Mycobacteria in the environment of pig farms in the Czech Republic between 2003 and 2007

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ABSTRACT: In the Czech Republic, most mycobacterial infections in pigs are caused by the *Mycobacterium avium* complex (*MAC*) and potentially pathogenic mycobacteria (PPM) derived from the environment. This study was undertaken after the isolation of PPM from various components of the environment of pig herds between 1996 and 2002 (Matlova et al., Veterinarni Medicina, 48, 2003, 343–357). Between 2003 and 2007, a total of 1114 environmentally-derived samples from 24 farms were examined. After staining according to Ziehl-Neelsen, acid-fast rods were found in 42 (3.8%) samples by direct microscopy, and PPM were isolated from 223 (20.0%) samples by culture. PPM occurred primarily in soil from the paddocks (53.8%), peat (53.2%), bedding (28.4%) and biofilm from the pipeline (21.0%). From *MAC, M. avium* subsp. *hominissuis* (*MAH*) genotype IS*901*– and IS*1245*+ was most frequent; *M. avium* subsp. *avium* (*MAA*) genotype IS*901*+ and IS*1245*+ and *M. intracellulare* genotype IS*901*– and IS*1245*– were isolated from one (0.4%) and three (1.3%) samples, respectively. The remaining isolates were identified as 19 other mycobacterial species: *M. gordonae* (*n* = 8), *M. triviale* (*n* = 6), *M. flavescens* (*n* = 3), *M. nonchromogenicum* (*n* = 3), *M. terrae* (*n* = 3), *M. xenopi* (*n* = 3), *M. fortuitum* (*n* = 2), *M. chelonae* (*n* = 2), *M. chitae* (*n* = 2), *M. abscessus* (*n* = 1), *M. gastri* (*n* = 1), *M. kumamotonense* (*n* = 1), *M. marinum* (*n* = 1), *M. parafortuitum* (*n* = 1), *M. peregrinum* (*n* = 1), *M. porcinum* (*n* = 1), *M. scrofulaceum* (*n* = 1), *M. smegmatis* (*n* = 1) and *M. simiae* (*n* = 1). The remaining 41 isolates of unidentified mycobacterial species did not contain the sequences IS*901* and/or IS*1245,* specific for medically important members of *MAC* (*MAA* and *MAH*); a further 44 isolates were not tested due to their contamination or loss of ability to grow *in vitro*. A farm where *MAH* was often detected in the lymph nodes of pigs and in the environment between 1996 and 2002 (Period I), was selected for further investigation between 2003 and 2007 (Period II). A comparison of the findings of mycobacteria on the investigated farm in Period I and in the following Period II showed a significant increase ($P < 0.01$) in the occurrence of mycobacteria other than *MAH*, especially in peat samples.

Keywords: mycobacteria other than tuberculosis (MOTT); ecology; avian tuberculosis; avian mycobacteriosis; environmental saprophytic mycobacteria; cultivation

The mycobacterial infection of pigs has resulted in severe financial losses to farmers in the Czech Republic. Between 1990 and 1999, the losses increased by 22 to 24% of slaughtered pig prices (Pavlik et al., 2003). Analysis of the occurrence of tuberculous lesions in pigs, performed in the period from 2000 to 2004, revealed that these were still being found in the tissues of slaughtered pigs. The most important causative agents of these tuberculous lesions were *Mycobacterium avium* complex (*MAC*) organisms (Pavlik et al., 2005). Between 1990 and 1992, the causative agent of avian tuberculosis *M. avium* subsp. *avium* (*MAA*) was found in 78.6%, 89.2% and 61.5% of tissues from the examined pigs during the respective years. These findings were considerably high in comparison to the causative

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agent of avian mycobacteriosis *M. avium* subsp. *hominissuis* (*MAH)*, which was only isolated from 18.3%, 9.7% and 30.3% of cases, during the respective years. However in the following years, 1993 and 1995 to 1999, the isolation rates of *MAH* tended to increase (Pavlik et al., 2003). These results were also confirmed by the study of all isolated members of *MAC* between 1996 and 2004 (Shitaye et al., 2006), by serotyping and IS*901* PCR (Pavlik et al., 2000; Bartos et al., 2006).

Molecular studies using a standardized IS*901* RFLP method (Dvorska et al., 2003) identified *MAA* isolates with similar RFLP types from pigs and contaminated peat (Matlova et al., 2005), domestic and wild birds (Dvorska et al., 2007; Moravkova et al., 2007; Shitaye et al., 2008a,b) and in one case, a horse (Pavlik et al., 2008).

Studies performed in the Czech Republic showed that bedding materials, various feed supplements and drinking water for animals, etc., were the sources of *MAH* rather than the infected animals themselves. In the first half of the 1990's, the source of *MAH* was deep bedding, usually composed of sawdust or other wood by-products, fermented by, e.g., ENVISTIM (Pavlik et al., 2003; Matlova et al., 2003a, 2004a). Results from the late 1990's, showed that increased tuberculous lesion formation in pigs was primarily elicited by *MAH* present in various feed supplements, of which the most important was peat (Matlova et al., 2003a, 2005; Pavlik et al., 2003) and kaolin (Matlova et al., 2003a, 2004b). In the following period (2000 to 2004), *MAH* was increasingly isolated from the examined pigs rather than *MAA* (Pavlik et al., 2005). Analysis of samples from the environment of pig herds between 1996 and 2002 determined that not only were the above-mentioned materials (above all sawdust used as bedding, peat and kaolin used as feed supplements) sources of *MAH*, but also drinking water, soil, invertebrate animals and dust, etc. (Matlova et al., 2003a).

