Mycobacteria isolated from the environment of pig farms in the Czech Republic during the years 1996 to 2002

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ABSTRACT: Sources of mycobacterial infections in 50 pig herds in the Czech Republic were investigated during the years 1996 to 2002. A total of 2 412 samples from the external environment (feeds, bedding materials, drinking water, biofilms on drinkers, scrapings from the walls, floors and pen barriers, dust, spider webs, peat, kaolin, faeces, organs of rodents, and birds, etc.) were examined. After staining by the Ziehl-Neelsen technique, acid-fast rods were detected in 95 (3.9%) samples by direct microscopic examination and mycobacteria were cultured from 575 (23.8%) samples. From *Mycobacterium avium* complex (*MAC*), *M. avium* subsp. *hominissuis* (genotype IS*901*-, IS*1245*+) of serotypes 4, 6, 8, and 9 (272; 47.0% isolates), *M. a. avium* (genotype IS*901*+, IS*1245*+) of serotype 2 (13; 2.2% isolates) and *M. intracellulare* (genotype IS*901*-, IS*1245*-) of different serotypes (2; 0.3% isolates) were detected most frequently. Other isolates from among 14 other mycobacterial species ranked as follows: 64 *M. gordonae*, 47 *M. fortuitum*, 17 *M. chelonae*, 14 *M. flavescens*, 11 *M. terrae*, seven *M. phlei*, seven *M. scrofulaceum*, three *M. diernhoferi*, three *M triviale*, three *M. smegmatis*, two *M. xenopi*, one *M. szulgai*, one *M. gastri*, and one *M. ulcerans*. The remaining 111 isolates of unidentified species did not contain specific sequences IS*901* and IS*1245* characteristic for the pathogenic members of *MAC* (*M. a. avium* and *M. a. hominissuis*). Peat, drinking water, biofilms on drinkers, bedding materials, feeds, free living birds, kaolin and charcoal were identified as potential sources of mycobacterial infections for pigs. Peat given to piglets as a feed supplement was the most important source of mycobacteria (65.1% positive of 327 examined samples); 81.2% of them were positive for *M. a. hominissuis* of serotypes 4, 6, 8, and 9. By contrast, mycobacteria of other species (*M. gordonae*, *M. fortuitum*, *M. chelonae*, *M. flavescens*, etc.) were the main isolates obtained from drinking water and biofilms on drinkers for pigs. By culture examination, the detection rate was higher in the biofilm samples (36.4%) than in the samples of drinking water (29.6%). The third group of sites with detected high levels (26.4%) of mycobacterial contamination were various types of beddings of woody material. *M. a. hominissuis* of serotypes 6, 8, and 9 were the most frequent isolates from sawdust; *M. a. avium* serotype 2 was sporadically detected. Mycobacterial findings in other samples from the external environment (wall and floor scrapings, dust, soil from the runs, and invertebrates) gave an indication of the pressure of infection in the herds. High contamination levels in faecal samples (15.6%) and in scrapings (18.4%) from respective parts of pens and stables indicated exposure of pigs to mycobacteria. In those materials, isolation of *M. a. hominissuis* of serotypes 4, 6, 8, and 9 prevailed. Mycobacteria were also detected in 7.9% of 430 samples of various invertebrate species. Various mycobacterial species were identified in the larvae and puparia of *Eristalis tenax* and *Musca* spp. and in imagoes of *Drosophila* spp., *Musca* spp., family Scatophagidae, *Stomoxys calcitrans*, *E. tenax*, and in earthworms. All of the constituents of the external environment that are potential sources of mycobacterial infections should be considered during implementation of preventative measures and the control of mycobacterial infections in pig herds.

Keywords: mycobacteria; pig; environment; avian tuberculosis; PCR

Pigs are highly susceptible to mycobacterial infections which bring about considerable economical losses to farmers. These losses result from limitation or prohibition of animal transfer to other farms outside the outbreak of infection, from early culling of infected animals and assessment of meat and organs of slaughtered animals as conditionally consumable (Alfredsen and Skjerve, 1993; Morita *et al.*, 1994a; Balian *et al.*, 1997; Cvetnic *et al.*, 1998; Komijn *et al.*, 1999; Offermann *et al.*, 1999; Kozak

Supported by the National Agency for Agricultural Research of the Ministry of Agriculture of the Czech Republic (Grant No. QC0195).

et al., 2003). Atypical or so called conditionally pathogenic mycobacteria (CPM) and *Rhodococcus equi* are frequently isolated from tuberculous lesions in pigs (Dvorska *et al.*, 1999). As CPM are often found in the external environment and in pig housing, they may complicate *intra vitam* (skin test with avian tuberculin and/or serological examinations) and *post mortem* diagnosis (patho-anatomical and histological examinations). However, repeated infection or exposure to heavy concentrations of the less virulent mycobacterial species is necessary to induce immunological responses or the development of patho-anatomical alterations in the host organism (Pavlas and Patlokova, 1985; Dvorska *et al.*, 1999; Pavlik *et al.*, 2002b, 2003).

