Mycobacterial and *Rhodococcus equi* infections in pigs in the Czech Republic between the years 1996 and 2004: the causal factors and distribution of infections in the tissues

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ABSTRACT: Between 1996 and 2004, tissue samples from 3 630 slaughtered pigs were examined by gross examination, microscopy after the Ziehl-Neelsen (ZN) staining of homogenised tissues for the detection of acid-fast rods (AFR) and by culture for the presence of mycobacteria and *Rhodococcus equi*: 1 781 head lymph nodes (ln), 1 123 mesenteric ln, 54 pulmonary ln, 32 inguinal ln, 562 non-identified ln and 78 samples of tissues from parenchymatous organs (liver, spleen and kidneys). Tuberculous/tuberculoid lesions were not detected in 249 (6.9%) animals slaughtered due to a positive response to avian tuberculin. Various gross lesions were detected in 3 381 (93.1%) animals as follows: adenopathy in 150 (4.1%), tuberculous lesions with caseation in 2 026 (55.8%) and tuberculous lesions with calcification in 1 205 (33.2%) of them. AFR were found in tissues from 2 047 (56.4%) animals: in 36 (14.5%) animals free from gross lesions, in 28 (18.7%) animals with adenopathy, in 801 (39.5%) animals with caseation and in 913 (75.8%) animals with calcified tuberculous lesions. Mycobacteria were isolated from the tissues of 289 (15.8%) of 1 852 animals without detected AFR and from the tissues of 1 290 (72.5%) of the 1 778 animals with detected AFR of various intensities. Of 1 579 mycobacterial isolates 1 493 (94.6%) were classified as *M. avium* complex (*MAC*) members: 469 (29.7%) *M. a. avium* (IS*901*+, serotypes 1, 2, and 3) and 891 (56.4%) *M. a. hominissuis* (IS*901*–) isolates of serotypes 4 (n = 1), 8 (n = 643), 9 (n = 74) and non-typed (n = 173). The other 52 (3.3%) isolates were members of other mycobacterial species: *M. chelonae* (n = 35), *M. smegmatis* (n = 4), *M. xenopi* (n = 3), *M. terrae* (n = 7), *M. aurum* (n = 1), *M. scrofulaceum* (n = 1), *M. fortuitum* (n = 1) and biochemically non-identified mycobacteria (n = 34). By examination of ZN stained homogenised tissues, AFR were detected significantly more frequently ($P < 0.01$) in samples from animals with caseated and/or calcified tuberculous lesions than in tissues from animals without tuberculous lesions. The detection rate of isolates from tissues with tuberculous lesions was likewise significantly higher ($P < 0.01$) than from tissues without tuberculous lesions.

Keywords: atypical mycobacteria; IS*901*; IS*1245*; PCR; serotyping; zoonosis; avian tuberculosis; economic losses; bovine tuberculosis

Among domestic animals, pigs are particularly susceptible to mycobacterial infections caused especially by members of the *Mycobacterium tuberculosis* complex (*MTC*; Thoen et al., 2006), *M. avium* complex (*MAC*; Pavlik et al., 2003b, 2005a), and conditionally pathogenic mycobacteria (Morita et al., 1994a,b; Thorel et al., 1997; Komijn et al., 1999; Offermann et al., 1999). The occurrence of tuberculous/tuberculoid lesions in pigs brings considerable economic losses (Berthelsen, 1974; Dey and

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Parham, 1993; Sigurdardottir et al., 1994; Margolis et al., 1994; Cvetnic et al., 1998). Twenty two to 24% of the total slaughter costs in the Czech Republic consist of slaughtered pigs with detected tuberculous/tuberculoid lesions (Pavlik et al., 2003b). The causes of granuloma formation may also be other bacterial species e.g. *Rhodococcus equi* (Dvorska et al., 1999; Flynn et al., 2001; Pavlik et al., 2005a).

In the Czech Republic bovine tuberculosis was put under control in 1968. In the following period, incidence of bovine tuberculosis in cattle and the other domestic animals including pigs was gradually decreasing (Pavlik et al., 1998, 2002a,d,e, 2005b; Pavlik, 2006). The last outbreak of bovine tuberculosis in cattle and domestic pigs was diagnosed in 1995 and was caused by *M. caprae* (Pavlik et al., 2002a,b; Erler et al., 2004). Bovine tuberculosis in free living animals and animals in captivity was diagnosed occasionally, and no contact with domestic animals occurred with registered in any of the infected free living animals (Pavlik et al., 2002c,e; Machackova et al., 2003; Trcka et al., 2006). Accordingly, the Czech Republic has been officially declared a bovine tuberculosis-free EU State (Anonymous, 2004; Pavlik, 2006).

Besides gross examination and microscopy (and histology in some cases) after the Ziehl-Neelsen (ZN) staining, culture examination for the presence of mycobacteria (Pavlik et al., 1998; Dvorska et al., 1999) was used for identification of pathogens causing tuberculous lesions in domestic animals, above all in cattle and pigs in the Czech Republic. Mycobacterial isolates classified as *MTC* members have been routinely identified by biological experiments in animals (above all in guinea pigs), by biochemical tests (Wayne and Kubica, 1986) and since 1994 by Accu-Probes (Gen-Probe Inc., San Diego, California, USA).

For identification of *MAC* members, the following methods were used: biological experiments in domestic fowl (*Gallus domesticus*; Pavlas and Patlokova, 1977) and serotyping according to Wolinsky and Schaefer (1973) modified by Sussland and Hrdinova (1976). Since 1996 the IS*901* PCR method (Pavlik et al., 2000) that allows identification of the causative agent of avian tuberculosis *M. a. avium* of serotypes 1, 2, and 3. Introduction of this method likewise allowed withdrawal of biological experiments in domestic fowl from use for the identification of the causative agent of avian tuberculosis (Dvorska et al., 2006).

