https://doi.org/10.17221/168/2020-VETMED

Detection of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples from infected Czech dairy herds

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Citation: Fichtelova V, Kralova A, Fleischer P, Babak V, Kovarcik K (2021): Detection of *Mycobacterium avium* subspecies *paratuberculosis* in environmental samples from infected Czech dairy herds. Vet Med-Czech 66, 1–7.

Abstract: The objective of the present study was to evaluate the suitability of environmental sampling to screen Czech dairy herds to detect *Mycobacterium avium* ssp. *paratuberculosis* (MAP) and to find the most convenient location for the MAP detection in the lactating cow area. Environmental samples (ES, *n* = 72) from milking parlour holding pens (*n* = 19), milking alleyways (*n* = 19) and free-stall alleyways (*n* = 34) from 19 herds were simultaneously tested to detect MAP by a quantitative PCR (qPCR) and bacterial culture. Eight and thirteen samples from the milking parlour holding pens, twelve and eleven samples from the milking alleyways and eleven and eighteen samples from the free-stall alleyways were qPCR and culture positive, respectively. A 4.6 times higher probability of being culture positive than qPCR positive was detected for the assessable MAP detection results from the free-stall alleyways [*P* = 0.008 6, odds ration (OR) = 4.572 8)] and no association was found between the results from the milking parlour holding pens (*P* = 0.191 4) and the milking alleyways (*P* > 0.999 9) and the diagnostic method used. The percentage of qPCR-positive samples in the tested locations was detected for the milking alleyways (63.2%), free-stall alleyways and milking parlour holding pens. The herd infectious status was in agreement with 16 (84.2%), 14 (73.7%) and 12 (63.2%) qPCR results from the milking alleyways, free-stall alleyways (32.4%) and milking parlour holding pens (42.1%), respectively. No statistically significant differences were detected for these results (*P* = 0.396 1). MAP was detected by the qPCR and bacterial culture in all three locations where the ES were collected. We suggest an environmental sampling followed by MAP detection by qPCR as an easy-to-perform time-saving protocol for MAP screening in Czech dairy herds. Although the milking alleyways seem to be the most convenient location for the environmental sampling, this assumption was not statistically supported.

Keywords: Johne's disease; lactating cow; sampling location; faecal culture; quantitative PCR

Bovine paratuberculosis (Johne's disease) is a chronic progressive granulomatous enteritis caused by the *Mycobacterium avium* subspecies *paratuberculosis* (MAP). It causes economic losses through a lower milk yield, the premature culling and reduced value of the culled animals (Garcia and Shalloo 2015). The infection is transmitted by ingestion of MAP in the milk and/or colostrum or by manure-contami-

Supported by the Ministry of Agriculture of the Czech Republic (Institutional Support No. MZE-RO0518) and by the National Agency for Agricultural Research (Project No. QK1820086).

https://doi.org/10.17221/168/2020-VETMED

nated teats or feed (Rathnaiah et al. 2017). The characteristic feature of the infection is a long incubation period of two or more years before the animals start to shed MAP bacilli in their faeces.

To reduce the negative consequences of MAP infections, control programmes are implemented in infected herds. At first, the herd MAP infection status has to be identified. Environmental sampling is one of the convenient screening methods to detect paratuberculosis in dairy herds without the laborious handling of each individual cow. Many environmental sampling protocols have been published so far, which differ in the number of environmental samples (ES) and also in the area and location of the sample collection (Raizman et al. 2004; Berghaus et al. 2006; Lombard et al. 2006; Pillars et al. 2009). ES from alleyways, pen floors or manure accumulation areas representing the living place of lactating, dry, sick or calving cows have frequently been collected. The primary manure storage area and common lactating cow area with manure accumulation were discovered to be the most likely MAP-positive locations (Smith et al. 2011; Wolf et al. 2015). Although, in many studies, five to six samples were required to be collected, other authors found fewer samples sufficient to determine the herd MAP infection status. The manure storage area and lactating cow area or three different locations were sampled in these studies (Raizman et al. 2004; Pillars et al. 2009; Donat et al. 2015; Corbett et al. 2018). An association has been detected between the positive environmental samples and the within-herd MAP prevalence (Raizman et al. 2004; Berghaus et al. 2006; Donat et al. 2015).

