Distribution of *Mycobacterium avium* subsp. *paratuberculosis* in tissues of naturally infected cattle as affected by age

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ABSTRACT: The objectives of this study were to investigate *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) distribution in various tissues, in faeces and in milk samples from 131 animals originating from one imported Jersey cattle herd from Denmark. *MAP* was detected by culture in 37.4% animals. Massive *MAP* growth was most often observed in the small intestines (48 animals). The lowest levels of *MAP* were found in spleen and mammary gland samples. *MAP* was detected in the faeces of 8.4% animals; however, milk samples were *MAP* negative by culture. The highest prevalence of *MAP* infection (42.9%) was in age Group B (1.6 to 3 years) and the lowest (6.1%) in group D (more than 8 years). Seven positive calves were detected in the youngest age group; one of them was less than one month of age, which could imply an intrauterine infection. *MAP* shedding in faeces by a five-month-old calf was also confirmed. The IS*900* RFLP (restriction fragment length polymorphism) type B-C1 was identified in all animals.

Keywords: Johne's disease; food safety; age groups

Mycobacterium avium subsp. *paratuberculosis* (*MAP*), the causative agent of paratuberculosis (Johne's disease), causes huge economic losses all over the world, primarily to dairy farmers (Hasonova and Pavlik, 2006).

In ruminants, the gastrointestinal tract is the primary site of infection, in particular the mucosa of the ileo-caecal junction including the adjacent mesenteric lymph nodes (Sweeney et al., 1992a,b, 2006; Rideout et al., 2003; Amemori et al., 2004; Antognoli et al., 2008). However, *MAP* dissemination to different extraintestinal tissues has been observed in some infected animals. In cows, *MAP* has been detected in the uterus (Sweeney et al., 1992b, 1996), supramammary lymph nodes and mammary parenchyma (Sweeney et al., 1992a, 1996; Antognoli et al., 2008; Brady et al., 2008). *MAP* identification in milk has been linked with developed disseminated *MAP* infection (Slana et al., 2008a). In bulls, *MAP* has been shown to be present in different parts of the reproductive organs and in sperm (Sweeney, 1996; Ayele et al., 2004; Buergelt et al., 2004). *MAP* has also been identified in the tonsils, lung, heart, liver, spleen, and kidneys of cattle (Pavlik et al., 2000a; Sweeney et al., 2006; Antognoli et al., 2008).

As *MAP* has been isolated from the uterus and amniotic fluid of red deer (Kopecna et al., 2008), and from the foetuses of infected cows (Seitz et al., 1989; Sweeney, et al., 1992b, 1996), vertical transmission *in utero* is a known fact (Buergelt et al., 2006; Whittington and Windsor, 2009). Clinical

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cases of paratuberculosis (PTB) are mostly connected with dissemination of *MAP*. Disseminations in extraintestinal tissues in subclinical cases of PTB have not been documented as well as clinical cases (Antognoli et al., 2008). Disseminated disease develops at terminal stages of infection as a result of uncontrolled multiplication and spread of *MAP* in the host organism due to its inability to react against mycobacterial antigens (Chiodini, 1996; Rideout et al., 2003). The probability of dissemination increases with the intensity of infection (Sweeney, 1996). Haematogenous or lymphatic spreads are potential routes for movement of *MAP* to extraintestinal tissues (Whittington and Windsor, 2009).

Until 2008, a PTB cattle control programme was in force in the Czech Republic. Between 1998 and 2008, it was required that the faeces of all animals older than 18 months be culture examined for the presence of *MAP* twice a year (Pavlik et al., 2000b; Anonymous, 2001). In the case of PTB control failure, the entire herd demonstrating high prevalence and clinical signs (> 10% of animals per year) is eradicated. This scenario occurred in the herd reported in this study. The herd of Jersey cattle (closed herd), including late-pregnant heifers from 29 farms, was imported from Denmark in 1994. Serological examination during the quarantine period and in the following two years did not reveal any signs of disease. Eight years after their import, PTB was first revealed in one clinically ill cow. As the *MAP* prevalence in the herd was higher than 15%, the whole herd was slaughtered.

The purpose of the present study (based on culture examinations) was to ascertain the prevalence of *MAP* infection in different age groups of an infected cattle herd and to assess the distribution of *MAP* in various tissues, including its shedding in faeces and milk.

MATERIAL AND METHODS

Examined samples (gastrointestinal tract, respiratory lymph nodes, reproductive organs, mammary tissue and the milk of lactating cows) originated from 131 animals born since 1992. The intestines and livers of 36 foetuses originating from 34 cows (twins in two cases) were investigated. Samples were collected in a slaughterhouse and frozen at –20°C.