For detailed identification of selected *MAC* isolates, IS*901* Restriction Fragment Length Polymorphism (RFLP) methods were used in the case of the causative agent of avian tuberculosis (Dvorska et al., 2003, Fischer et al., 2003, 2004a,b; Matlova et al., 2004a,b; Dvorska et al., 2004), IS*1245* RFLP and Mycobacterial Interspersed Repetitive Unit (MIRU) in the case of the causative agent of avian mycobacteriosis (Fischer et al., 2004b; Dvorska et al., 2004; Matlova et al., 2004b, 2005; Romano et al., 2005). The introduced IS*901* and IS*1245* PCR methods (Bartos et al., 2006) and biochemical tests according to Wayne and Kubica (1986) were performed. These were used for identification of *MAC* members during investigation of sources of mycobacterial infections from pigs with kaolin feed as a supplement (Matlova et al., 2004a), peat (Matlova et al., 2003, 2004a, 2005), from the stable and the external environment (Matlova et al., 2003).

Tuberculous/tuberculoid lesions, since the last case of bovine tuberculosis diagnosed in cattle and domestic pigs in the Czech Republic in 1995 (Pavlik et al., 2002b), were caused by *MAC* members (Pavlik et al., 2003b, 2005a) and *R. equi* (Dvorska et al., 1999; Pavlik et al., 2003b, 2005a), respectively. Of the *MAC* members, the causative agent of avian tuberculosis *M. a. avium* was predominantly isolated from tuberculous lesions in cattle (Pavlik et al., 2002d, 2005a; Dvorska et al., 2004). In contrast, isolation of the causative agent of avian mycobacteriosis *M. a. hominissuis* from tuberculous lesions prevailed in pigs (Dvorska et al., 1999; Pavlik et al., 2000, 2003b, 2005a).

From a veterinary aspect, the causative agent of avian tuberculosis *M. a. avium* (serotypes 1, 2, and 3), which is fully virulent to birds and causes miliary tuberculosis, is a more consequential pathogen (Yoder and Schaefer, 1971; Piening et al., 1972; Pavlik et al., 2000; Dvorska et al., 2003, 2004). In contrast, the other serotypes 4 to 28 were classified in the species formerly designated as *M. intracellulare* (Wayne and Kubica, 1986), which comprises subspecies *M. a. hominissuis* (Mijs et al., 2002) with serotypes 4 to 6, 8 to 11 and 21 (Bartos et al., 2006). These members formerly classified in an intermediary group (Piening et al., 1972) only cause tuberculous lesions at the site of inoculation after intramuscular infection (Pavlik et al., 2000; Bartos et al., 2006). The members of the remaining serotypes 7, 12 to 20 and 22 to 28 classified as species *M. intracellulare* are almost non-virulent to birds (Bartos et al., 2006). These members were occasionally isolated from pigs in the Czech Republic (Pavlik et al., 2003b).

Among other subspecies classified in species *M. avium* (Thorel et al., 1990), which cannot be serotyped and are not *MAC* members, *M. a. paratuberculosis* was occasionally detected in domestic pigs in Sweden (Pavlik et al., 1999) and in the USA (Thoen et al., 1975; Songem et al., 1980), in wild boars (*Sus scrofa*) in the Czech Republic (Machackova et al., 2003; Trcka el al., 2006) and in Spain (Alvarez et al., 2005). *M. a. silvaticum*, formerly designated as "wood pigeon" strains (mycobactin dependent isolates virulent to birds and isolated mainly from wild pigeons) has not been detected in pigs yet.

The purpose of the present study was to analyse the reliability of respective diagnostic methods used in the investigation of sources of tuberculous/tuberculoid lesions in domestic pigs and also to investigate the distribution of respective mycobacterial species in various tissues (above all *MAC* members) and *R. equi* between the years 1996 and 2004.

MATERIAL AND METHODS

Between the years 1996 and 2004, tissues from a total of 3 630 slaughtered pigs (only one tissue from each pig was examined) were delivered to the National Referral Laboratory (NRL) in Prague. The annual numbers of examined animals ranged between 266 in 1998 and 922 in 1996. Among 3 552 lymph node (ln) samples from pigs the following were examined: head ln from 1 781 (50%) animals, pulmonary ln from 54 (2.0%) animals, mesenteric ln from 1 123 (32%) animals, inguinal ln from 32 (1%) animals and non-identified ln from 562 (16%) animals (Tables 1 and 2). Among the organ samples from 78 pigs, the following were examined: liver from 53 (68%), spleen from 12 (15%), lungs from 5 (6%), kidneys from 6 (8%) and pancreas from 2 (3%) animals (Table 2).

Gross examination and sample collection

Gross examination. During meat inspection after slaughter, gross examination for the presence of tuberculous/tuberculoid lesions in respective lymph nodes was performed. Samples of tissues with tuberculous/tuberculoid lesions and lymph nodes from pigs with a positive response to the tuberculin test with avian tuberculin AVITUBAL (Bioveta Ivanovice na Hane, Czech Republic; Pavlik et al., 2003b) were collected for laboratory testing. Animals were classified in 4 groups according to the results of gross examination (Table 3):

PA 0 (without gross lesions). The vaccination against avian tuberculosis/mycobacteriosis was undertaken to determine the actual status of the prevalence of the disease in pig farms.