After elimination of bovine tuberculosis in the Czech Republic, the members of *Mycobacterium avium* complex (*MAC*) became the most significant causal agents of mycobacterial infections causing tuberculous lesions in the lymph nodes of domestic animals, particularly in cattle and pigs (Pavlik *et al.*, 2002a-d). According to the currently accepted taxonomy, *MAC* is divided into three groups, classified by 28 serotypes (Wolinsky and Schaefer, 1973). The first group consists of *M. avium* subsp. *avium* of serotypes 1, 2, and 3 and genotype IS*901*+ and IS*1245*+, which are highly virulent for birds (Pavlik *et al.*, 2000; Mijs *et al.*, 2002). The members of this subspecies cause miliary tuberculosis affecting lymph nodes and parenchymatous organs (liver, spleen, kidneys, and lungs). The main sources of the causal agents of this disease are free-living and domestic birds or small terrestrial mammals (Morita *et al.*, 1994b; Fischer *et al.*, 2000; Pavlik *et al.*, 2000).

By contrast, *M. a. hominissuis,* the second member of *MAC,* is most frequently isolated from tuberculous lesions in pig mesenteric, submandibular and, occasionally, inguinal lymph nodes. *M. a. hominissuis* (genotype IS*901*- and IS*1245*+ and serotypes 4 to 6, 8 to 11, and 21) is partially virulent for birds (Leinemann *et al.*, 1993; Morita *et al.*, 1994b; Nishimori *et al.*, 1995; Balian *et al.*, 1997; Ritacco *et al.*, 1998; Dvorska *et al.*, 1999; Komijn *et al.*, 1999; Offermann *et al.*, 1999; Pavlik *et al.*, 1999, 2000; Ramasoota *et al.* 2001; Mijs *et al.*, 2002; Pavlik *et al.*, 2003). These subspecies are most commonly found in the external environment – particularly water, soil, dust, and invertebrates (Horvathova *et al.*, 1997; Kazda, 2000; Pavlik *et al.*, 2000).

Isolates of the third member of *MAC* (genotype IS*901*-, IS*1245-)* of the remaining serotypes 7, 12 to 20 and 22 to 28, are designated as *M. intracellulare* and are not virulent for birds. They have been isolated only occasionally from tuberculous lesions in pig lymph nodes or parenchymatous organs (Wayne and Kubica, 1986; Dvorska *et al.*, 1999).

Other CPM (*M. fortuitum, M. gordonae, M. terrae, M. chelonae, M. smegmatis, M. phlei, M. scrofulaceum*, etc.) occurring in the external environment have been sporadically isolated from tuberculous lesions in pig lymph nodes (Wayne and Kubica, 1986; Horvathova *et al.*, 1997; Kazda, 2000; Fischer *et al.*, 2001; Pavlik *et al.*, 2003).

It is important to identify potential sources of infection to protect pigs from mycobacterial infections and to subsequently cut down economical losses sustained by farmers. Mycobacteria are able to survive under unfavourable conditions due to their lipo-poly-saccharidic bacterial cell wall that prevents them from dehydrating. CPM are almost ubiquitous in the ecosystem, e. g. soil, plants, water, dust, etc. CPM and non-pathogenic mycobacteria are capable of utilization of organic compounds found in those natural materials for their metabolism (Wayne and Kubica, 1986). Mycobacteria are purported to occur in the final stage of mineralization of organic compounds in the nature (Beerwerth and Popp, 1971; Beerwerth, 1973; Kazda, 2000). From the standpoint of temperature requirements for propagation, a range of CPM have adapted to the external environmental conditions and easily propagate at temperatures above 18°C (Kazda, 2000). Very few CPM require a strictly limited range of temperatures from 30 to 35°C (Beerwerth and Kessel, 1976b). Mycobacteria also contaminate the external environment via animal and human excrement (Pavlik *et al.*, 1994; Ayele *et al.*, 2001).

Water is the main reservoir of majority of CPM and surface-water plays a key role in circulation of mycobacteria in the external environment (Svorcova and Slosarek, 1984). Mycobacteria have been isolated from almost all types of water sources (brooks, rivers, ponds, wells, etc.) except for artesian spring water. A review of the literature (Horvathova *et al.*, 1997) found that more than 20 species of mycobacteria had been isolated from water with the following being the most widespread: *M. gordonae, M. flavescens, M. gastri, M. nonchromogenicum, M. terrae* a *M. triviale*. In addition, CPM *M. kansasii, M. xenopi, MAC, M. fortuitum* and *M. chelonae* were also isolated from water.

Nowadays, surface water is rarely given to pigs or used for feed preparation (Horvathova *et al.*, 1997). The most commonly reported sources of CPM for

domestic pigs have been contaminated drinking water (Beerwerth, 1973; Engel *et al.*, 1977), feed (Dalchow, 1988; Pavlik *et al.*, 2003), bedding (Brooks, 1971; Dalchow and Nassal, 1979; Songer *et al.*, 1980; Masaki *et al.*, 1982), soil in the runs (Horvathova *et al.*, 1997), waste water (Brooks *et al.*, 1984; Horvathova *et al.*, 1997), poikilothermic animals and invertebrates (Matlova *et al.*, 1998; Lescenko *et al.*, 2003) and other constituents of external environment (Songer, 1979; Windsor *et al.*, 1984; Gardner and Hird, 1989; Pavlik *et al.*, 2000).

Tap and well water, which is most commonly given to pigs, may be contaminated with small amounts of CPM. Pelletier *et al.* (1988) found that drinking water in certain areas was a continuous source of CPM that tolerated wide ranges of pH and temperatures. Mycobacteria may accumulate and subsequently propagate in water reservoirs, where they are most likely to be found in biofilms. Conditions for their propagation may be particularly favourable in the summer months (Kazda, 2000). Subsequently, mycobacteria spread to the whole water system.

