

***Mycobacterium avium* subsp. *paratuberculosis* and *M. a. avium* Detected by Culture, IS900 and IS901 Highly Sensitive PCR in Bulk Tank Milk from Dairy Herds in the Czech Republic between 2002 and 2004**

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Abstract

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In this study, the possible presence was monitored of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) and *Mycobacterium avium* subspecies *avium* (MAA) by means of culture examination and PCR in 251 bulk tank milk samples from dairy herds in the Czech Republic between 2002 and 2004. The detection of MAP and MAP DNA in repeatedly collected bulk tank milk (BTM) samples from the selected cattle farms (seven farms) was the second purpose of the study. By culture, MAP was detected in 5 (2.0%) and MAP DNA in 85 (33.9%) of the total of 251 BTM samples. MAA was detected by culture and by PCR in 1 (0.4%) of the 251 BTM samples. This study demonstrates the presence of MAP and MAA in dairy herds in the Czech Republic.

Keywords: Johne's disease; avian tuberculosis; food safety; zoonoses

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a causal agent of chronic granulomatous enteritis of ruminants, wild rabbits, and non-human primates being known as paratuberculosis, or Johne's disease (McCLURE *et al.* 1987; GREIG *et al.* 1997; AYELE *et al.* 2001). The MAP shedding is not limited only to the faeces, MAP cells having been cultured also from milk (MILLAR *et al.* 1996; MANNING, 2001; KHARE *et al.* 2004; AYELE *et al.* 2005; LOMBARD *et al.* 2006) and milk products (IKONOMOPOULOS *et al.* 2005). Due to this fact, the consumption of such infected milk and dairy products that could

be important vehicles of the MAP transmission is a danger to health (BEHR & KAPUR 2008). MAP is relatively resistant to high temperatures and was isolated from milk pasteurised at 72°C to 74°C, as reviewed by SLANÁ *et al.* (2008a).

As summarised in 2008 by SLANÁ *et al.* (2008a), the highest prevalence of MAP detected by culture (35.0%) was reported in Australia followed by the USA (28.6%). In Argentina, Ireland, and the UK, the culture revealed up to 10% of MAP positive samples. In the Czech Republic, MAP was detected by culture in 4 (1.6%) samples of commercially

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pasteurised milk at 72°C to 74°C from a cattle herd with unknown *MAP* occurrence. *MAP* was detected by culture in 2 (2.0%) samples of milk pasteurised locally at 72°C to 74°C coming from two infected herds.

Using the PCR method for the detection of *MAP* specific sequences in milk, the prevalence of *MAP* in bulk tank milk samples was found to range between 20% and 68% which was higher than the culture positivity (CORTI & STEPHAN 2002; STABEL *et al.* 2002; JAYARAO *et al.* 2004). *MAP* was also detected by PCR in milk from clinically healthy cows (SWEENEY *et al.* 1994). In recent years, a number of studies dealing with presence of *MAP*-specific DNA sequences in milk were performed in many countries as follows from the review by SLANÁ *et al.* (2008a).

Between 2002 and 2004, several studies focused on *MAP* detection in cow's milk and milk products were conducted in the Czech Republic with the use of different methods. Between 2002 and 2003, *MAP* was detected by culture in pasteurised cow's milk (AYELE *et al.* 2005). In 2003, *MAP* was detected by culture and highly sensitive IS900-PCR assay in various cheese types made from pasteurised cow's milk (IKONOMOPOULOS *et al.* 2005). In 2004, *MAP* was detected in powdered infant food made from cow's milk using culture, high sensitive IS900-PCR, and *F57* real-time PCR assay (HRUSKA *et al.* 2005).

Cattle are partially susceptible to the causative agent of avian tuberculosis caused by *Mycobacterium avium* subsp. *avium* (MAA), which contains specific insertion sequence IS901 (PAVLIK *et al.*, 2000). In the Czech Republic, this disease occurs particularly in domestic and free ranging birds (DVORSKA *et al.* 2007; SHITAYE *et al.* 2008a, b). MAA was occasionally found in the intestinal lymph nodes of cattle (PAVLIK *et al.* 2002; DVORSKA *et al.* 2004). MAA was cultured from cow's milk in different countries such as Germany (NASSAL 1961), Canada (JONES *et al.* 1966), and the USA (ROOSEVELT *et al.* 1966).