With regard to the fact that the farm environment is the primary source of *MAH,* attention was also paid to the occurrence of PPM in the environment of pig herds in the Czech Republic. The objectives of this study were established to analyse the occurrence of mycobacteria in 24 pig herds in the Czech Republic between 2003 and 2007. Beside that, the results for one of the examined pig herds previously investigated by Matlova et al. (2003a), were compared with earlier data to assess implications for the risk of infection.

MATERIAL AND METHODS

Examined biological material

A total of 1114 samples from the environment (Table 1) of 24 pig farms in the Czech Republic were examined between 2003 and 2007. The selection of the farms was done on the previous finding of tuberculosis lesions and positive reactions to avian tuberculin (data not shown). The samples were stored at +4°C for up to two days until they were analysed in the laboratory.

Microscopic examination

Before culture, samples were stained by the Ziehl-Neelsen (Z-N) technique and examined under an Olympus microscope at 1000× magnification for the presence of acid-fast rods (AFR). At least 100 fields of view were examined for each sample (Kubin et al., 1986).

Culture examination

Approximately 1 g of sample was homogenized and decontaminated by a previously described method (Fischer et al., 2000; Matlova et al., 2003a). A total volume of 100 µl of a decontaminated sample suspension was inoculated with sterile disposable tips and dispensed onto eight slopes of media: two tubes of egg-based media according to the method of Stonebrink (Stonebrink, 1978) four slopes of Herrold Egg Yolk Media (HEYM; two with, and two without Mycobactin J) and onto two tubes of liquid media according to Sula (Merkal et al., 1964; Kubin et al., 1986). Incubations were performed simultaneously at two temperatures: first set of the media at 25°C, and the second set at 37°C (i.e., every sample was cultured on four different media at each temperature). The cultures were checked for growth four times. The first reading was taken up to seven days for detection of fast-growing mycobacterial species; the subsequent readings were performed after 14 days, one, and finally, two months.

Identification of mycobacterial isolates by PCR

Z-N positive isolates were identified by PCR according to the method first described by Wilton and

Cousins (1992) and modified by Moravkova et al. (2008). According to the above methods, isolates were characterised as *M.* sp., *M. avium* sp. or members of the *MAC* (Moravkova et al., 2008).

Mycobacteria, other than those identified as *M. avium* species were identified by a biochemical method according to Wayne and Kubica (1986) or identification was based on *16S rRNA* gene sequencing using the broad-range primers 16S-27f (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16S-907r (5'-CCG TCA ATT CMT TTR AGT TT-3') according to Harmsen et al. (2003). Sequencing was performed at the MWG Biotech Company (Germany). Resulting sequences were analysed by Staden Package software (http://staden.sourceforge.net/) and compared with the sequences in two databases, GenBank using the BLAST utility (http://www.ncbi.nlm.nih.gov/BLAST/) and RIDOM (http://rdna2.ridom.de/).

Statistical analysis

Statistical analysis was applied to the positive results obtained from one farm between 2003 and 2007 (Period II). These positive samples from Period II were statistically compared to the positive samples examined previously by Matlova et al. (2003a) between 1996 and 2002 (Period I). 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.

RESULTS

From 1114 samples (collected from 24 farms), microscopic examination revealed AFR by Z-N staining in 42 (3.8%) samples in nine of the 17 sample groups. By culture, mycobacteria were detected in 223 (20.0%) samples from 14 of the 17 sample groups (Table 1).

Microscopic detection of acid-fast rods in samples

AFR were most frequently detected in samples of biofilm from drinkers (10 samples; 12.3%) and in faeces (five samples; 8.6%), whereas the detection rates in samples of feed, bedding, water from drinkers, dust and spider webs was lower (1.3 to 3.8%; Table 1).

Culture detection of mycobacteria in samples

Mycobacteria were most frequently detected by culture in soil from the paddocks (53.8%), peat (53.2%) and bedding (28.4%; Table 1).

Identification of mycobacterial isolates

From a total of 223 positive isolates, it was possible to identify 138 strains classified as 22 species of mycobacteria (including *MAA*). From the *MAC*, 92 (41.3%) isolates were identified as *MAH,* which is a total of 8.3% of *MAH* from the 1114 samples examined in this study. In contrast to this, only one (0.4%) of the examined *MAC* isolates was identified as *MAA*. Among the remaining PPM (223 isolates in total), *M. gordonae* was found most frequently (eight isolates, 3.6%), followed by *M. triviale* (six isolates, 2.7%) and *M. nonchromogenicum* (three isolates, 1.3%; Table 2).

Mycobacterial contamination of various samples from 24 farms

Water from the pipeline. Mycobacteria were detected in 35 (15.4%) samples (Table 1) with a wide species range; out of 22 detected mycobacterial species, nine where found to be contaminants of water. The most frequently found species were *MAH* (seven isolates, 20.0%) and *M. gordonae* (four isolates, 11.4%; Table 2).

Biofilm from the pipeline. Mycobacteria were detected in 17 (21.0%) samples (Table 1).