Animals (except foetuses) of four age groups (A, B, C and D) were used:

Group A: animals until 1.5 years; total: 44 animals including 32 calves (culture examination of faeces from this age group is not required by law);

Group B: 1.6 to three years; total: 30 animals (animals at the first production period);

Group C: 3.1 to eight years; total: 45 animals (the period of the highest production);

Group D: more than eight years; total: 12 animals (three of which were older than 12 years) including five from the original import from Denmark.

Tissue, faecal and milk samples were laboratory examined according to Pavlik et al. (2000a) and Kopecna et al. (2008). Briefly: Each tissue sample (about 1 g) was homogenised and decontaminated in 0.75% HPC (Hexadecylpyridinium chloride, Merck KGaA, Darmstadt, Germany) for 72 h. Faecal samples (approximately 1 g) were transferred into a 50 ml flask containing 30 ml of sterile distilled water and agitated in a horizontal shaker for 30 min. The sample flask was then left at room temperature for 30 min. Five ml of the supernatant was transferred into a 25 ml of 0.9% HPC and decontaminated for 72 h at room temperature. Samples of milk were centrifuged for 15 min at 2 500 rpm and then 5 ml of 0.75% HPC was added to the pellet. The sample was left undisturbed for 4 h at room temperature and then centrifuged for 15 min at 2 500 rpm. The pellet was then diluted in 1 ml of sterile distilled water. All tissue, faecal and milk samples were cultured on three slopes of different Herrold's egg yolk media (HEYM) and incubated at 37°C for three months.

Colony forming unit (CFU) counts were determined as the mean CFU of the three HEYM media used for the culture of one sample. The intensity of infection was described as: slight (+; less than 11 CFU), moderate (++; from 11–30 CFU) and massive (+++; more than 30 CFU per sample). After Ziehl-Neelsen (Z-N) staining, one randomly selected tissue isolate from each animal was examined using RFLP method (Pavlik et al., 1999; Moravkova et al., 2008).

For the statistical analysis of data, the GraphPad Prism 5.0 programme for Windows (GraphPad software, Inc., San Diego, CA, USA) was used. The purpose of the analysis was to assess whether the age of animals was a significant source of variability in *MAP* prevalence. Shapiro-Wilk's normality test was used for verification of data normality. The statistical significance of age effect on *MAP* prevalence was tested by means of the Kruskal-Wallis test. Subsequently, the statistical significance of differences between respective age groups of animals was tested by means of Dunn's post-test.

RESULTS

Faecal and milk samples

Out of 49 infected animals, *MAP* was isolated from the faeces of only 11 animals (22.4%). Slight shedding of *MAP* was observed in eight (72.7%) animals from the Groups A, B and C, while massive shedding was only detected in one animal from Group C (9.1%). All the animals from Group D, which comprised of the oldest animals, were classified as not shedding. Milk samples from 69 lactating cows were negative by cultivation (Tables 1 and 2).

Tissue samples

In each positive animal, *MAP* was presented in at least one tissue sample. In 77.6% of animals *MAP* was detected solely in tissue. The highest rate of *MAP* occurrence in tissue was seen in the B (21; 42.9%) and C (18; 36.7%) age groups. Seven (14.3%) and three (6.1%) animals from Groups A and D respectively, showed *MAP* in their tissues (Tables 1 and 2). Massive infections (+++) were most commonly demonstrated in Groups B (47.6% of animals) and C (55.6% of animals; Figure 1). The small intestine was the most frequent source of *MAP* (98.0% of animals with positive tissues), including most of the massive infections (38.8% of animals), as shown in Tables 1 and 2 and Figure 2. In contrast, spleen and mammary tissues yielded low numbers of *MAP*. From the bulk of examined tissues, only the small intestines and faeces were positive in the A Group (Table 1).

Foetuses

Of a total of 36 examined foetuses, 18 originated from 16 infected cows (two cows gave birth to twins) and another 18 originated from 18 cows with negative tissues and faeces cultivation. *MAP* was isolated from two foetuses of two infected dams. *MAP* was isolated from both the liver and intestinal mucosa of one of the foetuses (Table 3). This foetus originated from a Group B mother that had developed massive infection in many tissues (Table 1). It is noteworthy that *MAP* infection was also detected in another offspring in the history of this cow (unpublished data).