In the Czech Republic, MAA was detected in milk from cows that had been in contact with infected gallinaceous poultry (ROSSI *et al.* 1969) and a cow that had been orally infected with the liver of gallinaceous poultry affected by MAA (HEJLÍČEK & TREML 1995). However, nowadays the dairy cows in the Czech Republic are mostly kept on large scale farms (average size of a dairy herd is 150 head) where their contact with gal-

linaceous poultry is almost impossible (statistical data of the Ministry of Agriculture of the Czech Republic, personal observation). On some family farms, however, where the free-range gallinaceous poultry are kept, their contact with cattle is possible (personal observation).

The first purpose of the present study was to ascertain by culture examination whether the cultivable *MAP* had been found in non-pasteurised bulk tank milk (BTM) coming from dairy herds in the Czech Republic between 2002 and 2004; the presence of *MAP* DNA was detected by the highly sensitive IS900-PCR assay. The second purpose was to detect the occurrence of the cultivable *MAP* and *MAP* DNA in repeatedly collected BTM samples from the selected cattle farms, where the presence of *MAP* DNA either had or had not been detected by screening. The third purpose of the present study was to investigate the prevalence of the cultivable MAA in the above mentioned BTM using the culture method, and to ascertain by means of highly sensitive IS901 PCR whether MAA DNA was present in 10 selected BTM samples from family farms and large farms.

MATERIAL AND METHODS

Milk samples. A total of 251 BTM samples of fresh cow's milk were examined between 2002 and 2004.

Two hundred thirty seven BTM samples from 237 dairy cattle herds were examined only once. According to the PCR results, seven farms with the milk production from 3000 l to 4000 l per BTM were selected: *MAP* DNA was detected at the first examination on four farms designated A through D and was not detected on three farms designated E through G. BTM samples from these seven farms were again examined twice sequentially in two-week intervals.

All 251 BTM samples of fresh cow's milk were examined by culture for MAA presence. The following samples were examined once for MAA DNA presence by the highly sensitive PCR method: one BTM sample was taken from a family farm with MAA occurrence detected by culture, four randomly selected BTM samples were taken from other family cattle farms with the milk production between 100 l to 150 l per BTM, and five BTM samples were collected on large farms with the milk production of 4000 l to 5000 l milk.

Collection of milk samples. The milk samples were collected into sterile plastic 25 ml vials and transported to the laboratory at 4°C. The samples for cultivation were analysed immediately after the collection, the samples for PCR analysis were stored at –70°C before the testing.

Culture examination. Fifteen ml of raw milk were centrifuged at 2500 g for 15 min, the pellet was resuspended in 10 ml of 0.75% hexa-decylpyridinium chloride (HPC, Merck, Darmstadt, Germany). After 5 h of decontamination (at room temperature) and centrifugation, 250 µl aliquors of the resuspended pellet were inoculated onto three plates Herrold Egg Yolk Media (HEYM) containing 2 µl per ml of Mycobactin J (produced in Veterinary Research Institute, Brno, Czech Republic). To rule out the fast growing mycobacteria and early contamination of the cultures, the vials were observed during the first week of incubation. Further observation took place in every following two week interval until visible colonies appeared. The incubation proceeded for no less than eight months. With the primary cultures, the colony forming units (CFU) resembling mycobacteria were stained by the Ziehl-Neelsen (ZN) method for the presence of acid-fast bacilli (AFB). To distinguish *MAP* from other Mycobactin J non-dependent mycobacteria, CFUs were picked and subcultured on four HEYM slants, i.e. three HEYM containing and one HEYM non-containing 2 µl of Mycobactin J, and were cultured at 37°C for 3 months. The identification of the grown CFU was made by means of the conventional multiplex PCR as previously described by MORAVKOVA *et al.* (2008).

DNA extraction. To prepare a DNA sample for PCR amplification, a total of 200 µl of milk was taken. DNA was isolated by QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. A total of 200 µl of DNA was obtained from each milk sample.