Feed. Six species of PPM were detected in 25 (13.6%) samples (Table 1). *MAH* was detected most frequently (10 positive samples, 40.0%), followed by *M. flavescens* and *M. triviale,* each found in two (8.0%) samples (Table 2). By culture, feeding concentrates most frequently tested positive for PPM (22 positive samples, 17.9%), in comparison with feed supplements (Bentonit Zeo, Fibre cell and Sarbovet) and yeasts. In the feeding concentrates, five mycobacterial species were found, of which *MAH* was most frequent (eight isolates, 36.4%; Table 3). In other feed supplements, *MAH* was isolated only from the "Mistral" supplement. Only one positive sample (5.9%), which could not be precisely identified, was found in whey and milk (Table 1).

Table 1. Environmental samples from pig farms collected between the years 2003 and 2007

Samples examined[a]	Number	Z-N staining[b]		Culture	
		positive	%	positive	%
Water from pipeline	227	8	3.5	35	15.4
Feed[c]	184	7	3.8	25	13.6
Peat	156	4	2.6	83	53.2
Dust and spider webs[d]	88	3	3.4	9	10.2
Biofilm from pipeline[e]	81	10	12.3	17	21.0
Bedding[f]	81	3	3.7	23	28.4
Scrapings from stables[g]	79	1	1.3	8	10.1
Pig faeces[h]	58	5	8.6	6	10.3
Other samples[i]	43	0	0	1	2.3
Feed leftovers[j]	42	1	2.4	6	14.3
Whey and milk[k]	17	0	0	1	5.9
Free living birds[l]	17	0	0	0	0
Soil from paddocks	13	0	0	7	53.8
Kaolin[m]	9	0	0	0	0
Other feeding supplements[n]	9	0	0	1	11.1
Small terrestrial mammals[o]	5	0	0	1	20.0
Invertebrates[p]	5	0	0	0	0
Total	1114	42	3.8	223	20.0

[a]groups of samples designated according to the previously published results (Matlova et al., 2003a) during the years 1996 to 2002
[b]detection of acid-fast rods (AFR) after Ziehl-Neelsen (Z-N) staining
[c]feed samples collected from the troughs in the stables and from the storage tanks (for more details see Table 3)
[d]samples collected from the stables and feed stores
[e]biofilm samples collected from the drinking places and water-expanse reservoirs
[f]samples collected from the stables (for more details see in Table 4)
[g]scrapings collected in the stables: i.e. old pig faeces, feedstuff and dust from floors, wall, pen barriers and concrete paddocks and pen barriers
[h]collected from the floor in the boxes in the stables and paddocks
[i]included other samples, for example: waste water, samples from sewer, water filter, ventilation filter, skimming from the basin, rubbish mixture etc.
[j]samples of leftovers of feed concentrates were collected from the troughs
[k]samples originating from the storage tanks
[l]parenchymatous organs were examined (for more details see Table 5)
[m]kaolin was used as a feeding supplement for piglets under two months of age
[n]feeding supplements other than peat and kaolin were examined (Mistral, Univit CT-80, Hemasoft, Stalosanf, Staldrom and Heamstal)
[o]free living small terrestrial mammals were examined (more details in Table 5)
[p]for more details see Table 5

Feed leftovers. Mycobacteria were detected in 14.3% of samples of feed leftovers (Table 1). *M. flavescens, M. nonchromogenicum, M. gordonae* and *M. kumamotonense* were identified (Table 2).

Peat and kaolin. The second highest mycobacterial contamination was found in peat (Table 1). Out of 83 mycobacterial isolates, the following members of the *MAC* were most often detected: *MAH* (55 isolates), *MAA* (one isolate) and *M. intracellulare* that was not further typed (two isolates; Table 2). No mycobacteria were detected in samples of kaolin feed (Table 2). 5.9% of milk and whey samples were mycobacteria-positive (Table 1).

Bedding. The third highest mycobacterial contamination was found in bedding (28.4% positive samples; Table 1). Of seven mycobacterial species identified, *MAH* was the most frequent, being detected in 10 samples (43.5%; Table 2). Of all types of bedding, sawdust was the most often contaminated with mycobacteria (46.2% positive samples) followed by shavings (35.7% positive samples; Table 4).