PA 1 (adenopathy, usually with concurrent swollen lymph nodes as a consequence of effusion and congestion, pronounced trabeculae and developing lymphadenitis).

PA 2 (caseated tuberculous/tuberculoid lesions visible by non-aided eye).

PA 3 (calcified tuberculous/tuberculoid lesions visible by non-aided eye).

Sample collection. Samples were collected immediately after slaughter into sterile polyethylene bags and were delivered to the laboratory fresh or frozen (–20°C). They were kept frozen for up to 6 weeks before laboratory analysis.

Detection of mycobacteria

Microscopic examination. During the processing of samples for culture, imprint preparations were made and stained according to the ZN method and at least 100 microscopic fields were examined for AFR in each sample. Based on the results obtained by microscopy, the pigs were classified into four groups according to the amount of detected AFR (Table 3):

ZN 0 (AFR not detected)

ZN I (AFR detected occasionally – up to 10 AFR per 100 microscopic fields)

ZN II (few AFR detected – up to 100 AFR per 100 microscopic fields)

ZN III (frequently detected AFR – in each microscopic field at least one AFR detected)

Culture examination for mycobacteria. Approximately 1 g of tissue was homogenised with sterile sea sand in a mortar. The homogenate was processed with 1N HCl for 15 min and neutralised by 2N NaOH according to a previously described method (Fischer et al., 2000). One tissue sample was examined from each animal; no more than one isolate originated from each sample.

Identification of mycobacterial isolates

Accu-Probes (Gen-Probe Inc., San Diego, California, USA). Mycobacterial isolates were identified

Table 1. Mycobacteria isolation from lymph nodes and parenchymatous organs from 3 630 slaughtered pigs* between the years 1996 and 2004

Year	Total			Lymph nodes of														Parenchymatous organs**		
				head			lung			mesenterium			inguinum			non-identified					
	No.	+	%	No.	+	%	No.	+	%	No.	+	%	No.	+	%	No.	+	%	No.	+	%
Mycobacteria isolation																					
1996	922	439	48	318	137	43	9	5	56	365	188	52	2	0	0	213	102	48	15	7	47
1997	335	120	36	128	40	31	5	2	40	96	35	37	3	0	0	99	42	42	4	1	25
1998	266	109	41	99	39	39	3	1	33	83	36	43	0	0	0	74	31	42	7	2	29
1999	376	195	52	183	85	46	7	2	29	126	85	68	9	0	0	38	22	58	13	1	8
2000	290	138	48	174	67	39	5	2	40	92	60	65	3	0	0	8	4	50	8	5	63
2001	325	130	40	184	66	36	4	0	0	93	43	46	3	0	0	34	15	44	7	6	86
2002	316	120	38	186	52	28	7	0	0	76	42	55	3	0	0	36	21	58	8	5	63
2003	310	138	45	203	77	38	7	6	86	68	33	49	3	1	33	21	18	86	8	3	38
2004	490	190	39	306	84	28	7	1	14	124	79	64	6	0	0	39	22	56	8	4	50
Total	3 630	1 579	44	1 781	647	36	54	19	35	1 123	601	54	32	1	3	562	277	49	78	34	44
Rhodococcus equi isolation																					
1996	922	81	9	318	73	23	9	0	0	365	5	1	2	0	0	213	3	1	15	0	0
1997	335	26	8	128	22	17	5	0	0	96	1	1	3	0	0	99	3	3	4	0	0
1998	266	28	11	99	26	26	3	0	0	83	2	2	0	0	0	74	0	0	7	0	0
1999	376	65	17	183	55	30	7	0	0	126	6	5	9	0	0	38	4	11	13	0	0
2000	290	62	21	174	57	33	5	0	0	92	5	5	3	0	0	8	0	0	8	0	0
2001	325	78	24	184	76	41	4	0	0	93	0	0	3	0	0	34	2	6	7	0	0
2002	316	74	23	186	71	38	7	0	0	76	2	3	3	0	0	36	1	3	8	0	0
2003	310	83	27	203	80	39	7	0	0	68	0	0	3	1	33	21	3	14	8	0	0
2004	490	167	34	306	159	52	7	0	0	124	2	2	6	0	0	39	6	15	8	0	0
Total	3 630	664	18	1 781	619	35	54	0	0	1 123	23	2	32	1	3	562	22	4	78	0	0
Mycobacteria and *Rhodococcus equi* isolations***																					
1996	922	520	56	318	210	66	9	5	56	365	193	53	2	0	0	213	105	49	15	7	47
1997	335	146	44	128	62	48	5	2	40	96	36	38	3	0	0	99	45	46	4	1	25
1998	266	137	52	99	65	66	3	1	33	83	38	46	0	0	0	74	31	42	7	2	29
1999	376	260	69	183	140	77	7	2	29	126	91	72	9	0	0	38	26	68	13	1	8
2000	290	200	69	174	124	71	5	2	40	92	65	71	3	0	0	8	4	50	8	5	63
2001	325	208	64	184	142	77	4	0	0	93	43	46	3	0	0	34	17	50	7	6	86
2002	316	194	61	186	123	66	7	0	0	76	44	58	3	0	0	36	22	61	8	5	63
2003	310	221	71	203	157	77	7	6	86	68	33	49	3	1	33	21	21	100	8	3	38
2004	490	357	73	306	243	79	7	1	14	124	81	65	6	0	0	39	28	72	8	4	50
Total	3 630	2 243	62	1 781	1 266	71	54	19	35	1 123	624	56	32	1	3	562	299	53	78	34	44

*each sample originated from one animal; **parenchymatous organs include liver (n = 53), spleen (n = 12), lung (n = 5), kidney (n = 6) and pancreas (n = 2); ***in some animals dual infection with both mycobacteria and *Rhodococcus equi* was diagnosed; + = positive results of examinations (isolation of mycobacteria and/or *Rhodococcus equi*)

by Accu-Probes using a *MTC* probe for the detection of all *MTC* species, the following probes were used for the detection of *MAC* species: *MAC* probe for the detection of isolates of all 28 serotypes, *MA* probe for the detection of isolates of serotypes 1 to 6, 8 to 11, and 21, also the *MI* probe for the detection of isolates of the remaining serotypes 7, 12 to 20, and 22 to 28 (Bartos et al., 2006).

