant no. 524/03/1532 of Grant Agency of the Czech Republic.

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