Table 2. Mycobacterial species isolated from environmental samples

Mycobacterial isolates			*Mycobacterium*																					
Origin of isolates[a]	Number	*MAH*	*triviale*	*fortuitum*	*chelonae*	*gordonae*	*flavescens*	*xenopi*	*smegmatis*	*simiae*	*scrofulaceum*	*abscessus*	*porcinum*	*nonchromogenicum*	*intracellulare*[c]	*marinum*	*parafortuitum*	*terrae*	*kumamotonense*	*chitae*	*peregrinum*	*gastri*	spp.[d]	Sequencing not done[e]
Water from pipeline	35	7	1	1	1	4	–	1	–	–	–	–	–	1	–	1	–	–	–	–	1	–	15	2
Feed	25	10	2	1	–	–	2	–	–	–	–	1	–	–	1	–	–	–	–	–	–	–	2	6
Peat	83	57[b]	–	–	–	–	–	1	–	–	–	–	–	–	2	–	–	–	–	–	–	–	6	17
Dust and spider webs	9	4	1	–	–	–	–	–	–	–	–	–	–	1	–	–	–	1	–	–	–	–	–	2
Biofilm from pipeline	17	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	9	5
Bedding	23	10	1	–	1	–	–	1	1	–	1	–	–	–	–	–	1	–	–	–	–	–	4	3
Scrapings from stables	8	–	1	–	–	–	–	–	–	–	–	–	1	–	–	–	–	1	–	–	–	1	2	2
Pig faeces	6	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2	–	–	2	1
Other samples	1	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Feed leftovers	6	–	–	–	–	1	1	–	–	–	–	–	–	1	–	–	–	–	1	–	–	–	–	2
Whey and milk	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Soil from paddocks	7	3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	1	2
Other feeding supplements	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Small terrestrial mammals	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
Total	223	93	6	2	2	8	3	3	1	1	1	1	1	3	3	1	1	3	1	2	1	1	41	44
%		41.7	2.7	0.9	0.9	3.6	1.3	1.3	0.4	0.4	0.4	0.4	0.4	1.3	1.3	0.4	0.4	1.3	0.4	0.9	0.4	0.4	18.4	19.7

[a]groups of samples designated according to previously published results (Matlova et al., 2003a) during the years 1996 to 2002; for more details see Table 1 legend
[b]isolates consisted of one *MAA* isolate (genotype IS*901*+ and IS*1245*+) and one isolate which was not successfully typed further
[c]*M. intracellulare* (genotype IS*901*– and IS*1245*–)
[d]isolates not belonging to *MAC* after PCR examination (Moravkova et al., 2008) and by sequencing not successfully typed to the species
[e]isolates not belonging to *MAC* after PCR examination (Moravkova et al., 2008); sequencing was not done due to lack of growth in the subcultures or due to the contamination of the isolate with another bacterial or mould species

Scrapings from the stable and pig faeces. Mycobacteria contaminated 10.1% of samples of scrapings from floors in animal housing (Table 1), among which four species were identified (*M. triviale, M. porcinum, M. terrae* and *M. gastri*; Table 2). Mycobacteria were found in 10.3% of pig faecal samples (Table 1), where *MAH* and *M. chitae* were identified (Table 2).

Dust and spider webs. Mycobacteria were isolated from nine (10.2%) samples of dust and spider webs (Table 1) of which four isolates were identified as *MAH* (Table 2).

Soil from the paddocks. Mycobacterial contaminations were confirmed in 53.8% of samples of soil from the paddocks (Table 1). *M. terrae and MAH* were identified (Table 2).

Other samples. Mycobacteria were detected in 2.3% of the other samples from pig herds, including swabs from basins, water filters, ventilation filters, cleaning devices, sewage water and samples from sewers (Table 1). The isolate identified as *M. simiae* originated from a sample of the rubbish mixture from the floor (Table 2).

No mycobacteria were found in free living birds and invertebrates (Tables 1 and 5). Regarding the group of small terrestrial mammals, only one sample was found to be positive. However, its exact identification was not possible (Table 2).

Statistical analysis of results obtained on one farm in Periods I and II

Analysis of data from Periods I and II showed a significantly decreased frequency of occurrence of all positive isolates ($P < 0.05$) in comparison with all tested samples (Table 6). The same tendency was observed in *MAH* occurrence ($P < 0.01$; Table 7).

Mycobacterial contamination of different samples on the farm

Water from the pipeline. In this type of samples, a non-significant decrease in occurrence of positive samples was observed (Table 6). *MAH* was not detected in any isolate, which is a statistically non-significant difference in comparison with Period I (Table 7). In Period II, the highest percentage of *M. gordonae* was found (Table 8).

Table 3. Mycobacterial species isolated from feeding concentrates and feed supplements

Mycobacterial isolates					*Mycobacterium*						
Origin of examined samples	Number	Positive	%	*MAH*[a]	*flavescens*	*triviale*	*abscessus*	*intracellulare*[b]	*fortuitum*	spp.[c]	Sequencing not done[d]
Feed											
Feeding concentrates (finishing pigs)	123	22	17.9	8	2	2	1	–	1	2	6
Feeding concentrates COS 1 (piglets)	18	1	5.6	–	–	–	–	1	–	–	–
Feed supplement (Bentonit Zeo)	15	2	13.3	2	–	–	–	–	–	–	–
Feed supplement (Fibre cell M1)	3	0	0	–	–	–	–	–	–	–	–
Feed supplement (Sarbovet)	20	0	0	–	–	–	–	–	–	–	–
Yeast	5	0	0	–	–	–	–	–	–	–	–
Total	184	25	13.6	10	2	2	1	1	1	2	6
%	100	100		40.0	8.0	8.0	4.0	4.0	4.0	8.0	24.0

[a]*MAH* (genotype IS*901*– and IS*1245*+)
[b]*M. intracellulare* (genotype IS*901*– and IS*1245*–)
[c]isolates not belonging to the *MAC* after PCR examination (Moravkova et al., 2008) and by sequencing not successfully typed to the species level
[d]isolates not belonging to the *M. avium* complex after PCR examination (Moravkova et al., 2008); sequencing not done due to lack of growth in the subcultures or due to the contamination of the isolate with another bacterial or mould species