The frequency of *MAP* positive results obtained by cultivation of faecal, milk, tissue and foetus samples with regard to the age of examined animals are evaluated in Table 4. A significant difference was detected between the B and D (++, $P < 0.01$), A and B (+, $P < 0.05$) and C and D (+, $P < 0.05$) age groups. The difference between B and C Groups was not significant. In all tested cows, RFLP type B-C1 was identified.

DISCUSSION

The age of animals and culture detection of *MAP*

PTB infection is usually detected only in part of a herd (Chiodini, 1996). Thus it is likely that a majority of the non-infected animals can eliminate *MAP* from their tissues. In the present study, 14.3% of the youngest animals (until 1.5 years) were positive by culture. Slight *MAP* capture (mucosa

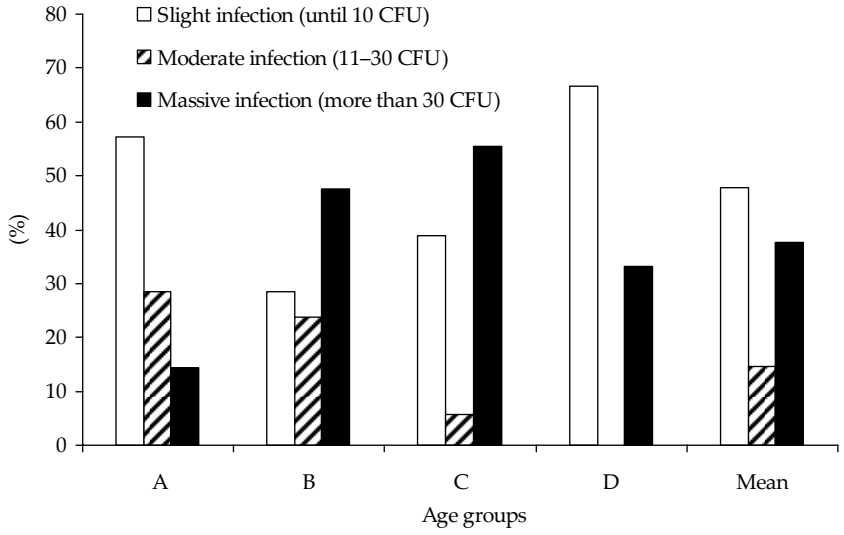

Figure 1. Proportions of *MAP*-positive animals in respective age groups depending on the intensity of infection

Table 1. *Mycobacterium avium* subsp. *paratuberculosis* in infected animals under the age of three years (age groups A and B)

Group	Lymph node		Spleen	Liver		Jejunum I		Jejunum II		Ileocaecum		Uterus	Foetus		Mammary			F
/No.	subm.	lung	tissue	tissue	LN	M	LN	M	LN	M	LN	tissue	liver	M	gland	LN	milk	
A/1	–	–	–	–	–	–	+	–	–	–	–	NA	NA	NA	NA	NA	NA	–
A/2	–	–	–	–	–	–	–	–	–	+	–	NA	NA	NA	NA	NA	NA	+
A/3	–	–	–	–	–	+	–	–	–	–	–	NA	NA	NA	NA	NA	NA	–
A/4	–	–	–	–	–	–	+	–	–	+	+++	–	–	–	–	–	NA	–
A/5	–	–	–	–	–	–	–	–	+	–	–	–	NA	NA	–	–	NA	–
A/6	–	–	–	–	–	++	–	–	–	–	+	–	NA	NA	–	–	–	–
A/7	–	–	–	–	–	–	–	–	++	–	–	–	NA	NA	–	–	NA	–
B/1	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	NA	–
B/2	–	–	–	–	–	–	++	–	++	++	–	–	NA	NA	–	–	–	–
B/3	–	–	–	–	–	–	–	–	+	+	++	–	NA	NA	–	–	–	–
B/4	–	–	–	–	–	+	–	–	–	–	–	–	NA	NA	–	–	–	–
B/5	–	–	–	–	–	–	–	+++	–	–	–	–	–	–	–	–	NA	–
B/6	–	–	–	–	–	–	–	–	++	+++	+	–	–*	–*	–	–	–	–
B/7	–	–	–	–	–	–	–	–	+	–	+++	–	NA	NA	–	–	–	–
B/8	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	NA	–
B/9	+++	–	–	–	+	–	–	+++	+++	–	+++	–	NA	NA	–	–	NA	–
B/10	–	+	–	+	+	+	++	+++	+++	+++	+++	+	+++	+++	–	+	–	+
B/11	–	–	–	–	–	+++	+	++	+++	–	–	–	NA	NA	–	–	–	–
B/12	–	–	–	–	–	+	+	–	+++	+++	–	–	NA	NA	–	–	–	+
B/13	–	++	–	–	–	–	+	–	–	+	+	–	–	–	–	–	–	–
B/14	–	–	–	–	–	+++	–	–	++	–	+++	–	NA	NA	–	–	–	+
B/15	–	–	–	–	–	–	+	–	+	++	+	–	NA	NA	–	–	–	–
B/16	–	–	–	–	+	+++	–	+	+++	+++	+++	–	NA	NA	–	–	–	–
B/17	–	–	–	–	–	–	+	+	+	–	–	–	NA	NA	–	–	–	–
B/18	–	–	+	+	+	–	++	+++	+++	+++	+	–	–	–	–	–	–	++
B/19	–	–	–	–	–	–	+	–	–	–	–	–	NA	NA	–	–	NA	–
B/20	–	–	–	–	–	–	–	+	+	+	+	–	NA	NA	–	–	NA	–
B/21	–	–	–	–	–	–	–	–	–	++	++	–	NA	NA	–	–	NA	–