IS900 PCR amplification. All the milk samples were isolated and PCR tested in duplicates. For the detection, the *MAP* specific insertion sequence IS900 was used. The PCR amplification was performed as follows: the PCR reaction mixture (40 µl) contained Hot Star Master Mix (QIAGEN) 20 pmol each of the IS900-P3N and IS900-P4N primers, 10⁴ copies of the internal amplification control (IAC) previously described by AYELE *et al.* (2005), and 4 µl of isolated DNA. The following program was used for the amplification on PTC 200 thermocycler (MJ Research, Waltham,

USA): initial denaturation at 96°C for 15 min, followed by 60 cycles consisting of denaturation at 94°C for 1 min, annealing at 67°C for 45 s, and the extension step at 72°C for 2 minutes. In the last step, 72°C for 2 min for the final extension was used. Ten microliters of the amplified products (257 bp for IS900 and 591 bp for IAC) were then separated by electrophoresis on 2% agarose gel (Serva, Heidelberg, Germany), stained with ethidium bromide, visualised by UV transillumination (TVR-312R Spectronic Corporation, USA), and photographed with a digital camera Canon (Canon, Westbury, USA).

High sensitivity IS901 PCR amplification. For the detection, the *MAA* specific insertion sequence IS901 was used. The PCR amplification was performed as follows: the PCR reaction mixture (20 µl) contained Hot Star Master Mix (QIAGEN) 5 pmol of the IS901-NP1 (5'-TTA ACA CGA TGA GTC ATG CG-3') and IS901-NP2 (5'-GCT TAT CGA TGT CCT TGA TC-3') in-house designed primers and 10 pmol in-house designed nested primers IS901-NP3 (5'-GTACCCGGCGAAGACCTG G-3') and IS901-NP4 (5'-AAG TCC AGC AGC CGT GCT G-3'), 10² copies of IAC and 2 µl of isolated DNA. The following program was used for the amplification on PTC 200 thermocycler (MJ Research): initial denaturation at 96°C for 10 min, followed by 16 cycles consisting of denaturation at 94°C for 1 min, annealing at 58°C for 45 s, and the extension step at 72°C for 1 min, and by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 68°C for 45 s, and the extension step at 72°C for 1 minute. In the last step, 72°C for 3 min for the final extension was used. Ten microliters of the amplified product (510 bp for NP1/NP2 primers, and 377 bp for nested NP3/NP4 primers) were then separated as described above.

RESULTS AND DISCUSSION

MAP in 251 bulk tank milk samples

By culture, *MAP* was detected in 5 (2.0%) and *MAP* DNA in 85 (33.9%) of the total of 251 BTM samples. All five Mycobactin J dependent *MAP* isolates were able to grow *in vitro* for up to 8 to 12 weeks (identification was confirmed by IS900 PCR testing). IS900 PCR amplification of DNA extracted from BTM samples and subsequent

agarose gel analysis of the amplified products showed a single band of 257 bp for each of the positive milk samples. The potential inhibition of PCR was checked by using IAC.

MAP DNA occurrence was confirmed by PCR in three out of five BTM samples where *MAP* had been tested positive by culture. Similar differences between the numbers of positive BTM samples detected by PCR and by culture had also been observed by other authors (PILLAI & JAYARAO 2002). Of 20 tested BTM samples, 50% and 5% were found to be positive by PCR and by culture, respectively (PILLAI & JAYARAO 2002). The discrepancy between the results might have been caused by a low concentration of *MAP*, which is often found in milk in clusters. That can cause non-homogeneous presence of *MAP* in milk. Due to this fact, the BTM sample taken for culture cannot be identical with that one taken for DNA isolation.

Of 251 BTM samples, 85 (33.9%) were tested IS900 PCR positive and *MAP* was detected by culture in three (3.5%) of them. The marked differences between the culture and IS900 PCR results can be explained by different sensitivity of the methods used. The number of IS900 copies in the *MAP* genome ranges between 12 and 18 (PAVLÍK *et al.* 1999; BULL *et al.* 2000) which ensures 10 times higher sensitivity of PCR in comparison with culture. Moreover, the detection of the *MAP* specific sequence is performed directly, without the use of decontamination or other agents that have to be added during the culture examination. That can also explain a higher proportion of positive milk samples tested by IS900 PCR. In addition, the decontamination procedures can cause

devitalisation of *MAP* (DUNDEE *et al.* 2001) and non-growing forms of *MAP* or devitalised *MAP* can also be present in milk (BERAN *et al.* 2006; SLANA *et al.* 2008b).