Table 4. Mycobacterial species isolated from the different components of bedding

Mycobacterial isolates					*Mycobacterium*							
Origin of examined samples	Number	Positive	%	*MAH*[a]	*triviale*	*chelonae*	*xenopi*	*smegmatis*	*scrofulaceum*	*paraffortuitum*	spp.[b]	Sequencing not done[c]
Straw	16	4	25.0	1	–	1	1	–	–	1	–	–
Wood-shaving	14	5	35.7	4	–	–	–	–	–	–	–	1
Sawdust	13	6	46.2	4	–	–	–	1	1	–	–	–
Hay	8	3	37.5	–	–	–	–	–	–	–	1	2
Mixed bedding	30	5	16.7	1	1	–	–	–	–	–	3	–
Total	81	23	28.4	10	1	1	1	1	1	1	4	3
%	100	100		43.5	4.3	4.3	4.3	4.3	4.3	4.3	17.4	13.0

[a]*MAH* (genotype IS*901*– and IS*1245*+)
[b]isolates not belonging to the *MAC* after PCR examination (Moravkova et al., 2008) and by sequencing not successfully typed to the species level
[c]isolates not belonging to the *M. avium* complex after PCR examination (Moravkova et al., 2008); sequencing not done due to a lack of growth in the subcultures or due to the contamination of isolate with another bacterial or mould species

Biofilm from the pipeline. In Period II, the occurrence of mycobacteria non-significantly decreased in comparison with Period I (Table 6). Among the isolates classified to the species level, *M. flavescens* and *M. terrae* predominated. *MAH* were not detected in the second period (Table 8).

Feed. The occurrence of mycobacteria increased non-significantly (Table 6). The situation was comparable for *MAH,* which was detected only in one case per investigation period (Table 7). In the second period, *M. flavescens* was identified in the majority of cases, whereas in the first period, it was *MAH* (Table 8).

Peat. In peat samples, the occurrence of mycobacteria other than *MAH* increased significantly ($P < 0.01$; Table 6). These were primarily *Mycobacterium* spp., which could not be identified. On the other hand, a significant decrease ($P < 0.01$) in the occurrence of *MAH* in peat was noted (Table 7). Species other than *MAH* were not detected in Period I. In Period II, *M. xenopi* was

Table 5. Mycobacterial species isolated from samples of birds, small terrestrial mammals and invertebrates

Group of samples	Source of samples	Number of examined		
		animals	samples	positive
Free living birds	sparrow (*Passer domesticus*)	5	10	0
	swallow (*Hirundo rustica*)	1	2	0
	black redstart (*Phoenicurus ochruros*)	1		0
	great tit (*Parus major*)	1	2	0
	faeces from birds (especially sparrows)	nk	1	0
Small terrestrial mammals	mouse (*Mus musculus*)	1	1	1[a]
	faeces from mice	nk	4	0
Invertebrates	darkling beetle (*Tenebrio* sp.)	2	2	0
	flies in the whey (*Musca domestica*)	3	3	0

nk = not known (mixture samples)
[a]isolate not belonging to the *MAC*, further typing was not possible due to contamination

Table 6. Comparison of mycobacteria detection between Period I (1996–2002) and Period II (2003–2007) on one farm

Samples examined	Period I[a]			Period II			Comparison Period I vs. II	
	number	positive	%	number	positive	%	trend	significance[b]
Water from pipeline	60	20	33.3	68	13	19.1	decrease	ns
Feed	49	1	2.0	78	8	10.3	increase	ns
Peat	268	136	50.7	33	29	87.9	increase	++
Dust and spider webs	30	1	3.3	13	0	0	decrease	ns
Biofilm from pipeline	229	87	38.0	38	12	31.6	decrease	ns
Scraping from stables	3	0	0	8	5	62.5	increase	ns
Pig faeces	55	5	9.1	12	3	25.0	increase	ns
Total	694	250	36.0	250	70	28.0	decrease	+

[a]published previously (Matlova et al., 2003a)
[b]ns = non-significant ($P > 0.05$), + = $P < 0.05$, ++ = $P < 0.01$ (P is two-sided P-value for Fisher's exact test)

identified and *M. intracellulare* was detected in two cases (Table 8).

Dust and spider webs. No positive samples were found in dust and spider webs in Period II. This was a non-significant decrease in comparison with Period I when one isolate was detected (Table 6). This isolate could not be identified at the species level (Table 8).

Scrapings from the stables and pig faeces. A non-significant increase in the occurrence of positive samples of scrapings was noted in Period II (Table 6). Positive isolates were only found in Period II and only one of them was identified at the species level, namely *M. triviale* (Table 8). In the samples of pig faeces, *MAH* was not detected in Period II. On the other hand, the occurrence of other mycobacterial species increased non-significantly (Table 6). These were not further identified (Table 8).

