The presence of *Mycobacterium avium* subsp. *avium* in common pheasants (*Phasianus colchicus*) living in captivity and in other birds, vertebrates, non-vertebrates and the environment

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ABSTRACT: Although avian mycobacteriosis is not prevalent among domestic fowl used for intensive husbandry, it has been described in both free living birds and birds in captivity, e.g., zoological gardens and small fowl flocks. In this study, we examined 305 pheasants from six flocks as well as 70 other birds belonging to 14 species and 97 other vertebrates caught in a closed area. We also investigated the prevalence of mycobacteria in non-vertebrates (earthworms) and soil in two pheasant flocks. *Mycobacterium avium* subsp. *avium* (*M. a. avium*) was isolated in four flocks from 17 (5.6%) pheasants. In one *M. a. avium*-infected pheasant co-infection with *M. a. hominissuis* was diagnosed. Granulomatous inflammatory lesions were observed in liver and spleen in only four *M. a. avium*-infected pheasants originating from two flocks. From the other 38 pheasants other mycobacterial species were isolated, such as *M. fortuitum*, *M. terrae*, *M. triviale*, *M. chelonae*, *M. scrofulaceum*, *M. smegmatis*, *M. flavescens*, *M. diernhoferi* and non-identifiable mycobacterial species. In the group of 70 birds of other species, we identified *M. a. avium* in two (2.9%) goshawks (*Accipiter gentilis*). We did not isolate *M. a. avium* from any of the other 97 vertebrates, the 391 environment samples or 97 earthworms.

Keywords: tuberculosis; *Mycobacterium avium* complex; zoonosis; food safety

Avian mycobacteriosis is a chronic disease of birds, usually characterised by the development of granulomatous inflammatory lesions in various tissues, a long incubation period depending not only on the physical health of the bird but also on the variation in virulence of the *M. a. avium* strain (Schrenzel et al., 2008). Several mycobacterial agents are responsible for this condition, mainly *Mycobacterium avium* complex members (*M. avium* subsp. *avium*, *M. a. hominissuis*, *M. intracellulare*; Napier et al., 2009; Shitaye et al., 2009; Kriz et al., 2010), or *M. genavense* (Manarolla et al., 2009; Shitaye et al., 2010).

However, *M. a. avium* is the most common cause of avian mycobacteriosis, also termed avian tuberculosis (Dvorska et al., 2007; Shitaye et al., 2008a; Pate et al., 2009; Kriz et al., 2010). Sporadically, other potentially pathogenic mycobacteria (PPM) are found in bird tissues and organs, e.g., *M. celatum* (Bertelsen et al., 2006), *M. simiae* (Travis et al., 2007), *M. gordonae* and *M. chelonae* (Silva et al., 2009), or *M. intermedium* (Kik et al., 2010). Unlike *M. a. avium,* other PPM are usually ubiquitous in the environment and induce granulomatous inflammatory lesions only in very rare cases (Pavlik et al., 2009a).

Birds infected with *M. a. avium* usually suffer from general weight lost, lethargy, and weakness, as well as a drop in egg production, ruffled feathers and finally death. During *post mortem* examination, microscopic or macroscopic granulomatous inflammatory lesions are found especially in liver,

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spleen, intestine, lung(s), bone morrow, gonads or kidneys (Tell et al., 2003; Shitaye et al., 2008a; Kriz et al., 2010). Histopathologically, the lesions can be diffuse or nodular with central caseous necrosis surrounded by lymphatic cells and multinucleated giant cells; capsules of connective tissue can also be present (Skoric et al., 2010).

Infected birds intermittently shed the causal agent through their faeces into the environment, posing a possible source of infection for other birds and animals, especially in confined spaces such as zoological gardens or small fowl flocks (Dvorska et al., 2007; Shitaye et al., 2008b). It is very difficult to assess the incidence or prevalence of avian tuberculosis among birds, mainly due to the lack of specific clinical symptoms and accurate diagnostic tests. However, according to macroscopic *post mortem* examination of wild birds, the prevalence of avian tuberculosis is estimated to be at least 1% (Hejlicek and Treml, 1993a).

According to data from experimental infections carried out on different bird species and reported cases, pheasants and domestic fowl appear to be the most susceptible birds to avian mycobacteriosis caused by *M. a. avium* (Hejlicek and Treml, 1993b, 1995; Prukner-Radovcic et al., 1998).