***MAP* detection in 237 bulk tank milk samples from 237 different cattle farms and in 14 repeated BTM samples from 7 farms**

By screening 237 BTM samples, *MAP* was detected by culture in four (1.6%) and *MAP* DNA in 82 (34.6%) of them (Table 1). In the second and third testing of BTM samples repeatedly collected on seven selected farms, *MAP* was tested positive by culture in one and by PCR in three out of the total of 14 of them, respectively. From four farms A through D where *MAP* had been detected in BTM samples by means of IS900 PCR during the screening period, *MAP* presence was not confirmed by culture in any of the 8 repeated samples. On the other hand, on three farms E through G, where *MAP* had not been detected during the screening period, *MAP* was detected by culture in one out of six BTM samples. In this sample, the presence of *MAP* DNA was also confirmed by the PCR method (Table 2). This is in accordance with the observations of other authors (TAYLOR *et al.* 1981; NASER *et al.* 2000; STABEL *et al.* 2002; AYELE *et al.* 2005).

The negative results with IS900 PCR in the repeated BTM samples collected on four infected farms indicated non-homogeneity of the samples and most likely irregular *MAP* shedding into milk as mentioned above (Table 2).

***MAA* in 251 bulk tank milk samples by culture examination and in 10 selected BTM by IS901 PCR**

MAA was detected by culture in one (0.4%) of 251 BTM samples. *MAA* DNA was detected in the sample by the highly sensitive IS901 PCR. The four and five BTM samples from other family farms and large farms, respectively, were found IS901 PCR negative. The *MAA* detection is not surprising, because *MAA* shedding into milk has been described above all during bovine tuberculosis control in the second half of the last century (NASSAL 1961; JONES *et al.* 1966; ROOSEVELT *et al.* 1966; ROSSI *et al.* 1969). However, *MAA* detected

Table 1. *Mycobacterium avium* subsp. *paratuberculosis* detection by culture and by PCR in 237 bulk tank milk samples from 237 different cattle farms

<i>MAP</i> detection by		Samples	
Culture	PCR	No.	%
+	+	2	0.8
+	–	2	0.8
–	+	80	33.8
–	–	153	64.6
Total examined		237	100

MAP – *Mycobacterium avium* subsp. *paratuberculosis*;
PCR – IS900 PCR

Table 2. *Mycobacterium avium* subsp. *paratuberculosis* detection in bulk tank milk collected three times¹ on seven-farms

Herd description		1 st examination		2 nd examination		3 rd examination		Total positive	
District	farm	PCR	culture	PCR	culture	PCR	culture	PCR	culture
I	A	+	–	–	–	+	+	2	1
II	B	+	–	–	–	–	–	1	0
III	C	+	–	+	–	–	–	2	0
IV	D	+	–	–	–	–	–	1	0
V	E	–	–	–	–	–	–	0	0
VI	F	–	–	–	–	+	–	1	0
VII	G	–	–	–	–	–	–	0	0

¹examination performed every second week: + positive examination, – negative examination

PCR – IS900 high sensitivity PCR (primers described by AYELE *et al.* 2005)

Culture – culture examination by 0.75% HPC sedimentation method as described by AYELE *et al.* (2005)

in the samples from a family farm may be risky because, according to the currently accepted legislation in the Czech Republic (Act No. 166/1999 Coll. on veterinary care and amendments of some related acts), non-heat-treated milk can be sold on family farms. Even though there should be a notice at the point of sale saying that the farm retails unpasteurised milk and the consumers should perform the heat-treatment before consumption, this is not accomplished in many cases (unpublished data). Hence, the consumers are at risk of infection with the causative agent of avian tuberculosis through the consumption of non-heat-treated milk because people are also susceptible to this pathogen (PAVLÍK *et al.* 2000).

CONCLUSIONS

This first study demonstrated that the prevalence of *MAP* DNA in raw cow's milk in the Czech Republic is higher than previously detected by culture. This is an alarming finding due to the fact that *MAP* is a possible candidate for aetiology of Crohn's disease. The detection of cultivable *MAA* and *MAA* DNA by culture and by PCR, respectively, in BTM from a family farm highlighted the risk of potential milk contamination with the causal agent of avian tuberculosis.

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