DISCUSSION

The environment is the main source of conditionally pathogenic mycobacteria (i.e., primarily serious pathogens of animals with the following most important two *MAC* members: *MAH*, *M. intracellu-*

Table 7. Comparison of *Mycobacterium avium* subsp. *hominissuis* detection between Period I (1996–2002) and Period II (2003–2007) on one farm

Samples examined	Period I[a]			Period II			Comparison Period I vs. II	
	number	positive	%	number	positive	%	trend	significance[b]
Water from pipeline	20	2	10.0	13	0	0	decrease	ns
Feed	1	1	100	8	1	12.5	decrease	ns
Peat	136	94	69.1	29	11	37.9	decrease	++
Dust and spider webs	1	1	100	0	0	0	nt	nt
Biofilm from pipeline	87	2	2.3	12	0	0	decrease	ns
Scrapings from stables	0	0	0	5	0	0	nt	nt
Pig faeces	5	1	20.0	3	0	0	decrease	ns
Total	250	101	40.4	70	12	17.1	decrease	++

[a]published previously (Matlova et al., 2003a)
[b]nt = not tested, ns = non-significant ($P > 0.05$), ++ = $P < 0.01$ (P is two-sided P-value for Fisher's exact test)

Table 8. Mycobacterial species isolated from environmental samples from one farm in Period I (1996–2002) and Period II (2003–2007)

Mycobacterial isolates	Number			*Mycobacterium*										
Origin of isolates[a]	Period I[b]	Period II[c]	*MAC*	*triviale*	*fortuitum*	*gordonae*	*flavescens*	*xenopi*	*szulgai*	*scrofulaceum*	*terrae*	*diernhoferi*	spp.[e]	Sequencing not done[f]
Water from pipeline	20	13	2/–	–/–	–/1	5/2	–/–	–/–	–/–	–/–	–/–	1/–	12/10	–/–
Feed	1	8	1/1	–/1	–/–	–/–	–/2	–/–	–/–	–/–	–/–	–/–	–/1	–/3
Peat	136	29	94[d]/13	–/–	–/–	–/–	–/–	–/1	–/–	–/–	–/–	–/–	42/2	–/13
Dust and spider webs	1	0	1/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–
Biofilm from pipeline	87	12	2/–	–/–	–/–	1/–	5/–	–/–	1/–	1/–	2/–	1/–	66/9	8/3
Scraping from stables	0	5	–/–	–/1	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/4	–/–
Pig faeces	5	3	1/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	4/2	–/1
Total	250	70	101/14	–/2	–/1	6/2	5/2	–/1	1/–	1/–	2/–	2/–	124/28	8/20
Period I	100		40.4	–	–	2.4	2.0	–	0.4	0.4	0.8	0.8	49.6	3.2
Period II		100	20	2.9	1.4	2.9	2.9	1.4	–	–	–	–	40	28.6

[a]groups of samples designated according to previously published results (Matlova et al., 2003a); for more details see Table 1 legend

[b, c]comparison of all *Mycobacterium* detection in Period I (1996–2002) published previously (Matlova et al., 2003a) and Period II (2003–2007)

[d]*Mycobacterium avium* complex isolates consisted of one *M. a. avium* isolate (genotype IS*901*+ and IS*1245*+) from peat, 92 isolates of *M. a. hominissuis* (genotype IS*901*– and IS*1245*+) and one isolate was not successfully typed further

[e]isolates not belonging to the *M. avium* complex after PCR examination (Moravkova et al., 2008) and by sequencing not successfully typed to the species level

[f]isolates not belonging to the *M. avium* complex after PCR examination (Moravkova et al., 2008), sequencing not done due to lack of growth in the subcultures or due to contamination of the isolate with another bacterial or mould species

lare, and also other species: *M. xenopi*, *M. chelonae* etc.; Kazda, 2000; Kazda et al., 2009). Of the *MAC* members, *MAH* predominated in our study, and was identified in a total of 93 (40.8%) isolates (Table 2). Also in other countries, *MAH* has been determined as the most frequent causative agent of tuberculous lesions in pigs (Dalchow and Nassal, 1979; Windsor et al., 1984; Dalchow, 1988; Alfredsen and Skjerve, 1993; Leinemann et al., 1993; Morita et al., 1994; Nishimori et al., 1995; Balian et al., 1997; Ritacco et al., 1998; Komijn et al., 1999; Offermann et al., 1999; Ramasoota et al., 2001; Mijs et al., 2002).

Between 1996 and 2002, the strong effect of a contaminated environment on the occurrence of tuberculin reactions to avian tuberculin and occurrence of tuberculous lesions in the lymph nodes of pigs at slaughter was investigated (Matlova et al., 2003b,c). One of the herds under investigation in the above-mentioned study was also included in the current study. Special attention in this part of our study was paid to collection of samples, which were identified as positive in the previous study (Matlova et al., 2003a). Emphasis was put on *MAH*-infected samples in the previous study (Matlova et al., 2003a) because *MAH* is an important swine pathogen. Accordingly, the purpose of the present study was to monitor the occurrence of mycobacteria in the environment of pig herds in the period between 2003 and 2007 and to compare the occurrence of *MAH* and other mycobacteria in Period I (1996–2002) described by Matlova et al. (2003a) with their occurrence in Period II (2003–2007).

The results of this study, which were in accordance with data from one farm investigated by Matlova et al. (2003a), have characterized the most important sources and risk factors, including new technologies, associated with mycobacterial infections of pigs in the Czech Republic.

Statistical analysis of a one farm analysed between Periods I and II

In the pig herd investigated in the present study between 1998 and 2001, considerable impact of contaminated environment on the occurrence of tuberculin reactions to avian tuberculin and occurrence of tuberculin lesions in the lymph nodes of slaughtered pigs was observed (Matlova et al., 2003b,c). Therefore, this herd was selected for a longitudinal study. The results of monitoring the occurrence of mycobacteria in this pig farm environment between 1996 and 2002 (Period I) were part of the study of Matlova et al. (2003a). Results obtained on this farm were also presented in the current study, because the most contaminated samples found in the previous period (especially with *MAH* frequently found in pigs) were selected for subsequent investigation. The purpose of the current study was to monitor the occurrence of mycobacteria in the environment of the pig farm in the following years, between 2003 and 2007, and to evaluate the occurrence of *MAH* and mycobacteria on the farm in Periods I (1996–2002) and II (2003–2007).