Forty-eight percent of all dairy cows in the Czech Republic are held in operations with more than 500 cows. Among Czech dairy herds, the true MAP prevalence is unknown, but a pilot study investigating 786 bulk tank milk (BTM) samples using an ELISA (enzyme-linked immunosorbent assay) to detect the specific antibodies discovered a relatively high number (11.5%) of herds with a high within-herd prevalence (Kovarcik and Kralova 2018). Recently, attempts have been made to introduce a voluntary MAP certification programme into dairy herds, and environmental sampling is intended to be one of the screening methods to evaluate the MAP herd status (Fleischer et al. 2018).

The main objective of this study was to evaluate the suitability of the environmental sampling for MAP screening in Czech dairy herds. Furthermore, we searched for the most convenient location out of the selected lactating cow areas for a rapid MAP detection by quantitative PCR representing a time-saving alternative to the bacterial culture.

MATERIAL AND METHODS

Herds

Nineteen free-stall dairy herds – sixteen Holstein ones and three Czech Fleckvieh ones (herd No. 7; 10; 15) – with a median herd size of 430 lactating cows (range from 130 to 920 cows) were included in the study (Table 1). Rubber mattresses (herd No. 18), composed bedding (herds No. 1, 2, 3, 9, 10, 12, 16, 17) and straw (the remaining ten herds) were used for the bedding. Manure was collected by mechanical scraping in all except one herd (No. 2) with a slatted floor.

The MAP control programme including the annual screening of each three-year-old and older cow during its dry-off period for antibodies by a serum ELISA (IDVet, Montpellier, France) was implemented in these herds (except herd No. 7). The number of ELISA-tested cows represented 54.4–125.1% (median 88.7%) of the lactating cows in the individual herds. The cumulative percentage of the tested ELISA-positive cows in the herds, as determined in 2019, is shown in Table 1.

Environmental samples (ES)

The environmental sampling in the herds was conducted from August to December 2019. The ES (n = 72) consisted of faecal material collected from three selected locations from the cow area:

1. The milking parlour holding pens (n = 19);
2. milking alleyways (paths for cows to come into the milking area close to the entrance into the milking parlour holding pens) (n = 19) and
3. free-stall alleyways (stall and feed alleys) (n = 34).

One sample from the milking parlour holding pen and one pooled sample representing a mixture of material of each milking alleyway were taken. Both locations (1 and 2) were sampled immediately after milking. One to four samples from the free-stall alleyways were collected depending on the num-

https://doi.org/10.17221/168/2020-VETMED

Table 1. Detection of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in the environmental samples from the individual locations and herds by the quantitative polymerase chain reaction (qPCR) and faecal culture (FC) and the percentage of the ELISA-positive three-year-old and older cows detected during the year of sampling

Herd	Lactating cows (*n*)	Milking parlour holding pens (*n* = 19)		Milking alleyways (*n* = 19)		Free-stall alleyways (*n* = 34)		ELISA-positive (%)
		qPCR	FC	qPCR	FC	qPCR	FC	
1	760	−	−	−	NA	− − − −	− − NA NA	0.8
2	430	−	+	−	+	−	+	1.7
3	535	−	−	+	−	+ −	+ +	3.4
4	322	−	+	+	+	+ −	+ +	3.5
5	613	+	+	+	+	+ − −	+ + +	3.9
6	470	+	+	+	+	+ +	+ +	10.5
7	140	+	+	+	+	+	+	ND
8	248	−	+	+	+	−	+	2.5
9	330	−	−	−	−	− −	+ NA	0.5
10	423	−	−	−	−	− −	− −	0.6
11	432	+	+	+	+	+	+	3.0
12	920	+	+	+	NA	+ − −	NA NA NA	1.5
13	435	−	+	+	+	+ − −	+ NA NA	2.5
14	228	−	+	−	NA	−	+	2.1
15	590	+	+	+	+	−	−	4.5
16	570	−	−	−	−	−	−	2.0
17	409	+	+	+	+	+	+	1.9
18	284	−	−	−	−	− −	− −	1.0
19	130	+	+	+	+	+	+	4.4
Σ_{Pos}	–	8	13	12	11	11	18	–

− = MAP was not detected in the sample; + = positive detection of MAP in the sample; Σ_{Pos} = sum of positive results; NA = not assessable FC due to contamination; ND = not done

ber of animals housed in a pen. One sample represented the living area of 250–350 housed cows. The sampling procedure was comprised of wiping approximately 20 × 30 cm of the floor area from six different sites by hand while wearing an examination glove. The glove was firmly tied and transported in a cooler to the laboratory. In the laboratory, the faecal material from the glove was mixed and transferred to a 60 ml plastic container and processed immediately or stored overnight at 4 °C.