Mycobacteria are usually ingested by animals and, after their passage through the digestive tract, they are detectable in faeces (Nassal *et al.*, 1974). In large pig farms, the aerial route of mycobacterial infection is also possible where pipelines are used for feeding liquid or powdery feed mixtures, where bedding in stable boxes is contaminated (particularly sawdust, wood shavings and straw) and in the means of animal transports. The mycobacterial wall is highly immunogenic in the majority of CPM and complicates the diagnosis of tuberculosis with bovine or avian tuberculins (Fodstadt, 1977; Pearson *et al.*, 1977; Monaghan *et al.*, 1994; Kazda, 2000). If the CPM dose is high, tuberculous lesions may be detected by veterinary-meat inspection, particularly in head and mesenteric lymph nodes of some animals (Dey and Parham, 1993; Margolis *et al.*, 1994; Morita *et al.*, 1994a; Thorel *et al.*, 1997; Pavlik *et al.*, 2003).

Since the early 1990s, the epizootiological situation regarding occurrence of tuberculous lesion in pigs in the Czech Republic changed several times due to various risk factors (Pavlik *et al.*, 2003). In the mid-1990s, the rate of detection of tuberculous lesions in lymph nodes increased due to the introduction of deep bedding technology for pigs. Particularly woody waste (primarily sawdust, wood shavings and occasionally bark) was used as bedding. Although that technology was favourable from the standpoint of ecology and economy and was more natural for pigs, tuberculous lesions caused high economic losses when the animals were sold for slaughter. It resulted from inappropriate storage of woody materials in a damp environment. CPM propagation can be expected particularly in the summer season with the temperatures above 18°C (Kazda, 2000).

In the beginning of 1998, peat began to be widely given to newborn piglets in the Czech Republic as a supplement because of its favourable dietetic qualities (Pavlik *et al.*, 1999, 2003). The low pH of peat (4.0 to 4.5) exerts bactericidal effects on coliforms and other species of intestinal microflora and thus reduces or prevents diarrhoeal diseases of piglets (Lenk and Benda, 1989; Kazda 2000; Framstad and Rein, 2001). Its high fibre content enhances water absorption from feed, reducing the volume of feed in the stomach with the result that feed intake increases and so does weight gain (Roost *et al.*, 1990; Fuchs *et al.*, 1995). In certain farms, peat was also used as bedding. Its structure and comparative softness helped reduce limb abrasions in piglets (Lysons, 1996; Dürrling *et al.*, 1998).

High concentrations of CPM were isolated from peat originating from natural sources. Their propagation in peat at the temperatures above 18°C was described in a series of studies (Kazda *et al.*, 1989; Kazda, 1990, 2000). Species *M. sphagni* was detected most frequently. However, *M. fortuitum, M. terrae, M. chelonae, M. gordonae, M. xenopi, M. phlei, M. marinum, M. flavescens, M. farcinogenes*, and *M. scrofulaceum* were also isolated (Kazda, 2000). These mycobacterial species were also demonstrated in pig organs (Bercovier and Vincent, 2001). Besides the above mentioned species of mycobacteria, high concentrations of *M. a. hominissuis* of serotype 8 were also repeatedly detected in peat waters (Kazda, 1973a,b).

The objective of our study was to describe the occurrence of respective mycobacterial species found in the external environments of herds of pigs infected with mycobacteria in the Czech Republic during the years 1996 to 2002.

MATERIAL AND METHODS

Examined material

A total of 2 412 samples from the external environment were examined. The samples originated from 50 farms in the Czech Republic with confirmed

mycobacterial infections in pigs. Positive skin reactions to avian tuberculin, assessed according to the previously described method (Pavlik *et al.*, 2003), had been found in 21 herds. Tuberculous lesions in lymph nodes of pigs had been detected in the remaining 29 herds. Based on these positive examination results, our laboratory was requested to search for the sources of mycobacteria. Samples of feeds, feed supplements (peat, kaolin, on occasion charcoal), water and scrapings of biofilms on drinkers, bedding, free living birds, small terrestrial mammals, and their faeces were collected. Samples of wall scrapings, pig faeces, dust and spider webs, soil from the runs and invertebrates (insects and earthworms) were also taken for determination of intensity of mycobacterial contamination in the environment. The samples were taken by sterile tongue depressors and put into sterile plastic bags. After collection in a farm, the samples were kept in a dark room at +6°C up to two weeks before analysis.

Laboratory examination

Microscopic examination. Before decontamination, imprint slides of external environment samples were stained by Ziehl-Neelsen (Z-N) technique for acid-fast rods (AFR) detection. At least 100 fields were viewed in every sample.

Culture examination. Approximately 1 g of each sample was homogenized and decontaminated by the method described previously (Fischer *et al.*, 2001). In a total, 40 µl suspensions were dispensed to two solid egg media according to Stonebrink, two solid Herrold Egg Yolk Media (HEYM), and two liquid serum media according to Sula (Merkal *et al.*, 1964; Kubin *et al.*, 1986). Incubations were performed in parallel, with one medium type each time, at 24°C and 37°C. Mycobacterial growth was checked after the first week of incubation and then every second week for two months.