Mycobacterial contamination of different samples

Water from the pipeline and biofilm from the pipeline. Water plays a significant role as a vehicle for the transmission of mycobacteria, and is regarded as a common source and milieu for multiplication under favourable conditions. In this study, the finding of *MAH* was most frequent (20%), followed by *M. gordonae* (11.4%; Table 2). Expansion vessels are at risk of the accumulation and subsequent multiplication of PPM, especially in the summer months (Kazda, 2000; Hilborn et al., 2006; Kazda et al., 2009). Furthermore, the highest risk for pigs is the uptake of surface water because it can be contaminated from different sources. Mycobacteria were isolated from replicate samples of, e.g., sediments of sewage water where concentrations reached up to 10×10^5 CFU/ml (Brooks et al., 1984). With regard to biofilm from the pipeline, as the detection rate of AFR by microscopy after Z-N staining is rather low (Margolis et al., 1994), this method was used here as an auxiliary tool for obtaining approximate results (Table 1). In many cases, mycobacteria cannot be isolated *in vitro* from Z-N positive samples (Matlova et al., 2003a), increasing the high risk of mycobacterial infection for pigs via water contaminated (Tables 1 and 2). Between 2000 and 2001, the occurrence of mycobacteria in the water main on one farm was investigated. Matlova et al. (2003b) revealed that the pH of the water decreased to 4.0 owing to disinfection and consequently, biofilm-containing mycobacteria was released into the water main. Consequently, this led to the formation of tuberculous lesions in up to 90% of slaughtered pigs from the above mentioned environment. Water and biofilm samples examined in the current study originated from the same water main, but were collected after this event. In both types of samples, the occurrence of mycobacteria (Table 6) non-significantly decreased from Period I (1996–2002) to Period II (2003–2007). This could be explained by regular mechanical cleaning of the drinkers, and steaming of the whole water main with hot water (70 to 80°C) for at least 20 min according to the recommendations (unpublished data).

Feed. Beerwerth and Schurmann (1969) isolated mycobacteria from 3.6% of 111 samples of unripe corn, as well as from 3.3% of 400 samples of feeding concentrates. They explained the relatively low detection rate by the distance of corn ears from the soil (up to 1 m) and a minimum risk of contamination of the ears with mycobacteria present in the soil. Even though mycobacterial contamination of feeds was not very high in the current study (13.6%), 40% of pathogens were identified as the clinically relevant *MAH* (Tables 1 and 2), followed by *M. flavescens* and *M. triviale* (Table 3). The occurrence of these mycobacteria can also be explained by their transmission from the environment. Accordingly, we can suppose that inadequate handling and storage of feeding concentrates can contribute to mycobacterial contamination of feeds. The present study documented that contamination of feed mixtures was non-significantly higher in Period II in comparison with Period I (Table 6). Therefore, we can assume that inappropriate handling practices or poor storage conditions could contribute to the contamination of these feed mixtures with mycobacteria.

Kaolin and other feed supplements. Extracted as a sterile material, kaolin is used for the same purposes as peat. However, it can be additionally contaminated with PPM during levigation and processing (Matlova et al., 2003a; 2004b; Trckova et al., 2004). In this study no mycobacteria were detected in kaolin samples (Tables 1 and 2).

Accordingly, if stored properly, kaolin can be a safe material used to prevent diarrhoea in weaned piglets (Trckova et al., 2009), in contrast to other feed supplements among which 11.1% of samples examined tested positive for mycobacteria (Tables 1, 2 and 3). However, farmers should be aware of the risks associated with the application of feed supplements.

Peat. In the mid-1990s, the feeding of piglets with peat as a supplement was introduced into some farms in the Czech Republic. Due to the fact that the occurrence of tuberculous lesions in pigs increased, farmers ceased feeding peat as a supplement in the late 1990s (Pavlik et al., 2003). An absolute ban on the use of antibiotic growth promoters came into force in 2006 (Trckova et al., 2009), and thus since that time, alternative supplements such as peat and kaolin have been used for the prevention of enteric diseases (Trckova et al., 2005; 2006a,b; 2009). In our study, peat was the second most contaminated material, with a predominating occurrence of *MAH* (Tables 1 and 2). Its application as a feed supplement often caused formation of tuberculoid lesions in the lymph nodes of pigs (Matlova et al., 2003b, 2005; Pavlik et al., 2007). However, natural peat constitutes an important source of atypical mycobacteria (Kazda, 2000; Kazda et al., 2009). Additionally, even though sterile upon extraction from the underground, it can be easily contaminated with mycobacteria afterwards. Accordingly, it should be stressed that this feed supplement is highly risky. In Period I, 69.1% of samples were contaminated with *MAH*. Between 1998 and 1999, *MAH* contaminated the peat on this farm, with significant implications for the formation of tuberculous lesions in the lymph nodes of pigs (Matlova et al., 2003b, 2005). In Period II, *MAH* occurrence declined by 31.2%, and conversely, the proportion of other mycobacterial species increased (Table 7). Hence, we can assume that tuberculous lesions were not formed in pigs in Period II owing to the decline in *MAH* occurrence in peat.