The breeding of common pheasants (*Phasianus colchicus*) for game shooting is widespread in the Czech Republic. Therefore, the aim of this work was to study the occurrence of *M. a. avium* and other mycobacteria in tissues of pheasants originating from six different breeding flocks (farms) using the culture method. To study the possible transmission of *M. a. avium* between pheasants and other birds or animals we also examined various other bird and animal species caught on the pheasant farms or in their immediate vicinity. Furthermore, on two farms with pheasants displaying both clinical symptoms (e.g., emaciation, ruffled feather, etc.) and pathoanatomical lesions suggesting mycobacteriosis, we examined samples of non-vertebrates (earthworms) and the environment (soil).

MATERIAL AND METHODS

Examined samples

In our study, 305 pheasants displaying emaciation, as well as weakness and ruffled feathers, or found dead, were chosen from six different pheasant flocks (A to F). The breeding flocks consisted of between two hundred and two thousand pheasants, which were housed in cages (aviaries) containing varying numbers of birds. The pheasants were kept on a floor made up partly of concrete. In each cage at least 50% of the floor was composed of soil with sand (Figure 1). Birds from each of the aviaries were separated according to their age and sex. The age distribution of the birds ranged from one to two and a half years old.

For this study, 70 other birds belonging to the following species: sparrow (*Passer* sp.; n = 22), turtle dove (*Streptopelia turtur*; n = 13), turkey (*Meleagris gallopavo*; n = 10), barn swallow (*Hirundo rustica*; n = 6), sparrow hawk (*Accipiter nisus*; n = 4), goshawk (*Accipiter gentilis*; n = 2), chaffinch (*Fringilla coelebs*; n = 3), great tit (*Parus major*; n = 2), blackbird (*Turdus merula*; n = 2), buzzard (*Buteo buteo*; n = 2), European magpie (*Pica pica*; n = 1), European robin (*Erithacus rubecula*;

Figure 1. Aviary with pheasants from the flock B

Figure 2. One of the dead pheasants found in the aviary of the flock A

n = 1), common cuckoo (*Cuculus canorus*; *n* = 1) and song thrush (*Turdus philomelos*; *n* = 1), as well as 97 other vertebrates: 92 small terrestrial mammals (46 brown rats – *Rattus norvegicus*, 46 house mouse – *Mus musculus*), one hedgehog (*Erinaceus* sp.) and four domestic cats (*Felis silvestris* f. *catus*) that could have come into contact with the tested pheasants or their droppings were included. Neither the birds nor the other animals suffered from any clinical symptoms of disease. The birds and other animals were randomly selected; the only criterion was their presence on the pheasant farms or in their immediate vicinity. Two goshawks were found dead due to an accident near one of the farms and therefore were also included in this study.

In two flocks (A and B) some dead pheasants were found and symptoms of emaciation, ruffled feathers, together with weakness and pathoanatomical lesions were observed (Figures 2 and 3). Therefore, these two flocks were studied more extensively. As well as other birds and vertebrates, samples of non-vertebrates such as earthworms (*n* = 97) and the environment such as soil (*n* = 391) were collected.

Post mortem examination and sample processing

Three hundred and five pheasants, 57 other birds and 97 vertebrates were necropsied and gross examination was performed. Samples of liver, spleen, bone marrow and the gastrointestinal tract (GIT) of birds along with samples of liver, spleen and the GIT of vertebrates and 97 earthworms were collected for culture examination. From 13 birds (other than pheasants) only individual faecal samples were collected.

Culture and identification of isolates

The tissue and environmental samples were individually processed using a decontamination method with hydrochloric acid and natrium hydroxide as described by Fischer et al. (2001). Processed samples were inoculated on two egg-based solid media (according to Herrold and Stonebrink) and into one liquid serum medium (according to Sula). Incubation was carried out simultaneously at two different temperatures (25 and 37 °C) for three months. All isolates positive by Ziehl-Neelsen were first tested using two PCR methods.

The first PCR test, described by Wilton and Cousins (1992) distinguishes between *M. avium*, *M. intracellulare* and other mycobacteria. The second multiplex PCR test discriminates among *M. a. avium/silvaticum*, *M. a. hominissuis* and *M. a. paratuberculosis* (Moravkova et al., 2008). Mycobacterial species other than *M. avium* and *M. intracellulare* were first identified using biochemical methods (Wayne and Kubica, 1986) and the Geno-type *Mycobacterium* CM/AS kits (HAIN life Science, GmbH, Germany). Isolates that could not be identified using these two tests were further examined by 16*S rRNA* sequencing (Harmsen et al., 2003).