Bacterial culture

The processing and culturing of the environmental samples were performed according to OIE (The World Organisation for Animal Health; OIE 2018). The samples were decontaminated with 0.9% 1-hexadecylpyridinium chloride (HPC) for 72 hours.

DNA extraction

The total DNA was extracted from 220 mg of the ES using a Genomic DNA kit from the stool samples (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Two modifications were incorporated into the isolation protocol according to Kralik (2011):

1. Instead of the NucleoSpin® Bead Tube A supplied with the isolation kit, 2 ml screw cap tubes prefilled with 350 mg of 0.1 mm zirconia-silica beads were used;
2. the samples were homogenised by a MagNA Lyser (Roche, Mannheim, Germany) at 7 000 rpm for 60 seconds.

The rest of the isolation procedure was performed according the manufacturer's instructions. The DNA was eluted in a final volume of 50 µl and the DNA samples were stored at −20 °C until used in the qPCR.

https://doi.org/10.17221/168/2020-VETMED

Quantitative PCR for detection of MAP DNA

The primer sequences IS*Mav2*-F, IS*Mav2*-R and the pIS*Mav2* probe were previously published by Schonenbrucher et al. (2008).

To design the TaqMan probe, the pIS*Mav2* was lengthened at the 5´end by 14 nucleotides and labelled at the 5´end with carboxyfluorescein (FAM) and at the 3´end by the Black Hole Quencher (BHQ1) (Table 2). The primers and probe were synthesised by Generi Biotech s. r. o. (Hradec Králové, Czech Republic).

A quantitative PCR was performed using the Luna® Universal Probe qPCR Master Mix (New England BioLabs, Ipswich, USA).

The reaction mix recipes were prepared according to the manufacturer's instructions with a final volume of 8 µl of the master mix and 2 µl of the sample DNA. The assay was carried out in the White LightCycler 480 Multiwell Plate 96 (Roche, Mannheim, Germany) and was run in the LightCycler 480 Instrument (Roche, Mannheim, Germany). The thermocycling was set according to the manufacturer's instructions. The fluorescence signals were measured at the end of each extension step. Each measurement was performed in duplicate.

A 163-base pair (bp) fragment amplified by the PCR using the primers IS*Mav2*-F and IS*Mav2*-R cloned into the pMiniT™ 2.0 Vector (New England BioLabs) was used to evaluate the MAP qPCR. A standard curve was established using the standard DNA that was 10-fold serially diluted.

The standard curve showed an efficiency of 1.92, a slope of −3.54 and an intercept of 44.96. The assay was linear over a range of 1×10^{10} to 1×10^{2} DNA copies and detected at least 2 MAP genomes.

Internal amplification control

The internal amplification control (IAC) was synthesised as described by Schonenbrucher et al. (2008) using the primers p17F/IS*Mav2* and p17R/IS*Mav2* (Table 2). A separate qPCR was performed with the TaqMan probe pUC19/17F/16RpVF (Table 2) and 10^4 DNA copies of the IAC. The rest of the PCR reaction mix recipe and cycling conditions remained the same as for the MAP qPCR.

Statistical data analysis

The association in the contingency tables was evaluated using the Fisher-Freeman-Halton exact test (FFH test) by software Statistica v13.2 (StatSoft Inc., Tulsa, OK, USA). Fisher's exact test function (R project, package stats) was used for this purpose. *P*-values < 0.05 were considered to indicate a statistical significance.

RESULTS

Environmental samples

The results of the MAP detection in the individual locations and herds are shown in Table 1. The MAP DNA was detected in eight (42.1%) samples from the milking parlour holding pens, twelve (63.2%) samples from the milking alleyways and eleven (32.4%) samples from the free-stall alleyways by qPCR. No PCR inhibition was detected in the DNA extracts as confirmed by the positive AIC amplification. Thirteen (68.4%), eleven (68.8%) and eighteen (69.2%) of nineteen, sixteen and twenty-six environmental samples with an assessable culture from the milking parlour holding pens, milking