Bedding. Wood products have been considered as the most risky bedding material (Kazda et al., 2009) and were found to be highly contaminated with mycobacteria when used as bedding, sawdust and wood shavings (Pavlas and Patlokova, 1985; Falkinham, 1989; Pavlas et al., 1991; Hanzlikova and Vilimek, 1992). In the present study, the detection rate of mycobacteria in bedding (above all in sawdust and shavings) was high (Tables 1, 2 and 3). In this group of samples, *MAC* members were most common, with a preponderant occurrence of *MAH* (Table 4). Mycobacteria can propagate in wood materials if they are stored under poor conditions in a moist environment, primarily in summer (Zorawski et al., 1983; Pavlas et al., 1991; Kazda, 2000). Studies strongly indicate that pig herds kept on deep litter bedding consisting of wood products are at a high risk.

Scrapings from the stables and pig faeces. Faeces and environment contaminated with faeces provide conditions suitable for the survival and further spread of conditionally pathogenic mycobacteria in pig herds, e.g., *M. triviale, M. porcinum, M. terrae* and *M. gastri* detected in our study (Table 2). Accordingly, the application of basic principles of animal hygiene is necessary. A non-significant increase in the frequency of occurrence of mycobacteria (Table 6) other than *MAH* occurred in both groups of these samples (Table 8). The scrapings comprised above all faeces, feeds, peat and other materials. Due to the fact that mycobacterial contamination was significantly increased in all of these types of samples (Table 6), it was also apparent here.

Dust and spider webs. The occurrence of mycobacteria in dust and spider webs is less common. Dust present in the environment of animal housing is closely associated with bedding and various activities on pig farms in general. Dust particles are often in motion and, as a vehicle for the transmission of mycobacteria, can easily contaminate water and feeds and also penetrate the respiratory tract of animals and humans (Kazda et al., 2009). Therefore, general preventive measures to minimise the formation and spread of dust which can be contaminated with mycobacteria should be adopted (Tables 1 and 2), as dust contaminated with mycobacteria can cause infection under certain conditions. In Period II, no dust and spider web samples were positive in the present study, which presents a non-significant decline in comparison with Period I (Table 6). Dust and spider webs are less important sources of infection for pigs. However, the concentration of mycobacteria in the environment is very important. Accordingly, it is necessary to keep swine in clean conditions.

Soil from the paddocks. Due to the fact that soil is also a reservoir of mycobacteria on pig farms (Kazda et al., 2009), it is necessary to prevent direct contact of the animals with soil in the paddocks (Tables 1 and 2) and soil-contaminated feeds. Moreover, the soil can become a source of

Rhodococcus equi that causes the formation of tuberculous lesions in pigs (Dvorska et al., 1999). This causative agent considerably complicates the veterinary meat inspection in the slaughterhouses because it causes the formation of tuberculoid lesions, especially in the head lymph nodes. These cannot often be discriminated by gross examination from tuberculous lesions caused by mycobacteria (Dvorska et al., 1999; Shitaye et al., 2006). All these results indicate that the external environment, especially soil, is a source of causative agents of tuberculous lesions in pigs.

Other samples. *M. simiae* was isolated from this group of samples (Tables 1 and 2), likewise from the faeces and parenchymatous organs of one wild boar (Machackova et al., 2003). Due to the fact that the boar was found in water (Kazda et al., 2009), this detection in organs and faeces is not surprising.

Free living birds, invertebrates and small terrestrial mammals. Even though wild birds constitute a large reservoir and vector of the causative agent of avian tuberculosis, *MAA*, their contact with pigs has been prevented in the majority of pig facilities. Thus, the risk of *MAA* spread to pig herds by birds is low (Tables 1, 2 and 5). Invertebrate animals can also become a source of mycobacterial infection for pigs (Fischer et al., 2001, 2003a,b, 2004a,b, 2006). Thus, regular implementation of disinsection procedures in the summer months is recommended. However, no positive isolate was found in this study. Although small terrestrial mammals constitute a low risk of mycobacteria spread into pig herds (Tables 1, 2 and 5), it is necessary to maintain rodent control in herds. As documented in a previous study performed in the Czech Republic (Fischer et al., 2000), these small free living animals, potentially contaminated with mycobacteria, can penetrate pig housing when there is a depletion of their natural food source.

It follows from our study that the environment poses the highest risk to pigs. The most important components in this regard are soil contaminated with mycobacteria, feed supplements such as peat, and bedding, especially wood materials. *MAH*, which poses a risk of mycobacterial infection to pigs, predominated in all kinds of these samples. However, we confirmed that the occurrence of this *MAC* subspecies on the investigated farm, where such mycobacterial infections were previously detected (Matlova et al., 2003b,c, 2005; Pavlik et al., 2007) decreased from Period I (1996–2002) to Period II (2003–2007). Accordingly, we can assume that this tendency will be maintained due to a general awareness of this problem among breeders and the relevant authorities. This prediction is supported by statistical data of the State Veterinary Administration of the Czech Republic, showing that the occurrence of tuberculous lesions in slaughtered pigs decreased during the period 2005–2008.

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