PCR. Primers 5´-GCA ACG GTT GTT GCT TGA AA-3´ and 5´-TGA TAC GGC CGG AAT CGC GT-3´ were used for the detection of IS*901* in all *M. avium* isolates (Kunze et al., 1992; Bartos et al., 2006).

Serotyping of *MAC* isolates. Most of the *MAC* isolates were examined by serotyping (Wolinsky and Schaefer, 1973), modified by Sussland and Hrdinova (1976).

Biochemical identification tests. Other mycobacterial species were identified biochemically where negative results were obtained by the above described methods (Wayne and Kubica, 1986).

Final identification. Based on these examinations, the isolates were identified as follows:

MAA: *M. a. avium*: Accu-Probes *MAC*+, *MA*+, and *MI*–; IS*901*+; serotypes 1, 2 and 3

MAH: *M. a. hominissuis*: Accu-Probes *MAC*+, *MA*+, and *MI*–; IS*901*–; serotypes 4–6, 8–11 and 21

MI: *M. intracellulare*: Accu-Probes *MAC*+, *MA*–, and *MI*+; IS*901*–; serotypes 7, 11–20 and 22–28

MAC: includes *MAA*, *MAH*, and *MI* isolates

Based on the previously described method (Pavlik et al., 2003b; 2005a), the ratio *MAA*/*MAH* was calculated for the infection of respective organs. In the cases of mixed infections, the infection caused by more consequential *MAC* members was taken into consideration: in animals affected with mixed infection caused by "*MAA + MAH*", "*MAA + MI*" or "*MAA + MAH + MI*", the *MAA* infection was taken into consideration, in animals affected with mixed infection caused by "*MAH + MI*", the *MAH* infection was taken into consideration (Table 2).

Rhodococcus equi detection

Prior to the decontamination of the homogenised lymph node samples were aseptically collected and cultured on blood agar supplemented with 5% of ram blood at 37°C for 48 hours. The *R. equi* isolates were detected on blood agar or on solid egg media for mycobacterial isolation (after the decontamination prior to the mycobacteria culture) and identified by biochemical tests (Goodfelow, 1986; Dvorska et al., 1999).

Statistical assessment

The chi^2-test (Stat Plus) was applied for the statistical evaluation of results (Matouskova et al., 1992).

RESULTS

Mycobacteria isolations

Examined tissue samples from different tissues. Mycobacteria were detected by culture in tissues from 1 579 (44%) of 3 630 animals. Culture positivity in respective years varied between 36% in 1997 and 52% in 1999. Culture positivity in 1999 was significantly increased ($P < 0.01$) in comparison with the years 1997, 1998, 2001, 2002 and 2004 (Table 1).

Examined tissue samples from lymph nodes (Table 1). The following positivity for mycobacteria was demonstrated in respective groups of lymph nodes:

(a) Mycobacteria were isolated from head ln samples from 647 (36%) of 1 781 animals. Culture positivity was significantly higher ($P < 0.01$) in 1999 than in 1997, 2002 and 2004.

(b) Mycobacteria were isolated from lung ln samples from 19 (35%) of 54 animals. Due to a low number of examined pigs (between 3 and 9 annually) statistical evaluation was not possible.

(c) Mycobacteria were isolated from ln samples from 601 (54%) of 1 123 animals. Culture positivity was significantly higher ($P < 0.01$) in 1999 than in 1996, 1997, 1998, 2001 and 2003.

(d) Mycobacteria were isolated from inguinal ln samples from 1 (3%) of 32 pigs. Due to a low number (between 0 and 9 annually) of examined pigs statistical evaluation was not possible.

(e) Mycobacteria were isolated from ln samples of unknown origin from 277 (49%) of 562 animals. Culture positivity was significantly higher ($P < 0.01$) in 2003 than in 1996, 1997, 1998 and 2001.

Culture positivity in mesenteric lymph nodes (54%) was significantly higher ($P < 0.01$) than in head (36%) and lung (35%) lymph nodes. Mycobacteria were detected in only 3% of inguinal lymph nodes,

Table 2. Distribution of different mycobacterial species in tissues originating from 3 630 pigs