Table 2. Nucleotide sequences of the primers and probes used in the study

Designation	Sequence (5´→3´)
IS*Mav2*-F	CGGCAAAATCGAGCAGTTTC
IS*Mav2*-R	TGAGCCGGTGTGATCATCTTT
pIS*Mav2*-VF	FAM-CACCGCAGTCTGCGCGCTGAGTTCCTTAG-BHQ1
p17F/IS*Mav2*	CGGCAAAATCGAGCAGTTTCCAGACAAGCTGTGACCGTCTCC´
p16R/IS*Mav2*	TGAGCCGGTGTGATCATCTTTACCTCTGACACATGCAGCTCC
pUC19/17F16RpVF	FAM-CTGTGACCGTCTCCGGGAGCTGCATGTGTCAG-BHQ1

A = adenine; BHQ1 = black hole quencher; C = cytosine; FAM = carboxyfluorescein; G = guanine; T = thymine

https://doi.org/10.17221/168/2020-VETMED

alleyways and free-stall alleyways, respectively, were culture-positive. In eleven (15.3%) samples, it was not possible to assess the culture results due to an overgrowth of the contaminating microflora.

In total, MAP was detected in twelve (63.2%) and fifteen (79%) herds by the qPCR and bacterial culture, respectively. The positive herd infection status as determined by qPCR agreed with the MAP detection from the milking alleyways. No MAP was detected by any of the two methods in four (21.1%) herds. The same results (either a positive or negative MAP detection) for all three locations were detected in fourteen (73.7%) herds by the qPCR and thirteen (68.4%) by the bacterial culture. At least one sample from all three locations was positive in ten herds (52.6%) by the bacterial culture and only seven (36.8%) by qPCR.

Association between sampling location and detection method

Out of the culture-positive samples, the highest percentage of qPCR-positive samples was detected for the milking alleyways (90.9%), while it was lower for the milking parlour holding pens (61.5%) and the lowest for free-stall alleyways (55.6%). Nevertheless, these differences were not statistically significant (P = 0.119 0).

When searching for an association between the sampling location and the methods used in the assessable MAP detection results, no statistically significant differences were detected for these results from the milking parlour holding pens and milking alleyways and method used (P = 0.191 4 and > 0.999 9, respectively). Statistically significant differences were observed for these results from the free-stall alleyways [P = 0.008 6, odds ration (OR) = 4.572 8], i.e., there was a 4.6 times higher probability for the results from the free-stall alleyways to be bacterial culture positive than qPCR positive.

Association between sampling locations and qPCR to determine herd infectious status

The infectious status of the herds was determined. The herd was considered to be infected when the MAP was detected in at least one ES. Fifteen (79%) infected and four (21.1%) non-infected herds were identified. The agreement between the herd infectious status and the qPCR results from a single sampling location was evaluated. The herd infectious status was in agreement with sixteen (84.2%), fourteen (73.7%) and twelve (63.2%) qPCR results from the milking alleyways, free-stall alleyways and milking parlour holding pens, respectively. No statistically significant differences were detected for these results (P = 0.396 1).

DISCUSSION

Milking parlour holding pens, milking alleyways and free-stall alleyways were chosen as the places to collect the ES in our study as the manure of the majority of the lactating cows accumulates there and, thus, the MAP detection probability increases. Despite the regular detection of MAP in samples from the manure storage area, it was omitted in our study due to hazardous sampling conditions on farms with lagoons. Previously, a sampling protocol that excluded this area has been published (Donat et al. 2015). A bacterial culture followed by PCR is commonly used to test the ES samples (Berghaus et al. 2006; Pillars et al. 2009; Wolf et al. 2015). To reduce the processing time, qPCR can be used instead of a bacterial culture (Donat et al. 2015). The critical step in the MAP detection by qPCR is the extraction of the DNA from the samples, because the PCR inhibitors present in the faecal material must be sufficiently removed during the extraction procedure (Chui et al. 2004). Amplification of the IAC in the presence of the sample DNA was used to monitor the potential PCR inhibition, and no PCR inhibition was observed in our study. A TaqMan instead of a TaqMan minor groove binder probe, as described by Schonenbrucher et al. (2008), was used in our study in the qPCR due to its better availability and lower price. This slight modification did not adversely affect the efficiency and sensitivity of the reaction. The amplification efficiency and detection limit were 97.8% and 57 DNA copies for the qPCR and 96% and 100 DNA copies for the qPCR, respectively, in the study published by Schonenbrucher et al. (2008).