Statistical analysis

Fisher's exact test, which is a part of the GraphPad Prism v5.02 programme (GraphPad Software, Inc., USA), was used for the statistical evaluation of the prevalence of *Mycobacterium* sp. in pheasants.

RESULTS

M. a. avium was isolated from 17 (5.6%) pheasants. These positive pheasants originated from four out of the six studied farms (A, B, C and D). On each of the farms C and D only one pheasant was found to be infected (Tables 1 and 2). Nodular granulomatous white to yellowish lesions were observed in the liver and/or spleen in only four pheasants from the 17 infected (Figure 3, Table 3).

In one pheasant from flock B, co-infection of *M. a. avium* with *M. a. hominissuis* was determined. *M. a. avium* was isolated from the liver and *M. a. hominissuis* from the spleen (Table 1). Non-*M. avium* complex PPM were isolated from a further 38 pheasants. *M. fortuitum* (*n* = 8), *M. terrae* (*n* = 3) and *M. trivial* (*n* = 4) were isolated from both the spleen and GIT. In one pheasant, *M. fortuitum* was isolated from both the bone marrow and the GIT. Other mycobacterial species: *M. chelonae* (*n* = 3), *M. scrofulaceum* (*n* = 3), *M. smegmatis* (*n* = 2), *M. flavescens* (*n* = 1), *M. diernhoferi* (*n* = 1) and non-identifiable *M*. species (*n* = 13) were only isolated from the GIT (Table 4).

Out of 70 birds belonging to 14 species captured from the surroundings of the pheasant farms (Tables 1 and 2), *M. a. avium* was isolated from the livers of two goshawks and from the intestine of one of the

Table 1. Detection of mycobacteria in two extensively examined infected pheasant flocks (A and B)

Flock	Origin	Examined			Identified mycobacterial species											
		n	positive	%	*M. a. avium*	*M. a. avium* + *M. a.hominissuis*	*M. a. hominissuis*	*M. chelonae*	*M. diernhoferi*	*M. flavescens*	*M. fortuitum*	*M. scrofulaceum*	*M. smegmatis*	*M. terrae*	*M. triviale*	*Mycobacterium* sp.
	Pheasants	226	45	19.9	7	0	0	3	1	1	8	3	2	3	4	13
	Other birds	33	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Other vertebrates	66	4[c]	6.1	0	0	0	0	1	0	0	0	0	1	0	2
A	Earthworms	86	15	17.4	0	0	0	0	1	0	2	1	1	0	0	10
	Environment (soil)	307	23	7.5	0	0	2	1	0	0	2	3	0	0	0	15
	Subtotal	718	87	12.1	7	0	2	4	3	1	12	7	3	4	4	40
	%		100		8.1	0	2.3	4.6	3.5	1.2	13.8	8.1	3.5	4.6	4.6	46.0
	Pheasants	15	8	53.3	7	1[a]	0	0	0	0	0	0	0	0	0	0
	Other birds	8	2	25.0	1	1[b]	0	0	0	0	0	0	0	0	0	0
	Other vertebrates	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	Earthworms	11	2	18.2	0	0	1	0	0	0	0	0	0	0	0	1
	Environment (soil)	84	3	3.6	0	0	0	0	0	0	0	0	0	1	0	2
	Subtotal	118	15	12.7	8	2	1	0	0	0	0	0	0	1	0	3
	%		100		53.3	13.3	6.7	0	0	0	0	0	0	6.7	0	20.0
Total		836	102	12.2	15	2	3	4	3	1	12	7	3	5	4	43
%				100	14.7	2.0	2.9	3.9	2.9	1.0	11.8	6.9	2.9	4.9	3.9	42.2

[a]in one pheasant co-infection of *M. a. avium* and *M. a. hominissuis* was detected
[b]in one goshawk co-infection of *M. a. avium* and *M. a. hominissuis* was detected
[c]mycobacteria were isolated from 4 (8.7%) out of 46 brown rats (*Rattus norvegicus*)

animals. Furthermore, *M. a. hominissuis* was found in a lung sample taken from one of the goshawks (Table 1). No other PPM were isolated from any of the studied birds (Tables 1 and 2).