Identified mycobacterial isolates			Examined lymph nodes (total No.)						Parenchymatous organs (total No.)					
			head	lung	mesenteric	inguinal	Uk	total	liver	spleen	lung	kidney	pancreas	total
Species	No.	%	(1 781)	(54)	(1 123)	(32)	(562)	(3 552)	(53)	(12)	(5)	(6)	(2)	(78)
MAA serotype 2	421		127	6	170	1	97	401	18	2	0	0	0	20
serotype 3	16		4	1	5	0	4	14	2	0	0	0	0	2
non-serotyped[a]	32		15	0	6	0	11	32	0	0	0	0	0	0
Subtotal	469	29.7	146	7	181	1	112	447	20	2	0	0	0	22
MAH serotype 4	1		1	0	0	0	0	1	0	0	0	0	0	0
serotype 6	0		0	0	0	0	0	0	0	0	0	0	0	0
serotype 8	643		290	6	256	0	85	637	6	0	0	0	0	6
serotype 9	74		26	0	37	0	9	72	1	1	0	0	0	2
mixed or non-serotyped[a]	173		82	2	64	0	25	173	0	0	0	0	0	0
Subtotal	891	56.4	399	8	357	0	119	883	7	1	0	0	0	8
MI non-serotyped[a]	17	1.1	4	1	4	0	8	17	0	0	0	0	0	0
Mixed: *MAA+MAH*	53		18	3	17	0	14	52	1	0	0	0	0	1
MAA+MI	24		7	0	10	0	6	23	1	0	0	0	0	1
MAH+MI	38		12	0	19	0	5	36	1	1	0	0	0	2
MAA+MAH+MI	1		0	0	1	0	0	1	0	0	0	0	0	0
Subtotal	116	7.8	37	3	47	0	25	112	3	1	0	0	0	4
MAC Subtotal	1 493	94.6	586	19	589	1	264	1 459	30	4	0	0	0	34
MAA/MAH (No.)	547/983		171/429	10/11	209/394	1/0	132/138	523/972	22/9	2/2	0/0	0/0	0/0	24/11
(ratio)[b]	0.6		0.4	1.0	0.5	0	1.0	0.5	2.4	1.0	0	0	0	2.2
M. chelonae	35	2.2	27	0	4	0	4	35	0	0	0	0	0	0
M. smegmatis	4	0.3	2	0	1	0	1	4	0	0	0	0	0	0
M. terrae	7	0.4	5	0	1	0	1	7	0	0	0	0	0	0
M. aurum	1	0.1	1	0	0	0	0	1	0	0	0	0	0	0
M. xenopi	3	0.2	0	0	3	0	0	3	0	0	0	0	0	0
M. scrofulaceum	1	0.1	0	0	1	0	0	1	0	0	0	0	0	0
M. fortuitum	1	0.1	1		0	0	0	1	0	0	0	0	0	0
M. species[c]	34	2.2	25	0	2	0	7	34	0	0	0	0	0	0
Subtotal	86	5.4	61	0	12	0	13	86	0	0	0	0	0	0
Total	1 579	100	647	19	601	1	277	1 545	30	4	0	0	0	34
%	100		41.0	1.2	38.1	0.1	17.5	97.8	1.9	0.3	0	0	0	2.2

[a]non-serotyped isolates; [b]ratio of *MAA* and *MAH* (for more details see chapter Material and Methods); [c]*Mycobacterium* species; Uk = unknown tissues which were examined; *MAA = Mycobacterium avium* subsp. *avium,* Accu Probe *MAC*+, *MA*+ and *MI*–, IS*901*+, serotypes 1, 2 and 3; *MAH = Mycobacterium avium* subsp. *hominissuis,* Accu Probe *MAC*+, *MA*+ and *MI*–, IS*901*–, serotypes 4–6, 8–11 and 21; *MI = Mycobacterium intracellulare,* Accu Probe *MAC*+, *MA*– and *MI*+; IS*901*–; serotypes 7, 11–20 and 22–28; *MAC = Mycobacterium avium* complex includes *MAA*, *MAH*, and *MI* isolates

which was significantly less ($P < 0.01$) than in the other lymph nodes (Table 1).

Examined parenchymatous organs (Table 1). Parenchymatous organs from 78 animals were examined and mycobacteria were isolated from 34 (44%) of them. Due to a low number of examined pigs (4 to 15 annually), statistical evaluation was not possible. No significant differences were found between the detection rate of mycobacteria in parenchymatous organs and in various lymph nodes. The detection rate of mycobacteria was significantly higher ($P < 0.01$) in parenchymatous organs in comparison with only inguinal lymph nodes.

Gross examination and isolation of mycobacteria

Generalised or miliary forms of tuberculosis was not detected in any of 3 630 tested animals; tissues free from gross lesions (group PA 0) were found in 249 (7%) animals, tissues with adenopathy (group PA 1) in 150 (4%) animals, tissues with caseated tuberculous lesions (group PA 2) in 2 026 (56%) animals and tissues with calcified tuberculous lesions (group PA 3) in 1 205 (33%) animals. The highest detection rate of mycobacteria (57%) determined by culture was recorded in group PA 3. That was significantly higher ($P < 0.01$) than in the remaining groups. The second highest culture positivity was found in group PA 2 (39%). That was significantly higher ($P < 0.01$) and ($P < 0.05$) in comparison with groups PA 0 and PA 1, respectively (Table 3).

Gross examination and AFR detection

AFR were found in ZN stained tissues from 36 (15%) animals of group PA 0, 28 (19%) animals of group PA 1, 801 (40%) animals of group PA 2 and 913 (76%) animals of group PA 3. The difference in frequencies of animals with AFR detected in the tissues were always significant ($P < 0.01$) between all four groups from PA 0 to PA 3. No significant differences were found between groups PA 0 and PA 1. A rather high number of detected AFR (animal groups ZN II and ZN III) were significantly more frequently ($P < 0.01$) recorded in samples of tissues with tuberculous lesions (animal groups PA 2 and 3) in comparison with samples from animals showing adenopathy (animal group PA 1) and animals without gross lesions (animal group PA 0). The differences were significant ($P < 0.05$) in two groups: PA 2 and PA 1 (Table 3).

Gross examination and mycobacterial species detection

***MTC* members** (Table 3). *MTC* members were not detected in any of examined tissue samples during the whole study period.