Isolate identification. All the AFR positive isolates were examined by the PCR method for the detection of *dna*J gene specific for *Mycobacterium* genus using primers 5′-GGG TGA CGC GAC ATG GCC CA-3′ and 5′-CGG GTT TCG TCG TAC TCC TT-3′ (Nagai *et al.*, 1990). For IS*901* detection, primers 5′-GCA ACG GTT GTT GCT TGA AA-3′ and 5′-TGA TAC GGC CGG AAT CGC GT-3′ (Kunze *et al.*, 1992) and for IS*1245* detection, primers 5′-GCC GCC GAA ACG ATC TAC-3′ and 5′-AGG TGG CGT CGA GGA AGA-3′ (Guerrero *et al.*, 1995; Van Soolingen *et al.*, 1998; Dvorska *et al.*, 2001) were employed. All *MAC* isolates were serotyped according to the system of Wolinsky and Schaefer (1973) modified by Sűssland and Hrdinova (1976). Mycobacterial isolates which were not classified as *MAC* were assessed by biochemical methods (Wayne and Kubica, 1986).

RESULTS

From 2 412 samples, mycobacteria were detected in 95 (3.9%) and 575 (23.8%) samples by microscopy and by culture, respectively (Table 1).

Mycobacteria detection in the environmental samples

Microscopic detection of mycobacteria. AFR were most frequently detected in biofilms on drinkers (27.2%). Low rates of AFR (less than 3.9%) were found in drinking water, peat, bedding, pig faeces, dust and spider webs, feeding and scrapings (Table 1).

Isolation of mycobacteria by culture. Mycobacteria were detected by culture in 14 of 15 groups of various constituents of the external environment. Two to three mycobacterial species were simultaneously detected from the samples of three free living birds, so a total of 579 isolates were identified. Mycobacteria were isolated most frequently from peat, samples of biofilms on drinkers, drinking water, and bedding (Table 1).

Identification of mycobacterial isolates

M. a. hominissuis (47.1%) and *M. a. avium* (2.2%) isolates predominated. The most frequently isolated CPM were *M. gordonae*, *M. fortuitum*, *M. chelonae*, *M. flavescens* and *M. terrae*. In 111 (19.2%) biochemically unidentified isolates, insertion sequences IS*901* and IS*1245* specific for *M. a. avium* and *M. a. hominissuis* were not detected by the PCR method (Table 2).

Mycobacterial contamination of respective samples

Peat. Mycobacteria were most frequently isolated from peat (65.1%). Among eight identified species,

Table 1. Examined biological material from pig farms infected with mycobacteria

Biological samples examined		Positive examination for mycobacteria by[b]			
		microscopy[a]		culture	
Origin	No.	No.	%	No.	%
Invertebrates[c]	430	0	0	34	7.9
Peat[d]	327	10	3.1	213	65.1
Feed concentrates[e]	270	2	0.7	28	10.4
Bedding[f]	231	7	3.0	61	26.4
Biofilm from the pipeline[g]	217	59	27.2	79	36.4
Pig faeces[h]	179	5	2.8	28	15.6
Stable scrapings[i]	174	1	0.6	32	18.4
Dust and spider webs[j]	117	2	1.7	9	7.7
Water from the pipeline	233	9	3.9	69	29.6
Kaolin[d]	88	0	0	6	6.8
Free living birds[k]	60	0	0	8[m]	13.3
Charcoal[d]	28	0	0	4	14.3
Birds nests in the stables	20	0	0	0	0
Soil and moss from the paddocks	19	0	0	3	15.8
Small terrestrial mammals[l]	19	0	0	1	5.3
Total	2 412	95	3.9	575	23.8
Total No. of isolates				579[m]	

Explanations: [a]detection of acid-fast rods (AFR) after the Ziehl-Neelsen staining; [b]microscopy and culture examinations; [c]larvae, pupas and imagoes from invertebrates trapped in the stables (more details see in Table 5), results partially published previously (Fischer *et al*., 2000, 2001, 2003); [d]used as a feed supplement for piglets under two months of age; [e]from the troughs in the stables and from the storage tanks; [f]straw, hay, silage, sawdust and bark; [g]biofilm from the drinking places and water-expanse reservoirs; [h]from the floor in the boxes in the stables and paddocks; [i]samples consisted from the mixture of old pig faeces, feedstuff and dust from internal (floors, walls, pen barriers) and external (concrete paddocks and pen barriers) parts of the stables; [j]from the stables and feed storages; [k]bird parenchymatous organs from free living birds (more details see in Table 4); [l]parenchymatous organs (n = 16) and faecal samples (n = 3) from free living small terrestrial mammals from the stables (more details see in Table 4); [m]from 8 birds, 12 mycobacterial isolates were received (more details see in Table 4)

isolates of *M. a. hominissuis* and *M. a. avium* constituted 81.2% and 1.4%, respectively (Tables 1 and 2).

Water biofilm and drinking water. By culture examination, mycobacteria were detected in 36.4% of biofilm samples and in 29.6% of drinking water samples. Among 13 species of identified mycobacterial isolates, eleven and ten were detected in sediments and drinking water, respectively. The majority of isolates were species *M. gordonae* and *M. fortuitum*. *M. a. hominissuis* represented 24.6% isolates from drinking water and only 2.5% isolates from biofilms (Tables 1 and 2).

Bedding. Among the samples of bedding, sawdust was frequently contaminated (43.6%) by subspecies *M. a. avium* and *M. a. hominissuis*, which comprised 58.8% of the isolates from sawdust. Except wood shavings, mycobacteria were also isolated from straw, hay, and in bran. Moreover, besides predominating *MAC* members, species *M. terrae*, *M. smegmatis*, *M. fortuitum*, *M. chelonae*, *M. phlei* and other unidentified CPM were isolated (Table 3).