PPM such as *M. diernhoferi* (n = 1), *M. terrae* (n = 1) and *M*. sp. (n = 2) were isolated from four brown rats; however, no pathological lesions were observed in any of the rats. Samples of earthworms

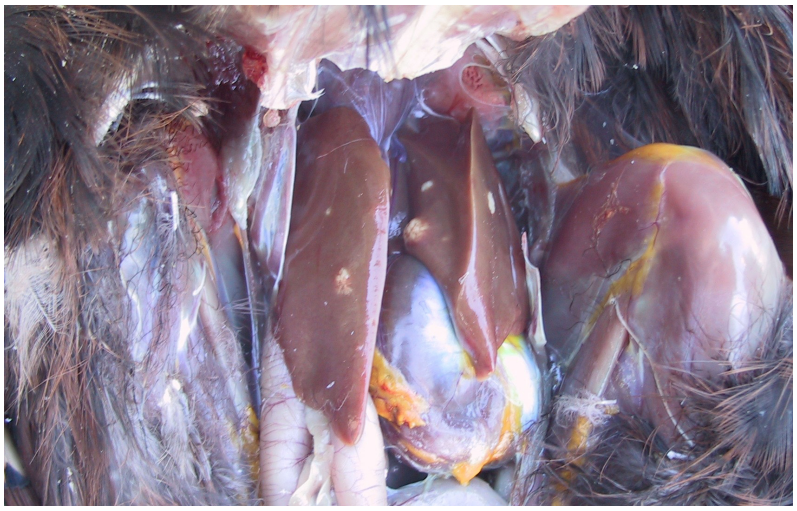

Figure 3. Nodular granulomatous lesions in the liver of one pheasant from the flock A

Table 2. Detection of *Mycobacterium avium* subsp. *avium* in pheasants, other birds and vertebrates in four examined pheasant flocks (C, D, E and F)

Flock	Origin	Examined			*M. a. avium*
		n	positive	%	*n*
C	Pheasants	45	1	2.2	1
	Other birds	9	0	0	0
	Other vertebrates	2	0	0	0
	Subtotal	56	1	1.8	1
D	Pheasants	6	1	16.7	1
	Other birds	15	0	0	0
	Other vertebrates	3	0	0	0
	Subtotal	24	1	4.2	1
E	Pheasants	7	0	0	0
	Other birds	4	0	0	0
	Other vertebrates	7	0	0	0
	Subtotal	18	0	0	0
F	Pheasants	6	0	0	0
	Other birds	1	0	0	0
	Other vertebrates	18	0	0	0
	Subtotal	25	0	0	0
Total		123	2	1.6	2

yielded *M. fortuitum* (n = 2), *M. a. hominissuis* (n = 1), *M. scrofulaceum* (n = 1), *M. diernhoferi* (n = 1), *M. smegmatis* (n = 1) and *M.* sp. (n = 11). Soil samples were found to contain *M. scrofulaceum* (n = 3), *M. fortuitum* (n = 2), *M. a. hominissuis* (n = 2), *M. chelonae* (n = 1), *M. terrae* (n = 1) and *M.* sp. (n = 17), as described in Table 1.

The prevalence of other PPM in pheasant flock A was significantly higher (P-value for Fisher's exact test < 0.01) than in flocks B, C, D, E and F.

DISCUSSION

Avian mycobacteriosis is usually suspected in a flock upon the observation of emaciated and/or dead birds and infection is subsequently diagnosed on the basis of the presence of macroscopic granulomatous lesions and acid fast organisms upon histopathological examination (Witte et al., 2008; Manarolla et al., 2009; Millan et al., 2010). However, due to the long incubation period required to ob-

Figure 4. Stagnant water and mud on the floor of a pheasant aviary of the flock A

Table 3. Distribution of *Mycobacterium avium* subsp. *avium* in 17 infected pheasants

Flock/Number of pheasants examined	Bird ID	Liver	Spleen	GIT	Bone marrow
A/226	1	+[PA]	+	–	+
	2	+[PA]	+[PA]	–	–
	3	+	+	–	–
	4	+	+	–	–
	5	+	–	–	–
	6	+	–	–	–
	7	–	–	+	–
B/15	8	+[PA]	–	–	+
	9	+[PA]	–	–	+
	10	+	+	+	+
	11	+	+	–	+
	12	+	+	–	–
	13	+	–*	–	–
	14	–	–	–	+
	15	–	–	–	+
C/45	17	–	–	+	–
D/6	16	–	+	–	–
Total	17	12	8	3	7
%		70.6	47.1	17.6	41.2
PA present		4	1	0	0
%		23.5	5.9	0	0

GIT = gastrointestinal tract

+ = *M. a. avium* isolation

[PA]pathological lesions were found

**M. a. hominissuis* was isolated

serve clinical symptoms and pathoanatomical lesions combined with the relatively short lifespans of birds kept on breeding farms, mycobacterial infection can easily be missed. Therefore, to increase the probability of *M. a. avium* detection, it would be more suitable to use culture or direct PCR tests for bird tissue examinations (Shitaye et al., 2008a; Silva et al., 2009; Kaevska et al., 2010).