***MAC* members** (Table 3). *MAC* members were significantly ($P < 0.01$) most frequently detected in all four groups classified according to gross lesion incidence (PA 0 to 3). Among them, *M. a. avium* was significantly ($P < 0.01$) more frequently isolated from lymph nodes of animals without gross lesions (group PA 0) than from the remaining three groups (PA 1, 2, and 3) with gross lesions.

Conditionally pathogenic mycobacteria (Table 3). Among conditionally pathogenic mycobacteria, *M. chelonae* (35 isolates) was most frequently isolated. It was detected in 0.5 to 1.5% of animals from all four groups (PA 0 to PA 3). Among the other conditionally pathogenic mycobacteria, species *M. smegmatis*, *M. xenopi*, *M. terrae*, *M. aurum*, *M. scrofulaceum* and *M. fortuitum* were occasionally isolated. It was not possible to biochemically identify 34 mycobacterial isolates that were negative by Accu-Probes and did not contain IS*901*; their occurrence was only occasional.

Microscopic detection of AFR and detection of mycobacterial species

***MAC* members** (Table 3). *MAC* members were detected in 16% of animals in the group without AFR (ZN 0). Mycobacteria were detected in 69%, 83%, and 74% of animals in groups ZN I, ZN II and ZN III, respectively. Differences between culture detection of *MAC* in samples with AFR (animal groups ZN I, ZN II and ZN III) and the group without detected AFR (ZN 0) were significant ($P < 0.01$).

M. a. avium (Table 3). The detection rates of the causative agent of avian tuberculosis *M. a. avium* were gradually increasing in respective groups of animals from 18% in group ZN 0 to 46% in group ZN III; the differences were significant ($P < 0.01$). Differences between groups ZN II and ZN III were non-significant. A comparable gradual increase in the detection rates was recorded in the group of

Table 3. Gross and laboratory (direct microscopy and culture) examinations of tissue samples from 3 630 pigs

Examination		Animals			*MAA*		*MAH*		*MI*		Mixed		*M. che-lonae*	*M. smeg-matis*	*M. xenopi*	*M. terrae*	*M. aurum*	*M. scrofu-laceum*	*M. fortu-itum*	*M.* species	*R. equi*	
PA[a]	ZN[b]	No.	+	%	+	%	+	%	+	%	+	%									+	%
0	0	213	33	16	15	46	15	46	0	0	0	0	2	0	0	0	0	0	1	0	7	3
	I	31	18	58	12	67	5	28	1	3	0	0	0	0	0	0	0	0	0	0	1	3
	II	5	3	60	3	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	III	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Subtotal		249	54	22	30	56	20	37	1	1	0	0	0	0	0	0	0	0	1	0	8	3
1	0	122	23	19	7	30	14	61	0	0	0	0	1	1	0	0	0	0	0	0	6	5
	I	22	17	77	5	29	10	59	1	5	0	0	0	0	0	0	0	0	0	1	1	5
	II	5	4	80	2	50	2	50	0	0	0	0	0	0	0	0	0	0	0	0	2	40
	III	1	1	100	0	0	1	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Subtotal		150	45	30	14	31	27	60	1	1	0	0	1	1	0	0	0	0	0	1	9	6
2	0	1 225	189	15	19	10	64	34	6	1	55	5	22	1	2	4	1	1	0	14	564	46
	I	615	450	73	106	24	294	65	3	1	39	6	1	0	0	0	0	0	0	7	31	5
	II	156	137	88	45	33	88	64	2	1	0	0	1	1	0	0	0	0	0	0	6	4
	III	30	17	57	6	35	9	53	0	0	0	0	1	0	0	1	0	0	0	0	1	3
Subtotal		2 026	793	39	176	22	455	57	11	1	94	5	25	2	2	5	1	1	0	21	602	30
3	0	292	44	15	11	25	22	50	3	1	3	1	4	0	0	1	0	0	0	0	24	8
	I	638	418	66	142	34	250	60	1	1	12	2	2	1	1	1	0	0	0	8	17	3
	II	244	197	81	81	41	104	53	0	0	7	3	1	0	0	0	0	0	0	4	4	2
	III	31	28	90	15	54	13	46	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Subtotal		1 205	687	57	249	36	389	57	4	1	22	1	7	1	1	2	0	0	0	12	45	4
0 to 3	0	1 852	289	16	52	18	115	40	9	1	58	3	29	2	2	5	1	1	1	14	601	33
	I	1 306	903	69	265	29	559	62	6	1	51	4	3	1	1	1	0	0	0	16	50	4
	II	410	341	83	131	38	194	57	2	1	7	2	2	1	0	0	0	0	0	4	12	3
	III	62	46	74	21	46	23	50	0	0	0	0	1	0	0	1	0	0	0	0	1	2
Total		3 630	1 579	44	469	30	891	56	17	1	116	3	35	4	3	7	1	1	1	34	664	18.3

[a]gross examination: 0 (no gross lesions were found), 1, 2 and 3 (tuberculoid/tuberculous lesions were found): 1 (adenopathy), 2 (caseation) and 3 (calcification); [b]staining according to Ziehl-Neelsen (ZN) for the detection of acid fast rods (AFR) was carried out: 0 (AFR not detected), I (AFR detected occasionally – up to 10 AFR per 100 microscopic fields), II (few AFR detected – up to 100 AFR per 100 microscopic fields), III (frequently detected AFR – in each microscopic field at least one AFR detected); [c]IS*6110*/IS*901* PCR and Accu-Probes with *M. avium* complex, *M. avium* and *M. intracellulare* probes negative biochemically, not identified mycobacterial isolates confirmed by ZN staining; *MAA* = *M. a. avium*: Accu-Probe *MAC*+, *MA*+ and *MI*–, IS*901*+, serotypes 1, 2 and 3; *MAH* = *M. a. hominissuis*: Accu-Probes *MAC*+, *MA*+ and *MI*–, IS*901*–, serotypes 4–6, 8–11 and 21; *MI* = *M. intracellulare*: Accu Probe *MAC*+, *MA*– and *MI*+, IS*901*–, serotypes 7, 11–20 and 22–28. Mixed includes *MAA*, *MAH* and *MI* isolates

animals with caseated tuberculous lesions (PA 2) where the detection rate of *M. a. avium* isolates was gradually increasing with increasing numbers of detected AFR (groups ZN 0 to ZN III).