No. = number of animals, subm. = submandibular, LN = lymph node, M = mucosa, I = cranial part of intestine, II = caudal part of intestine, F = faeces, A = age Group A (until 1.5 years), B = age Group B (1.6–3.0 years), + = slight infection (less than 11 CFU), ++ = moderate infection (11–30 CFU), +++ = massive infection (more than 30 CFU), NA = not available

*two foetuses were examined

Table 2. *Mycobacterium avium* subsp. *paratuberculosis* in infected animals older than three years (age groups C and D)

Group /No.	Lymph node		Spleen tissue	Liver		Jejunum I		Jejunum II		Ileocaecum		Uterus tissue	Foetus		Mammary			F
	subm.	lung		tissue	LN	M	LN	M	LN	M	LN		liver	M	gland	LN	milk	
C/1	–	–	–	–	–	–	–	–	+	+	–	–	NA	NA	–	–	–	+
C/2	–	–	–	–	–	–	–	–	–	–	+	–	+++	–	–	–	–	–
C/3	–	–	–	–	–	–	+	–	+	–	++	–	NA	NA	+++	–	–	–
C/4	–	–	–	–	–	–	–	+	+	++	–	–	NA	NA	–	–	NA	–
C/5	–	–	–	–	–	+	+	–	–	–	–	–	NA	NA	–	–	–	–
C/6	–	–	–	–	–	–	+	–	+	+++	–	–	NA	NA	–	–	–	–
C/7	–	–	–	–	–	–	–	–	+	+++	+++	–	–	–	–	–	–	+
C/8	–	–	–	–	–	–	–	–	–	–	+	–	NA	NA	–	–	–	–
C/9	–	–	–	–	–	–	–	+	+++	–	+++	+	–	–	–	–	–	++
C/10	–	–	–	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–
C/11	–	–	–	–	–	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	+++
C/12	+	–	–	–	–	–	+	–	+	–	–	–	–*	–*	–	–	NA	+
C/13	–	–	–	–	–	++	+++	+	++	++	+++	–	NA	NA	–	–	–	–
C/14	–	–	–	–	–	+++	++	+++	+	+++	–	–	–	–	–	–	–	–
C/15	–	–	–	–	–	–	–	–	+	–	–	–	NA	NA	–	–	–	–
C/16	–	–	–	–	–	–	+	–	–	+	–	–	NA	NA	–	–	–	–
C/17	–	–	–	–	–	–	++	+	++	–	+++	–	NA	NA	–	–	–	–
C/18	–	–	–	–	–	+++	–	–	+	+++	–	–	NA	NA	–	–	–	+
D/1	–	–	–	–	–	–	+	–	–	–	–	–	NA	NA	–	–	NA	–
D/2	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
D/3	–	+++	–	–	–	–	–	–	–	–	–	–	NA	NA	–	–	–	–

No. = number of animals, subm. = submandibular, LN = lymph node, M = mucosa, I = cranial part of intestine, II = caudal part of intestine, F = faeces, C = age Group C (3.1–8.0 years), D = age Group D (more than 8.0 years), + = slight infection (less than 11 CFU), ++ = moderate infection (11–30 CFU), +++ = massive infection (more than 30 CFU), NA = not available

*two foetuses were examined

Table 3. *Mycobacterium avium* subsp. *paratuberculosis* in 131 Jersey cattle from four age groups