Our results suggest a relatively high occurrence of *M. a. avium* in pheasant flocks. In the Czech Republic pheasants are bred for hunting and consumption of their meat. For that reason the detection of *M. a. avium* infection in pheasants might represent a health risk for consumers.

Prukner-Radovcic et al. (1998) reported on what was most probably *M. avium* infection in one pheasant flock. One month after the first clinical signs of the disease, such as depression, anorexia and weight loss, manifested, a bird mortality rate of one to five per day was observed. Examination of the diseased or dead pheasants revealed creamy granulomatous nodules of varying sizes in all the birds (Prukner-Radovcic et al., 1998). In our study, we also found dead pheasants due to *M. a. avium* infection and observed granulomatous lesions in four pheasants originating from flocks A and B (Figure 2 and 3, Table 1).

The majority of the mycobacteria (other than *M. a. avium*) were isolated almost exclusively from the GIT (Table 4), which might be due to passive transport. We were not able to establish definite infection, because histopathological examinations were not performed. However, some of these mycobacterial species were reported by other authors to be causal agents of mycobacterial infections or diseases in animals, e.g., *M. chelonae* in a brown caiman (Slany et al., 2010) and in domestic fowl (Silva et al., 2009), or *M. scrofulaceum*, *M. smegmatis* and *M. flavescens* in wild boars (Trcka et al., 2006).

M. fortuitum, *M. triviale* and *M. terrae* were isolated from the spleen and in one pheasant *M. fortuitum* was isolated from both the bone marrow and GIT. These mycobacterial species most probably penetrate the GIT and are spread via the blood stream to the organs (Dvorska et al., 2007). Both *M. fortuitum* and *M. a. hominissuis* are among the most common causes of mycobacterial infections in both animals and humans (Cvetnic et al., 2007; Kaevska and Hruska, 2010a,b; Lai et al., 2010; Blahutkova et al., 2011) and have also been isolated from birds (Keymer et al., 1982; Hoop et al., 1996; Shitaye et al., 2009). On the other hand, *M. triviale* and *M. terrae* are rarely isolated from humans or animals (Pavlik et al., 2009b).

Notably, PPM were isolated from the tissues of pheasants from flock A with prevalent *M. a. avium* infection and clinical symptoms of mycobacteriosis, but not in the infected flock B and in the other four flocks, where *M. a. avium* was only isolated from two pheasants and clinical symptoms of mycobacteriosis were not observed.

As mycobacterial infection elicits a mainly cellular type of immune response, a potential explanation of this phenomenon might be that virulent *M. a. avium* can inhibit the production of chemokines while invading intestinal cells to evade detec-

Table 4. Distribution of potentially pathogenic mycobacteria other than *Mycobacterium avium* subspecies *avium* and *Mycobacterium avium* subspecies *hominissuis* in tissues from 305 dissected pheasants

Mycobacterial species	Positive pheasants		Positive tissues from 305 pheasants			
	n	%	liver	spleen	GIT	bone marrow
M. fortuitum	8	2.6	0	1	7	1*
M. triviale	4	1.3	0	1	3	0
M. terrae	3	1.0	0	1	2	0
M. chelonae	3	1.0	0	0	3	0
M. scrofulaceum	3	1.0	0	0	3	0
M. smegmatis	2	0.7	0	0	2	0
M. flavescens	1	0.3	0	0	1	0
M. diernhoferi	1	0.3	0	0	1	0
Mycobacterium sp.	13	4.3	1[PA]	0	12	0
Total positive	38	12.5	1	3	34	1*
Percentage out of 305 birds	12.5		0.3	1.0	11.1	0.3

GIT = gastrointestinal tract

[PA]pathological lesions were found

*in one pheasant *M. fortuitum* was isolated from both the bone marrow and GIT

tion (Sangari et al., 1999). Such manipulation of the host immune system might facilitate the invasion of host tissues by other less virulent PPM. However, "exhaustion" of a bird's immune system by virulent *M. a. avium* infection might also be responsible for the increased sensitivity to less virulent PPM. More research is needed to elucidate the relationship between various mycobacterial species during such a co-infection. The discrepancy in the isolation of other PPM between flocks A and B with high prevalences of *M. a. avium* infection would seemingly run counter to the above notion.