M. a. hominissuis (Table 3). In contrast, the causative agent of avian mycobacteriosis *M. a. hominissuis* was detected in all three groups of animals (ZN I to ZN III) with detected AFR. These ranged between 50 and 62% in comparison with ZN 0 without detected AFR where the positivity was 40% only; differences between this group and groups ZN I and ZN II were significant ($P < 0.01$).

Of 1 579 mycobacterial isolates, 1 493 (94.6%) were the following *MAC* members:

469 (29.7%) *M. a. avium* (IS*901*+, serotypes 1, 2 and 3)

891 (56.4%) *M. a. hominissuis* (IS*901*–) of serotypes 4 ($n = 1$), 8 ($n = 643$), 9 ($n = 74$) and 173 non-serotyped isolates

17 (1.1%) *M. intracellulare*

The remaining 52 (3.3%) isolates were other mycobacterial species: *M. chelonae* ($n = 35$), *M. smegmatis* ($n = 4$), *M. xenopi* ($n = 3$), *M. terrae* $n = 7$), *M. aurum* ($n = 1$), *M. scrofulaceum* ($n = 1$), *M. fortuitum* ($n = 1$) and biochemically non-identified mycobacteria ($n = 34$; Table 2).

R. equi isolation

Examined tissue samples from different organs. *R. equi* was detected in tissues from 664 (18%) of 3 630 animals. In respective years, culture positivity ranged between 8% in 1997 and 34% in 2004. Culture positivity between 2001 and 2004 was significantly higher ($P < 0.01$) than between 1996 and 1998 (Table 1).

Gross examination and *R. equi* isolation. *R. equi* was isolated from tissues of 8 (3%), 9 (6%), 602 (30%) and 45 (4%) animals from groups PA 0, PA 1, PA 2 and PA 3, respectively. The detection rate of *R. equi* was the highest in group PA 2; it was significantly higher ($P < 0.01$) in comparison with the remaining three groups (Table 3).

AFR detection and *R. equi* isolation. *R. equi* was isolated significantly more frequently ($P < 0.01$) from tissues without detected AFR (group ZN 0; positivity 33%) in comparison to animals with detected AFR (groups ZN I, ZN II and ZN III: 4%, 3% and 2% positivity, respectively). *R. equi* was significantly more frequently ($P < 0.01$) isolated from the animal group ZN 0 without detected AFR with caseated/tuberculoid lesions (PA 2) that reached 46% in comparison to all the remaining groups of animals ZN I to ZN III. Comparable differences between ZN 0 and the three groups of animals ZN I to ZN III were found for the group of animals PA 3 (Table 3).

Detection of *R. equi* from different organs

Lymph nodes (Table 1). *R. equi* was isolated from 619 (35%) of 1 781 animals whose head ln were examined. Culture positivity detected between 2001 and 2004 was significantly higher ($P < 0.01$) than between 1996 and 1997. Different positivity for *R. equi* was detected in respective groups of lymph nodes (Table 1). Culture positivity in head lymph nodes (35%) was significantly higher ($P < 0.01$) in comparison with all the other lymph nodes.

Parenchymatous organs (Table 1). *R. equi* was not isolated from any of parenchymatous organs from 78 animals.

Mixed infection with mycobacteria and *R. equi*

Mycobacteria and *R. equi* were detected together by culture in tissues from 2 243 (62%) of 3 630 animals. Culture positivity in respective years varied between 44% in 1997 and 73% in 2004. Culture positivity was significantly lower ($P < 0.01$) in 1997 than in all the remaining years except for 1998 (Table 1).

Culture positivity in head lymph nodes (71%) was significantly increased ($P < 0.01$) in comparison with all the other ln. In contrast, the detection rate in inguinal lymph nodes was only 3%; that was significantly lower ($P < 0.01$) than in all the other lymph nodes (Table 1).

Mycobacteria and *R. equi* were together isolated from 34 (44%) of 78 animals whose parenchymatous organs were examined. That is significantly less ($P < 0.01$) than from head lymph nodes; however, it is significantly more ($P < 0.01$) than from inguinal lymph nodes.

DISCUSSION

The prevalence of avian tuberculosis or mycobacteriosis in pigs is assumed to be related to direct or indirect exposure to infected animals, birds and/or contaminated feed. In the Czech Republic since the early 1990s the epizootiological dynamics corre-

sponding to the observation of tuberculous lesions in pigs is variable (Pavlik et al., 2003b). The main reasons were:

(1) Transformation of the agricultural system connected with the impaired standard of animal hygiene and the frequent contact of domestic pigs with game birds.
(2) Application of different bedding materials.
(3) Widely used peat as a feed supplement.

Above all, using peat as a feed supplement for piglets in the end of 1998 and in 1999 resulted in a significant increase ($P < 0.01$) in the prevalence of tuberculous lesions in slaughtered pigs in 1999 compared with the years 1997, 1998, 2001, 2002 and 2004.