Organ system	Sample origin	Age Group A (until 1.5 years)			Age Group B (1.6–3.0 years)			Age Group C (3.1–8.0 years)			Age Group D (more than 8.0 years)			Total A to D		
		No.	Pos.	%	No.	Pos.	%	No.	Pos.	%	No.	Pos.	%	No.	Pos.	%
Respiratory tract	submandibular LN	44	0	0	30	1	3.3	45	1	2.2	12	0	0	131	2	1.5
	tracheobronchial LN	44	0	0	30	2	6.7	45	0	0	12	1	8.3	131	3	2.3
Intestinal tract	spleen tissue	44	0	0	30	1	3.3	45	0	0	12	0	0	131	1	0.8
	liver tissue	44	0	0	30	2	6.7	45	1	2.2	12	0	0	131	3	2.3
	liver LN	44	0	0	30	4	13.3	45	0	0	12	0	0	131	4	3.1
	jejunum I M	44	2	4.6	30	6	20.0	45	5	11.1	12	0	0	131	13	9.9
	jejunum I LN	44	2	4.6	30	10	33.3	45	9	20.0	12	1	8.3	131	22	16.8
	jejunum II M	44	0	0	30	8	26.7	45	6	13.3	12	0	0	131	14	10.7
	jejunum II LN	44	2	4.6	30	14	46.7	45	14	31.1	12	0	0	131	30	22.9
	ileocaecal valve M	44	2	4.6	30	11	36.7	45	10	22.2	12	1	8.3	131	24	18.3
	ileocaecal LN	44	2	4.6	30	13	43.3	45	7	15.6	12	0	0	131	22	16.8
	faeces	44	1	2.3	30	4	13.3	45	6	13.3	12	0	0	131	11	8.4
Genital tract	uterus	4	0	0	25	1	4.0	44	1	2.3	12	0	0	85	2	2.4
	foetus (intestine)	1	0	0	11	1	9.1	19	0	0	5	0	0	36	1	2.8
	foetus (liver)	1	0	0	11	1	9.1	19	1	5.3	5	0	0	36	2	5.6
Udder	mammary gland	4	0	0	27	0	0	44	1	2.3	12	0	0	89	1	1.1
	supramammary LN	4	0	0	27	1	3.7	44	0	0	12	0	0	89	1	1.1
	milk	2	0	0	18	0	0	39	0	0	12	0	0	69	0	0
Total		544	11	2.0	479	80	16.7	749	62	8.3	202	3	1.5	1974	156	7.9

No. = number of samples, Pos. = number of positive samples, LN = lymph node, M = mucosa, I = cranial part of intestine, II = caudal part of intestine

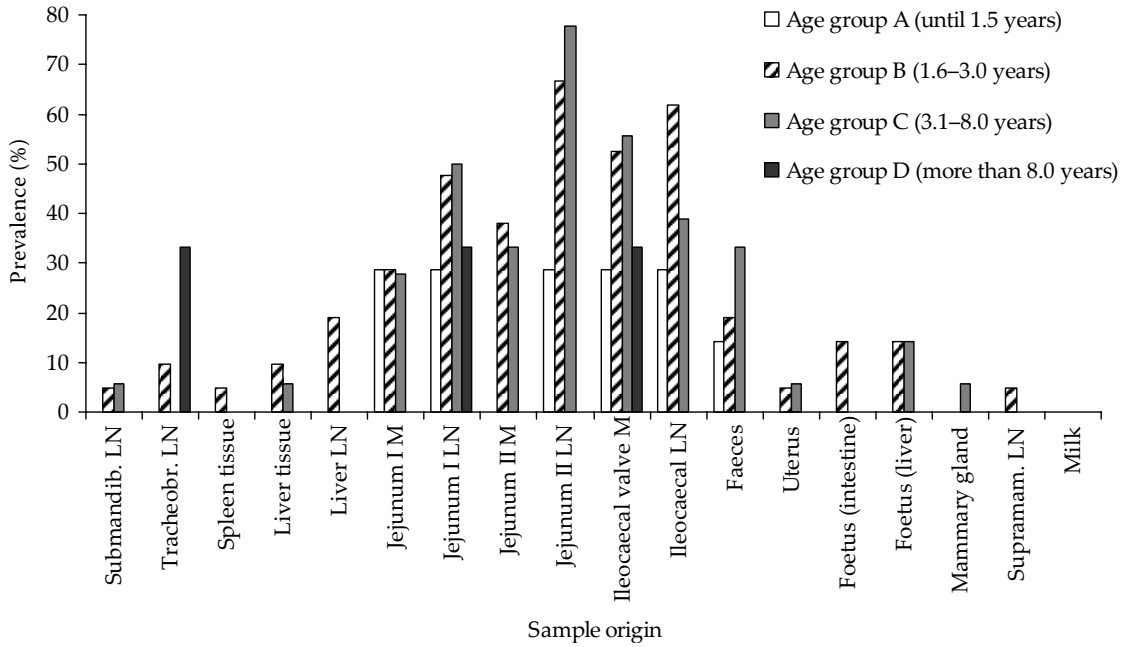

Figure 2. *MAP* prevalence in samples of different origin and from respective age groups