Free living birds and small terrestrial mammals. Mycobacteria were isolated from 8 (13.3%) of 60 free living birds of 7 species: six house sparrows, one

Table 2. Mycobacterial species isolated from the environmental samples

Mycobacterial isolates		*Mycobacterium*																	
Origin	No.	*avium*[k]	*hominissuis*[l]	*intracellulare*[m]	*diernhoferi*	*chelonae*	*flavescenc*	*fortuitum*	*gastri*	*gordonae*	*phlei*	*scrofulaceum*	*smegmatis*	*szulgai*	*triviale*	*terrae*	*ulcerans*	*xenopi*	spp.[n]
Invertebrates[a]	34	0	16	0	0	8	0	6	0	0	0	1	0	0	0	0	0	0	3
Peat[b]	213	3	173	0	0	1	1	3	0	2	0	0	0	0	0	2	0	1	27
Feed concentrates[c]	28	0	12	0	0	0	1	2	0	1	0	0	0	0	2	1	0	0	9
Bedding[d]	61	7	24	1	0	1	0	5	0	0	2	0	1	0	0	1	0	0	19
Biofilm from the pipeline[e]	79	0	2	0	1	3	7	12	0	36	2	3	1	1	0	2	0	0	9
Pig faeces[f]	28	0	10	0	1	1	0	2	0	1	0	1	0	0	0	0	0	0	12
Stable scrapings[g]	32	1	12	0	0	0	1	2	0	1	2	0	1	0	0	2	0	0	10
Dust and spider webs[h]	9	0	3	0	0	0	0	2	0	0	0	0	0	0	0	0	0	1	3
Water from the pipeline	69	0	17	1	1	2	4	9	0	22	0	1	0	0	1	2	0	0	9
Kaolin[b]	6	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Free living birds[h]	12	2	1	0	0	1	0	3	1	1	1	1	0	0	0	0	1	0	0
Charcoal[b]	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
Soil and moos[i]	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2
Small terrest. mammals[j]	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Total	579	13	272	2	3	17	14	47	1	64	7	7	3	1	3	11	1	2	111
%		2.2	47.0	0.3	0.5	2.9	2.4	8.1	0.2	11.1	1.2	1.2	0.5	0.2	0.5	1.9	0.2	0.3	19.2

Explanations: [a]larvae, pupas and imagoes from invertebrates trapped in the stables (more details see in the Table 5), results partially published previously (Fischer *et al.*, 2000, 2001, 2003); [b]used as a feed supplement for piglets under two months of age; [c]from the stables and feed storage; [d]straw, hay, silage, sawdust and bark; [e]biofilm from the drinking places and water-expanse reservoirs; [f]from the floor in the boxes in the stables and paddocks; [g]from the troughs in the stables and from the storage tanks; [h]bird parenchymatous organs from free living birds: from 8 birds, 12 mycobacterial isolates were received (more details see in Table 4); [i]from the paddocks; [j]parenchymatous organs ($n = 16$) and faecal samples ($n = 3$) from free living small terrestrial mammals from the stables (more details see in Table 4); [k]*Mycobacterium avium* subsp. *avium*, genotype IS*901*+ and IS*1245*+; [l]*Mycobacterium avium* subsp. *hominissuis*, genotype IS*901*– and IS*1245*+; [m]*Mycobacterium intracellulare*, genotype IS*901*– and IS*1245*–; [n]isolates without IS*901* and IS*1245* specific for *M. a. avium* and *M. a. hominissuis*, unidentified by biochemical tests

Table 3. Mycobacteria isolated from different components of bedding

Origin of examined samples	No. of samples			*Mycobacterium*							
	examined	positive	%	*avium*[a]	*hominissuis*[b]	*intracellulare*[c]	*terrae*	*smegmatis*	*fortuitum*	*chelonae*	*phlei*	spp.[d]
Sawdust	78	34	43.6	6	14	0	1	1	0	0	0	12
Wood shavings	20	0	0	0	0	0	0	0	0	0	0	0
Straw	52	7	13.5	0	4	0	0	0	0	0	0	3
Hay	9	2	22.2	1	1	0	0	0	0	0	0	0
Bran	37	4	10.8	0	2	1	0	0	0	0	0	1
Mixed bedding	35	14	40.0	0	3	0	0	0	5	1	2	3
Total	231	61	26.4	7	24	1	1	1	5	1	2	19
%		100		11.5	39.3	1.6	1.6	1.6	8.2	1.6	3.3	31.1

Explanations: [a]*Mycobacterium avium* subsp. *avium*, genotype IS*901*+ and IS*1245*+; [b]*Mycobacterium avium* subsp. *hominissuis*, genotype IS*901*– and IS*1245*+; [c]*Mycobacterium intracellulare*, genotype IS*901*– and IS*1245*–; [d]isolates without IS*901* and IS*1245* specific for *M. a. avium* and *M. a. hominissuis*, unidentified by biochemical tests

white wagtail and one starling. From three of these infected birds, distinct mycobacterial species were isolated from respective organs. From 19 small terrestrial mammals, mycobacterial infection caused by *M. fortuitum* was diagnosed only in one common vole (Table 4).

Feed concentrates and supplements - kaolin and charcoal. Six of the 17 mycobacterial species detected in the external environment were found in those materials (Table 2).

Dust and spider's webs, soil and moss, scrapings from the stables and pig faeces. Mycobacterial isolates originating from these biological materials comprised 11 species including *M. a. avium* and *M. a. hominissuis.* (Table 2).