Our retrospective study in the years 1996 to 2004 showed that 1 579 (44%) and 664 (18%) of the examined pigs were found to be infected with the *MAC* members and *R. equi* isolates respectively (Table 1). Both agents were predominantly found in the head and mesenteric lymph nodes; it follows that the route of mycobacterial and *R. equi* infections of pigs is the digestive tract, as mycobacteria penetrate there after ingestion of contaminated feed or bedding materials (Dvorska et al., 1999; Matlova et al., 2003, 2004a,b). However, pigs themselves could be the source of infection, as they are shedding mycobacteria through faeces (Nasal et al., 1974; Matlova et al., 2003). Despite this, contamination of peat and bedding materials could have occurred during transportation and storage that resulted from contact with potentially infected free living birds and small terrestrial mammals (Fischer et al., 2000; Pavlik et al., 2000).

This long-term analysis showed a significant and high prevalence of avian tuberculosis and mycobacteriosis in the pig farm industry. A wide use of sawdust litter may also be an important risk factor, as it allows multiplication of mycobacteria (Kazda, 2000). Hence, constant exposure of pigs to contaminated litter both before and after weaning may account for the high prevalence of mycobacterial lesions (Rezinkov et al., 1971; Matlova et al., 2004b).

M. a. hominissuis (56.4%) and *M. a. avium* (29.7%) were the major dominant species, whilst *M. intracellulare* (1.1%) has been rarely detected (Table 2); this may show that *M. intracellulare* is not virulent to pigs. However, it was identified together with both species. In comparison with the head and mesenteric lymph nodes, even with the parenchymatous organs, the detection rate of *MAC* isolates from the lung and inguinal lymph nodes was significantly ($P < 0.01$) lower.

Other isolated conditional pathogenic mycobacterial species such as *M. chelonae, M. smegmatis, M. terrae, M. xenopi, M. aurum, M. scrofulaceum* and *M. fortuitum* may affect the health status of animals, in particular, during unfavourable conditions (Wayne and Kubica, 1986). *M. a. avium* was most commonly isolated from birds and cattle (Pavlik et al., 2000, 2002d, 2005a,b; Dvorska et al., 2004), however, in our analysis of mycobacterial infection in pigs, it was found to share a considerable figure with *M. a. hominissuis* in causing tuberculous lesions.

The frequent detection of *MAC* members and atypical mycobacteria from pigs implies that mycobacterial infection was contracted from contaminated external and stable environments and different contaminated feed supplements (Windsor et al., 1984; Gardner and Hird, 1989; Horvathova et al., 1997; Durrling et al., 1998; Pavlik et al., 1999, 2000; Matlova et al., 2003, 2004a, 2005; Trckova et al., 2004).

Furthermore, recent molecular studies showed that humans can acquire the *MAC* infection from pigs or both may be infected from a similar environment (Komijn et al., 1999; Pavlik et al., 2000; Dvorska et al., 2002). This report and our result bring to surface the importance of avian tuberculosis and mycobacteriosis in pigs in terms of economy and epidemiology.

Identification of *MAC* isolates predominantly showed the presence of *M. a. hominissuis* isolates of serotypes 8 and 9. This is evident that *M. a. hominissuis* species are the most common causes of tuberculous lesions particularly in pigs (Windsor et al., 1984; Alfredsen and Skjerve, 1993; Nishimori et al., 1995; Dvorska et al., 1999; Komijn et al., 1999; Pavlik et al., 2000, 2003b). Tuberculous lesions caused by *M. a. avium* and even *R. equi* have been detected by other authors (Alfredsen and Skjerve, 1993; Dvorska et al., 1999).

No isolates of serotype 1 of *M. a. avium* and isolates of serotype 6 of *M. a. hominissuis* have been isolated; this may be associated with the low virulence of a respective serotype to pigs or with their absence in the environment and/or in host organisms.

Soil contaminated with horse faeces is thought to be the most common source for *R. equi* bacteria (Takai and Tsubaki, 1985; Prescott, 1991; Dvorska et al., 1999). The population of horses in the Czech Republic has decreased (O.I.E., 1999). In contrast, however, *R. equi* was isolated from lymph nodes of pigs with an increasing trend especially from the

year 2002 to 2004. This may be due to the capacity of the *R. equi* to survive for a long time in the contaminated soil and other environmental sources and/or affiliating itself to other susceptible hosts. For example, *R. equi* has also been isolated from cattle, though it was rare compared to pigs (Dvorska et al., 1999; Pavlik et al., 2003a,b) and it was able to cause formation of tuberculoid lesions. In both species (cattle and pigs), cervical lymphadenitis can be caused by the host acquiring the pathogen from the soil (Quinn et al., 1994).

Unlike the *MAC* isolates, *R. equi* species has not been detected in parenchymatous organs, in which *R. equi* may limit itself to the intestine, adjacent lymph nodes in pigs (Takai and Tsubaki, 1985; Prescott, 1991) and the head lymph nodes as the predilection site.

Culture was found to be the reliable method for making a definitive diagnosis in the examined samples. However, the acid fast staining (ZN) technique has also showed (48%) maximum agreement with the culture (44%) which has also been significantly valuable. The tuberculous/tuberculoid lesions were observed in 93% of examined tissues which were found to be less sensitive in the detection of the mycobacterial lesions. Development of granulomas in animals is related to the reaction of the immune response of the host against infections, which can even be of non mycobacterial origin (Takai, 1997). Hence, this finding strengthens our suggestion that based on pathological lesions a presumptive diagnosis can be done, if not otherwise making a definitive diagnosis remains troublesome.

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