of jejunum) was observed in a one month old calf. Similarly, *MAP* (a slight infection) was isolated from the faeces and mucosa of the ileocaecal valve in a five month old calf. However, mothers of these calves were cultivation negative and therefore a mode of transmission other then intrauterine is likely. The colonization of the gastrointestinal tract and faecal shedding of *MAP* in the latter case indicated a current infection. On the other hand, a four month old calf was slightly positive in the lymph node of the jejunum. An intrauterine infection is assumed, as its mother was culture positive in faeces and in tissue taken from the gastrointestinal tract. Although only a few animals in the youngest group were found to be culture positive, we assume that the number of infected individuals was higher due to the low amount of detectable *MAP* cells in the positive animals.

Table 4. Significance of the differences in *Mycobacterium avium* subsp. *paratuberculosis* prevalence between four age groups of animals

Age group (years)	A (≤ 1.5)	B (1.6–3.0)	C (3.1–8.0)	D (≥ 8.1)
A (≤ 1.5)	NT	+	–	–
B (1.6–3.0)	+	NT	–	++
C (3.1–8.0)	–	–	NT	+
D (≥ 8.1)	–	++	+	NT

+ = $P < 0.05$ (statistically significant), ++ = $P < 0.01$ (statistically significant), – = $P > 0.05$ (statistically non-significant), NT = not tested

Young animals are generally most susceptible to infection. According to the meta-analysis of Windsor and Whittington (2009) calves older than six months are more susceptible to *MAP* infection than adult cattle, but at the same time, they are less susceptible than calves younger than six months of age. It is considered that the intestinal mucosa of newborn calves is highly permeable and thus it allows *MAP* to penetrate through the mucosal barrier (Sweeney, 1996). It is therefore possible, that a functional rumen in adult animals has a harmful effect on *MAP* before it reaches the intestine (Windsor and Whittington, 2009). In the PTB control program operating throughout the Czech Republic, culture examination of faeces of calves was not required by law at that time. These animals could shed *MAP* in their faeces and contaminate the environment without being detected and thus posed a serious threat to other health animals.

A development of the infection associated with growing number of detectable *MAP* appears with increasing age (Windsor and Whittington, 2009). This trend was also demonstrated in the present study, where the highest amount of culture positive animals was found in the B and C age groups. In Group B (1.6 to 3 years), 42.9% of the animals were found to be infected. At this age, animals

are entering the first production period and at the same time they are exposed to strong stress factors. According to Johnson-Ifearulundu et al. (2000), a rapid increase of milk production after calving poses a critical period not only for infected, but also for healthy cows. At this time, a cow's organism is most likely exposed to the occurrence of a negative energy balance (Johnson-Ifearulundu et al., 2000). The influence of other stress factors such as nutrition or lactation, are described by Windsor and Whittington (2009). Stress factors can contribute to the weakening of cell-mediated immunity, which can lead to shedding of *MAP* in faeces (van Roermund et al., 2007; Windsor and Whittington, 2009). A combination of stress factors, infective dose of agents and immunological state of an individual animal then determine if exposure leads to infection, regression, recovery or disease (Windsor and Whittington, 2009).

A higher number of animals tested positive (36.7%) in Group C (aged 3.1–8 years) compared to the oldest Group D (6.1% of animals). This is most likely due to the early slaughter of the majority of *MAP* infected animals which would now belong in age Group D (unpublished data). Five Group D cows, originally imported from Denmark, were negative by cultivation and therefore excluded from further investigations. These animals were unlikely to be exposed to *MAP* in the early postnatal period. Alternatively, age-related resistance, which is regarded as a significant factor in PTB, might have protected these cows in adulthood and after their transfer to a new herd (Chiodini, 1996; Ayele et al., 2001; Hasonova and Pavlik, 2006; Windsor and Whittington, 2009).