Invertebrates. Mycobacteria were isolated from 34 (7.9%) of the invertebrate samples. *M. a. hominissuis* was isolated most frequently. *M. chelonae*, *M. fortuitum*, and *M. scrofulaceum* were also isolated. According to the species composition of invertebrates, mainly the members of family Scatophagidae, stable flies, flies *Musca* spp., fruit flies *Drosophila* spp., drone flies and earthworms were contaminated with mycobacteria (Table 5).

DISCUSSION

Recently, infections of humans and animals with the *MAC* members have become serious, particularly because of their frequent detection in immunocompromised patients (mainly in *HIV/AIDS* patients) (Sax and Weinberger, 1995; Tumballero *et al*., 2001; Salama *et al*., 2003). Based on molecular-biological studies, pigs were found to be an important potential source of *MAC* infection for humans, or both the groups of hosts (pigs and humans) may be infected from the same source – environment (Komijn *et al.* 1999; Pavlik *et al*., 2000; Dvorska *et al*., 2002).

The causal agent of avian tuberculosis is an economically and epidemiological important agent of infectious disease. In our study, *M. a. avium* was detected in only 2.2% of isolates. However, detection of these isolates in beddings, peat, and in scrapings from pig's stables must also be considered important due to the high virulence for pigs (Table 2). Contamination of bedding and peat during transportation and subsequent storage might have resulted from potentially infected free living birds and small terrestrial mammals contaminating these sites (Pavlik *et al*., 2000; Fischer *et al*., 2000).

Table 4. Mycobacterial isolates from free-living birds and small terrestrial mammals

Source of samples	No. of animals			No. of isolates	*Mycobacterium*								
	examined	positive	%		*avium*	*hominissuis*	*fortuitum*	*ulcerans*	*scrofulaceum*	*chelonae*	*gordonae*	*phlei*	*gastri*
Free-living birds													
Tree sparrow[a]	0	0	0	3	0	0	0	0	0	0	0	0	0
House sparrow[b]	6	18.8	6	32	2	1	2	1[l]	1[l]	0	0	0	0
Pigeon[c]	0	0	0	19	0	0	0	0	0	0	0	0	0
White wagtail[d]	1	100	2	1	0	0	0	0	0	1[m]	1[m]	0	0
Starling[e]	1	100	3	1	0	0	1[n]	0	0	0	0	1[n]	1[n]
Collared turtle-dove[f]	0	0	0	3	0	0	0	0	0	0	0	0	0
Red-backed shrike[g]	0	0	0	1	0	0	0	0	0	0	0	0	0
Total	8	13.3	11	60	2	1	3	1	1	1	1	1	1
Small terrestrial mammals													
Common vole[h]	1	100	1	4	0	0	1	0	0	0	0	0	0
Domestic mouse[i]	0	0	0	10	0	0	0	0	0	0	0	0	0
Domestic shrew[j]	0	0	0	2	0	0	0	0	0	0	0	0	0
Faeces[k]	0	0	0	3	0	0	0	0	0	0	0	0	0
Total	1	5.3	1	19	0	0	1	0	0	0	0	0	0

Explanations: [a]*Passer montanus*, [b]*Passer domesticus*, [c]*Columbia livia* f. *domestica*, [d]*Motacila alba*, [e]*Sturnus vulgaris*, [f]*Streptopelia decaocto*, [g]*Lanius colurio*, [h]*Microtus arvalis*, [i]*Mus musculus*, [j]*Sorex araneus*, [k]faeces from small terrestrial mammals from the stables; [l]from one *Passer domesticus, M. ulcerans* and *M. scrofulaceum* were isolated from stomach and spleen, respectively; [m]from one *Motacila alba, M. chelonae* and *M. gordonae* were isolated from stomach and intestine, respectively; [n]from one *Sturnus vulgaris, M. fortuitum*, *M. phlei* and *M. gastri* were isolated from liver, spleen and intestine, respectively

From the other *MAC* members, 47.1% of *M. a. hominissuis* of serotypes 4, 6, 8, and 9 were identified (Tables 2 to 5). That subspecies is the major causal agent of mycobacterial infections in pigs. During the years 1990 to 1999, 7 246 mycobacterial isolates from slaughtered pigs were obtained in the Czech Republic; 55.7% and 39.2% of them were classified as *M. a. avium* and *M. a. hominissuis*, respectively. Only 5.1% of isolates ranked among atypical mycobacteria as follows: *M. fortuitum, M. terrae, M. chelonae*, and *M. phlei* (Pavlik *et al.*, 2003).

The remaining 50.4% isolates in our study ranged across 15 CPM species (Table 2). As majority of these CPM may also take an active part in development of infection both in animals and humans (Matlova *et al.*, 1998; Bercovier and Vincent, 2001; Fischer *et al.*, 2001; Pavlik *et al.*, 2003), attention was focused also on their incidence (Tables 3 to 5).