A bacterial culture is assumed to be "the gold standard" for MAP detection in faecal samples. Only culture-positive ES were considered to be true MAP positive despite the positive herd MAP infection status determined by the detection of ELISA-positive cows in the study herds (Pillars

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et al. 2009). The culture-positive samples were then used to look for an association between the qPCR results of the samples and their sampling locations. A relatively high extent of differences was detected between the percentage of the qPCR-positive samples and the sampling location. The highest percentage of qPCR positive samples was detected for the samples from the milking alleyways (63.2%). Nevertheless, the differences among the locations were not statistically supported (P = 0.119 0), suggesting that MAP detection by qPCR is not affected by the sampling location. Furthermore, in order to evaluate the suitability of the individual sampling locations to detect MAP in the infected herds by qPCR, the herd infectious status was compared with the results of the MAP detection in these locations. Twelve (80%) out of fifteen MAP-positive herds could be identified by the positive qPCR results from the milking alleyways and the overall agreement between the determined herd infectious status and the qPCR results from this location was 82.4%. A smaller numbers of herds, i.e., ten (66.7%) and eight (53.3%), were detected to be MAP-infected based on the qPCR results from the free-stall alleyways and the milking parlour holding pens, respectively. The qPCR results from the milking alleyways seem to be more suitable to identify the herd as MAP-positive or MAP-negative than the other two locations, although the differences among them are not statistically significant (P = 0.396 1). However, our findings may be adversely affected by the small number of samples tested.

Taking the assessable MAP detection results into account, it was found that there was a 4.6 times higher probability for the samples from the free-stall alleyways to be bacterial culture positive than qPCR positive. This observation is in contrast to previously published data suggesting a comparable sensitivity of the bacterial culture and qPCR to detect MAP in faecal material (Alinovi et al. 2009). Only one to three MAP colonies were detected in the culture-positive qPCR-negative samples from the free-stall alleyways suggesting the presence of a very low number of MAP cells in the tested material. The bedding material present in the free-stall samples diluted the number of bacteria in the ES and because a lower amount of input material is used in qPCR (220 mg) than in the bacterial culture (1 g), this may lead to negative results for the qPCR only. Further bacterial growth during the cultivation may lead to positive culture results, even for samples with low MAP concentrations. The qPCR-negative results of the MAP detection in the ES may, thus, reflect the low numbers of MAP cells in the samples approaching the detection limit of the qPCR used.

No MAP was detected in four herds concurrently with low percentages of ELISA-positive cows (0.8–2.0%) detected during the year of sampling. These results agree with previously published data indicating that environmental sampling is not appropriate to discover a positive infection status in low prevalence herds (Smith et al. 2011; Donat et el. 2015).

As environmental sampling is intended to be used in the herd classification programme, the sampling procedure should be easy to perform. Samples from milking parlour holding pens and milking alleyways are easy to collect and faecal material covering these areas after milking has well homogenised faecal material from the majority of the adult cows held in the herd. A relatively smaller area of milking alleyways further reduces the need for the skills of a person collecting the ES. On the contrary, the free-stall alleyway sampling is prone to the collection of individual faecal pats as the bedding material (especially straw) holds individual pats in place and, thus, prevents their thorough homogenisation. Furthermore, free-stall alleyway sampling is more laborious as the alleyways from all holding pens should be sampled, which further increases the cost. Last, but not least, the method of the ES collection from the free-stall alleyways is more difficult to standardise due to the different bedding and manure collection systems in Czech dairy herds. From this point of view, milking alleyway pens and milking parlour holding pens seem to be preferable areas for the environmental sampling to free-stall alleyways.

It can be assumed from the results of our study that qPCR is capable of detecting MAP in the ES from Czech free-stall dairy herds. Considering the assessable MAP detection results, the bacterial culture seems to be more sensitive to detect MAP in samples from free-stall alleyways, while the proportion of MAP-positive samples from milking parlour holding pens and milking alleyways was not significantly influenced by the method used. However, the qPCR seems to be a time-saving alternative to the bacterial culture for MAP detection in environmental samples and could be used as a convenient herd screening method in a national herd classification programme.

https://doi.org/10.17221/168/2020-VETMED

Acknowledgement

Authors wish to thank Dr. Faldikova for correcting and proofreading of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Received: August 26, 2020
Accepted: December 2, 2020