Culture examination of faeces

A low amount of faeces-positive results were obtained in the examined cattle herd (Table 3). Kim et al. (2004) assumed that faecal culture was an ineffective method for the detection of low bacterial shedders and according to Whitlock et al. (2000) the sensitivity of the method for such animals may be as low as 33%. Giese and Ahrens (2000) established 100 CFU/g faeces as a detection limit for culture examination. Negative culture examinations of faeces have been described in a bull with clinical signs of PTB (Buergelt et al., 2004) and in a cow with positive *MAP* findings in its tissue samples (Whitlock et al., 1996). The most important factor contributing to the failure of faecal culture probably lies in the low numbers of *MAP* presented in the faeces of intermittent or low shedding animals (de Lisle et al., 1980). Furthermore, repeated thawing and freezing of faecal samples may also contribute to decreased viability and subsequent cultivability of *MAP* (Richards and Thoen, 1977).

Faecal shedding of *MAP* occurs most frequently in adult animals in the clinical stage of disease or shortly before it (Antognoli et al., 2007). In our study, *MAP* was demonstrated in the faeces of a five-month-old calf (Table 1). A slight shedding of *MAP* in faeces of eight year old heifers was described (Antognoli et al. (2007). McDonald et al. (1999) described the shedding of *MAP* in the faeces of one to 18-month-old animals; nevertheless, they considered isolation from animals above 16 months as more common. According to Weber et al. (2005), young animals become faecal positive when breeding in the herds with high prevalence of infection.

Shedding of *MAP* in faeces of young animals may be underestimated because most of the investigations are focused only in adult animals. Furthermore, as naturally infected calves are shedding *MAP* intermittently and in low amounts (under detection limit), early diagnosis is difficult (Antognoli et al., 2007). Van Roermund et al. (2007) suggested a "pattern" for the shedding of *MAP* by infected calves. According to the study, a peak occurs shortly after *MAP* intake or infection, than it declines to zero for a relatively long time before increasing again. In the Czech Republic, the faecal cultivation was only performed in animals older than 18 months. Thus, a spread of *MAP* by younger animals poses a certain risk to the rest of a herd (Pavlik et al., 2000b).

MAP in various tissues

Little is known about the dissemination of *MAP* in subclinical cases of PTB compared to clinical cases (Antognoli et al., 2008). However, information about the infection location or its intensity may contribute to a better understanding of infection pathogenesis and the prevention of food product contamination with infectious agents.

The gastrointestinal tract was *MAP* positive in 77.6% of animals, confirming this tract as a primary site of PTB infection (Sweeney et al., 1992b). *MAP* was also isolated from the extraintestinal tissues of 18.4% animals. In three cases, this was the pulmonary lymph node, which is noteworthy as some authors

have suggested the airborne transmission of *MAP* (Corner et al., 2004). According to this hypothesis, cattle are exposed to live *MAP* bacteria present in aerosol and dust particles. Infectious particles can therefore reach the pulmonary alveoli and after being engulfed by alveolar macrophages, enter the pulmonary lymphatic system and then the whole organism via blood circulation (Ayele et al., 2001). Waters et al. (2003) documented the instillation of *MAP* into the tonsillar crypts of neonatal calves, which resulted in the colonization of tonsils and some tissues of gastrointestinal tract, faecal shedding and both a humoral and cellular immune responses. The detection of *MAP* in submandibular lymph nodes in our study possibly indicates *MAP* invasion through the injured mucosa of the oral cavity. Further studies are needed for confirmation of such assumptions.

In regards to the youngest group of animals, *MAP* was only isolated from the gastrointestinal tract tissues. In the study of Sweeney et al. (2006) based on oral infection of neonatal calves, the small intestine is the primary portal of *MAP* entry into the host, with extension to the mesenteric lymph nodes. Nevertheless, the authors mentioned an importance of the infectious dose. In general, the majority of *MAP* positive samples in our study originated from the posterior jejunal lymph nodes (61.2%) and from the mucosa of the ileo-caecal junction (49.0%; Figure 2). Some papers considered the important role of ileal tissue (mostly lymph nodes than mucosa) as a reservoir of infection (Coussens, 2004; Wu et al., 2007). Therefore, these tissues are highly suitable for confirmation of infection (Amemori et al., 2004).

Methods for differentiation or subtyping of *MAP*, such as IS900 multiplex PCR (Bull et al., 2000), RFLP – random fragment length polymorphism (Pavlik et al., 1999), AFLP – amplified fragment length polymorphism (Motiwala et al., 2003) or MLSSR – multilocus short sequence repeat (Amonsin et al., 2004) have been previously developed. RFLP analysis of the examined tissue isolates identified RFLP type B-C1 in all cases. B-C1 represents the most common RFLP type observed in cattle and wild ruminants in the Czech Republic (Pavlik et al., 2000c; Machackova et al., 2004; Kopecna et al., 2008) as well as in other countries (Whipple et al., 1990; Djonne et al., 2005).