Sources of mycobacterial infection for pigs

Free living birds and small terrestrial mammals. The major reservoir and vector of *M. a. avium* are birds (Pavlik *et al.*, 2000; Dvorska *et al.*, 2003). However, from parenchymatous organs of small terrestrial mammals originating from free nature, *M. a. avium* have been isolated both from insec-

Table 5. Mycobacteria isolated from invertebrates

Stages of development	Source of samples[c]	No. of samples			*Mycobacterium*				
		examined	positive	%	*hominissuis*[a]	*fortuitum*	*scrofulaceum*	*chelonae*	spp.[b]
Imagoes (n = 185)	fruit fly[d]	19	2	10.5	2	0	0	0	0
	cockroach[e]	1	0	0	0	0	0	0	0
	fly[f]	51	4	7.8	2	2	0	0	0
	scatophaga[g]	1	1	100	0	1	0	0	0
	little house fly[h]	2	0	0	0	0	0	0	0
	stable fly[i]	5	1	20.0	0	1	0	0	0
	drone fly[j]	77	1	1.3	1	0	0	0	0
	drone fly[k]	1	0	0	0	0	0	0	0
	blowfly[l]	2	0	0	0	0	0	0	0
	blowfly[m]	4	0	0	0	0	0	0	0
	hover fly[n]	1	0	0	0	0	0	0	0
	non-identified Diptera	3	0	0	0	0	0	0	0
	earthworms	18	1	5.6	0	0	1	0	0
Larvae (n = 206)	fly[f]	20	5	25.0	1	0	0	1	3
	drone fly[j]	186	16	8.6	8	1	0	7	0
Pupas (n = 39)	fly[f]	5	2	40.0	1	1	0	0	0
	drone fly[j]	34	1	2.9	1	0	0	0	0
Total		430	34	7.9	16	6	1	8	3
%			100		47.1	17.6	2.9	23.5	8.8

Explanations: [a]*Mycobacterium avium* subsp. *hominissuis*, genotype IS*901*– and IS*1245*+; [b]mycobacterial isolates without IS*901* and IS*1245* specific for *M. a. avium* and *M. a. hominissuis*; [c]larvae, pupas and imagoes from invertebrates trapped in the stables, results partially published previously (Fischer *et al.*, 2000, 2001, 2003); [d]*Drosophila* spp.; [e]*Blatella germania*, [f]*Musca* spp.; [g]Scatophagidae; [h]*Fannia* spp.; [i] *Stomoxys calcitrans;* [j]*Eristalis tenax;* [k]*Eristalis nemorum;* [l]*Calliphora vomitoria;* [m]*Calliphora vicina;* [n]*Syritta pipiens*

tivores and rodents (Fischer *et al*., 2000). In our study, both *M. a. avium* and *M. a. hominissuis* and the other mycobacterial species were isolated from birds' organs (Table 5).

Infection with two and even three species of mycobacteria was detected in two and one bird, respectively (Table 4). Our results are consistent with those obtained by Beerwerth and Kessel (1976a), who extensively investigated occurrence of mycobacteria in bird faeces. Based on systemic examination of some hundreds of samples, they found that even clinically normal birds without pathologically-morphological alterations of organs may be vectors not only of *M. a. avium* (serotypes 2 and 3), but also CPM. Therefore it is desirable to protect animal herds from penetration of these small vertebrates to the stables and prevent pigs from contact with the environment contaminated with faeces of birds and small terrestrial mammals. It is also necessary to control the contact of free living birds and small terrestrial mammals to prevent contamination of bedding, voluminous fodder, feed supplements, and drinking water with their faeces or corpses.

Peat. Peat was the material of external environment which was most contaminated with mycobacteria in our study, with nearly two-thirds of samples being culture positive (Table 1). Low pH of peat provides favourable environment for survival of mycobacteria (Kazda *et al*., 1989; Kazda, 2000). A number of CPM species present in peat are also pathogenic for pigs. They may cause formation of tuberculous lesions in their lymph nodes (Pavlik *et al*., 2000, 2003).

Unlike peat originating from natural deposits in which a lot of non-pathogenic and CPM were detected, incidence of *M. a. hominissuis* of serotypes 4, 6, 8, and 9 prevailed in piglets given peat. Likewise, three isolates of *M. a. avium* of serotypes 2 and 3 which cause avian tuberculosis in birds were also detected (Table 2).

Drinking water and biofilms. Water is a common reservoir of mycobacteria (Horvathova *et al*., 1997). It is evident from Table 1, that a very high incidence (36.4%) of CPM in the samples of biofilms and in drinking water (29.6%) was detected also in our study. As mycobacteria cannot be detected by microscopic examination in many cases, we used that technique as a preliminary or additional examination (Margolis *et al*., 1994). However, in samples of biofilms, detection of AFR by microscopy reached up to 27.2% and mycobacteria could be isolated from about one half of these samples (Table 1). Most frequent findings of CPM species in water environment are as follows: *M. fortuitum, M. chelonae, M. gordonae, M. marinum, M. terrae*, which may – in high levels – induce non-specific skin reactions in pigs at avian tuberculin tests; in some cases they may lead to the development of tuberculous lesions in lymph nodes of pigs (Pearson *et al*., 1977; Fodstad, 1977; Gardner and Hird, 1989; Balian *et al*., 1997; Dvorska *et al*., 1999; Bercovier and Vincent, 2001; Pavlik *et al*., 2003).