Pheasants belong to the taxonomic order "Galliformes", and are characterised by their feeding habits of digging up soil and consuming roots and non-vertebrates acquired from the soil, which may be a source of many different mycobacterial species (Fischer et al., 2001, 2003; De Groote et al., 2006). Another explanation for the high level of infection with other PPM in flock A could be elevated levels of stress due to the higher density of birds, which was about 2000 individuals, as well as free contact with stagnant water and mud in aviaries (Figure 4). Moreover, young pheasants were bred on sawdust bedding (Figure 5). It has been

Figure 5. Breeding of young pheasants on sawdust bedding in the flock A

reported that sawdust can be a source of PPM for animals (Matlova et al., 2003; Krizova et al., 2010).

Moreover, in two birds (pheasant and goshawk) we observed co-infection of *M. a. avium* with *M. a. hominissuis*. Previous examples of co-infection have already been described by Shitaye et al. (2008a) who isolated *M. a. avium* together with *M. diernhoferi*, as well as *M. gastri* and *M. chelonae* from domestic fowl tissues and Dvorska et al. (2007) who isolated *M. a. avium* together with *M. a. hominissuis* from nine captive water birds. These findings suggest that *M. a. avium* infection might facilitate infection with less virulent mycobacterial species.

The second part of this work focused on various bird species that could have come into contact with the infected pheasants; most of these were sparrows (*Passer* sp.), turtle-dove (*Streptopelia turtur*) or turkeys (*Meleagris gallopavo*). In spite of the fact that avian mycobacteriosis has been described in these birds previously (Hejlicek and Treml, 1995; Gerhold and Fischer, 2005; Saggese et al., 2008) we did not isolate *M. a. avium* or other PPM from any of the studied birds. The only exceptions were two goshawks (mentioned above) from which *M. a. avium* was isolated from the liver and in one of them also from the intestine (Table 1).

This was surprising because raptors are usually considered to be more resistant than, for example, song birds or turkey, from which we were unable to isolate any mycobacterial species in contrast with Witte et al. (2008). Nonetheless, there are reports which describe *M. a. avium* in raptors. Skoric et al. (2010) described a case of avian mycobacteriosis in one Ruppell's griffon vulture (*Gyps ruppellii*) kept in a zoo collection. Millan et al. (2010) found lesions of mycobacteriosis and confirmed *M. a. avium* infection in 2.4% of examined raptors, in most cases in kestrels (*Falco tinunculus*).

Goshawks and kestrels have different eating habits; goshawks largely consume small birds whilst the kestrels' diet consists mainly of small vertebrates. *M. a. avium* has previously been isolated from the organs of both small birds and small vertebrates (Hejlicek and Treml, 1993c; Fischer et al., 2000). In our previous study we have isolated mycobacteria, including *M. a. avium*, from insectivores and rodents obtained from swine or cattle farms affected by mycobacterial infection (Fischer et al., 2000). In the present study, we did not find *M. a. avium* in any of the vertebrate samples; however, we were able to isolate *M. diernhoferi*, *M. terrae* and *M.* sp. from four (8.7%) out of 46 brown rats (Table 1).

In the flocks A and B with the most widespread *M. a. avium* infection, we also examined soil samples and earthworms. Contrary to our previous study (Fischer et al., 2003), and our expectations, we did not isolate *M. a. avium* from any soil sample or earthworm (Table 1). These results can be explained by the fact that isolation of *M. a. avium* from soil is more complicated than from other sample types and depends heavily on the method that is used, as well as the composition of the soil. During the decontamination procedure of soil, many *M. a. avium* bacteria can be killed; however, insufficient decontamination usually leads to the overgrowth of contaminants.

The isolation of mycobacteria is also influenced by the adsorption of bacteria to soil particles (Dhand et al., 2009). Another possible explanation for the results obtained is that according to our previous reports, the shedding of *M. a. avium* from infected birds is highly irregular and the amount of bacteria shed at any one time is dependent on the progression of the infection in the host (Dvorska et al., 2007; Shitaye et al., 2008b). In the present study we isolated *M. a. avium* from the GIT in only three pheasants (Table 3). Therefore, the likelihood of environmental contamination was low.

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