Foetuses

Two foetuses were found to be positive by culture. One of them originated from a slightly infected cow and the other from a cow massively infected in various organs and shedding *MAP* in its faeces (Tables 1 and 2). Lambeth et al. (2004) showed *MAP* in most sheep foetuses obtained from clinically infected sheep, but only one positive foetus (among 54) originated from a sheep with a subclinical disease. The assumed probability of *MAP* infection in foetuses from clinically ill cows is about three times higher when compared to subclinically infected mothers (Seitz et al., 1989; Whittington and Windsor, 2009). It follows that the probability of foetal infection grows with increasing intensity of infection and onset of clinical symptoms in dams.

Sweeney et al. (1992b) demonstrated the infection of foetuses originating only from mothers massively shedding *MAP* in faeces and not in dams without clinical symptoms. In our case, seven foetuses originated from six cows shedding *MAP* in faeces. However, only one foetus from a cow slightly shedding *MAP* in faeces was infected and thus we cannot confirm the association between massive *MAP* faecal shedding and an increased probability of foetal infection. It is possible that the number of infected foetuses could be underestimated because *MAP* has been mainly found in foetal kidneys (Sweeney et al., 1992b). Also, cultivation using solid media could decrease the real prevalence of foetal *MAP* infection according to Whittington and Windsor (2009). In any case, as the faecal-oral route is believed to be one of the principal pathways of *MAP* transmission, *in utero* transmission represents another important possibility and should be considered in the control of the disease (Buergelt et al., 2006).

Milk

Some studies mention low numbers of culturable *MAP* in raw milk (Giese and Ahrens, 2000; Lynch et al., 2007) and state 100 CFU/ml of milk as a detection limit of *MAP* cultivation (Giese and Ahrens, 2000). Together with intermittent shedding of *MAP* (Corti and Stephan, 2002), these factors may explain the negative milk culture results in this study. HPC decontamination and antibiotic supplementation of culture media can also be reasons for cultivation failure (Gao et al., 2005; Lynch et al., 2007). Besides that, absence of standardized methodology for *MAP* isolation from milk complicates the determination of the number of *MAP* cells in naturally infected milk (Grant et al., 2005; Slana et al., 2008b).

The mechanism of the shedding of *MAP* into milk has not yet been well investigated (Slana et al., 2008b). It seems that *MAP* could get into milk directly from lymphatics draining into the mammary gland or indirectly via faecal contamination during the milking process. According to Grant et al. (2001), the latter is more probable. The technique of collecting of milk samples eliminated a possibility of contamination in our study. *MAP* in milk is mostly found in animals in advanced stages of disease or those heavily shedding *MAP* in faeces comparing to cows in the subclinical stage of infection (Sweeney et al., 1992a; Streeter at al., 1995). Unfortunately, only one heavily shedding cow was identified in the present study.

According to Grant (2006) there are about four to eight animals in the subclinical stage of infection regularly shedding *MAP* in their faeces (eventually in milk), for every one clinical case of PTB. This is consistent with our findings that the asymptomatic cows outnumbered the cows with clinical symptoms. The occurrence of subclinically ill animals does not only pose a risk of *MAP* spreading in the herd, but also to food safety. *MAP* in naturally infected milk survives pasteurization (Grant et al., 2001, 2002, 2005). As *MAP* (not only viable, but dead as well) is supposed to contribute to the development of Crohn's disease, this could represent a potential risk for human health (Chamberlin and Naser, 2006; Uzoigwe et al., 2007; Behr and Kapur, 2008).

CONCLUSIONS

In the present study, *MAP* was detected by culture in the tissues, faeces and foetuses of cows originating from one herd. The highest prevalence of *MAP* infection in the animals was detected at the beginning of their production. On the other hand, the lowest prevalence was found among the oldest animals, which was probably associated with "age resistance". The causative agent was mainly present in the small intestines of 98% of animals that tested *MAP* positive, and this was the site where massive infection was most commonly detected. Even though cows of reproductive age were the highest shedders of *MAP* in faeces, animals younger than 18 months of age were also faecal positive. Without clinical symptoms, these animals can pose a risk of contamination to the environment.

MAP detection in tissues other than the digestive tract is important. After *MAP* dissemination, the female reproductive organs and mammary gland can become potential reservoirs of infection for foetuses and newborn animals (vertical transmission of *MAP*). This is the primary reason why the offspring of infected cows should not be used for breeding. It is highly probable that they are infected and will later spread the causative agent to the environment. In our study, no *MAP* positive milk samples was detected by culture which can be explained by intermittent shedding of *MAP* and the presence of low numbers of viable *MAP* cells in milk.

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