Bedding. Woody products appear to be risk when used as bedding (Beerwerth and Popp, 1971; Brooks, 1971; Fodstad, 1977; Pavlas and Patlokova, 1985; Cvetnic *et al*., 1998). In our study, the highest rate of isolation of mycobacteria from bedding materials was from sawdust (43.6%). Similarly as in the previous studies, *M. a. hominissuis* was the most common isolate bedding comprising 39.3% isolates obtained from that material (Table 3). The isolation of *M. a. avium* from bedding suggests that it may be an important route of exposure to pigs. Contamination of both sawdust and hay with the causal agent of avian tuberculosis could originate from faeces of wild birds and small terrestrial mammals (Table 4; Beerwerth and Kessel, 1976a; Fischer *et al*., 2000; Pavlik *et al*., 2000). The species composition of isolated mycobacteria (Table 3) was similar as in previous studies. Pavlas and Patlokova (1985) isolated the species *M. terrae*, *M. gordonae*, *M. triviale*, *M. flavescens* and *M. a. hominissuis* of serotypes 4, 6, 8, and 9 from hay. That material contamination resulted most likely from their contact with the soil and dust particles during harvest, additional contamination during transport, storage and further handling. Species composition of isolated CPM gives evidence of that fact (Tables 2 and 3).

Feed concentrates. Although mycobacterial contamination of feeds was not very high in our study (10.4%), almost a half of the detected isolates were *M. a. hominissuis* (Tables 1 and 2) which is hazardous for pig's health. According to the authors Nassal *et al*. (1974), Dimov and Gonzales (1986), and Beerwerth and Schurmann (1969), the levels of mycobacterial contamination of grain and feed mixtures are usually low. Nassal *et al*. (1974) suggest that it most likely resulted from the great distance between ears of grain and the soil surface. It follows that the ear surface does not usually get contaminated with soil containing high levels of mycobacteria. Beerwerth and Schurmann (1969) isolated mycobacteria from 3.6% of 111 samples of

unripe corn examined and from 3.3% of 400 grain feeds sold to farmers.

However, if the handling and storage conditions of feeds are not of a high standard they may become contaminated with mycobacteria and may contribute to the development of tuberculous lesions in mesenteric lymph nodes. The period of summer months may be particularly critical as mycobacteria quite rapidly propagate under the conditions of increased humidity in feed mixtures. However, we have also isolated other CPM species (*M. flavescens*, *M. fortuitum*, *M. gordonae*, *M. triviale*, and *M. terrae*), which may affect the health status of animals (Table 2). E.g. *M. terrae* has been isolated previously from pellets intended for the feeding of heifers in the Czech Republic (Hejlicek *et al*., 1982). That causal agent was later isolated from other samples of external environment, and after biological experiments in pullets, it was found to be the cause of para-alergic reactions to avian tuberculin in dermal tests in cattle.

Kaolin and charcoal. Feed supplements kaolin and charcoal may be classified as unusual sources of mycobacteria. Some farmers began giving these to piglets with diarrhoea as feed supplements because of their good absorbent (Matlova *et al*., 2003). Contamination with mycobacteria may result from the processing because surface water is used for levigation from surface mines, or from manipulation with charcoal and its superficial contamination with dust. In our study, mycobacteria were detected in 6.8% and 14.3% of kaolin and charcoal samples, respectively (Table 1). Despite that it is necessary to consider these raw materials contaminated outside and/or inside of the farm as hazardous for pig infection.

Pig faeces and scrapings from the stable walls and floors. Isolations of mycobacteria from pig faeces in our study were rather frequent (15.6%) and thus give evidence of mycobacterial contamination in the herds with mycobacterial infections. Pig faeces seem to be the most important source of the stable wall and floor contaminations, as in samples of scrapings comprising particularly combination of faeces, feeds, water, and other materials mycobacteria were detected by Z-N microscopy and culture in 18.4% samples (Table 1). However, it is well known that large quantities of atypical mycobacteria detected in animal faeces can propagate in the contents of digestive tract (Nassal *et al*., 1974). Species composition of CPM (with prevailing *M. a. hominissuis* of serotypes 4, 6, 8, and 9) also gives evidence that these constituents of external environment may be hazardous for the other non-infected animals (Table 2).

Soil. Another natural reservoir of mycobacteria in the pig farms is soil with prevailing incidence of CPM and non-pathogenic mycobacterial species (Beerwerth and Kessel, 1976b). Besides one isolate of *M. terrae,* other two isolates did not contain sequences IS*901* and IS*1245* specific for *M. a. avium* and *M. a. hominissuis*. In the biologically active layer of soil, mycobacteria are often detected already by microscopic examination and their number is estimated to be 100 to 100 000 mycobacteria/1g of soil (Beerwerth and Kessel, 1976b). Therefore it is necessary to protect pigs in the farms from direct contact with the soil in the runs or with contaminated feed. Anyway, the soil may also be the source of *Rhodococcus equi*, which likewise produces tuberculous lesions in pigs (Dvorska *et al*., 1999). Anyhow, runs with soil are used on pig farms in the Czech Republic very rarely.

Dust and spider's webs. Dust and spider's webs are less important sources of mycobacteria. In our study, mycobacteria were isolated only in 7.7% of samples taken from farms with animals affected by mycobacteriosis. AFR detection by microscopy gave evidence of contamination of the stable environment by mycobacteria (Table 1). Failure of their culture detection may have been related to their devitalisation in the day light during their drying up in the dust and spider's webs.

Invertebrates. According to the current knowledge, invertebrates may participate in spreading causal agents of mycobacterial infections (Fischer *et al*., 2001, 2003a,b). In our study, only 34 (7.9%) of 430 samples were contaminated with mycobacteria (Tables 1 and 5). Almost a half of isolates (47.1%) was *M. a. hominissuis* (Tables 2 and 5). Therefore, during implementation of preventative measures and control of mycobacterial infections, it is necessary to consider all the constituents of external environment which are potential sources of mycobacterial infection.

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Received: 03–05–07
Accepted after corrections: 03–11–03

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