International Journal of Food Microbiology 151 (2011) 314–318

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Correlation of *Mycobacterium avium* subsp. *paratuberculosis* counts in gastrointestinal tract, muscles of the diaphragm and the masseter of dairy cattle and potential risk for consumers

Radka Pribylova, Iva Slana, Petr Kralik, Alena Kralova, Vladimir Babak, Ivo Pavlik *

Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic

ARTICLE INFO

Article history:
Received 5 April 2011
Received in revised form 16 September 2011
Accepted 24 September 2011
Available online 1 October 2011

Keywords:
Johne's disease
Tissue
qPCR
Cultivation
Food safety
Meat inspection

ABSTRACT

Tissues of cattle intended for human consumption can be contaminated by *Mycobacterium avium* subsp. *paratuberculosis (MAP)*. Although different studies attribute varying roles of *MAP* in Crohn's disease progression it is thought that the exposure of humans to this bacterium should in any case be minimised. In this study, we have collected samples of intestine, mesenteric lymph nodes, muscles of diaphragm (*musculus diaphragma*) and masseter muscles (*musculus masseter*) from twenty-five cows in a slaughterhouse. The infectious status of all animals was confirmed by culture of faeces. *MAP* was found in almost all the intestines and mesenteric lymph nodes examined, including three faecal culture-negative animals indicating intermittent shedding. As intestine is used for the traditional production of sausages, it is alarming that 84.2% of intestine samples were positive for *MAP. F57* and IS*900* real time PCR revealed *MAP* in 40 to 68% of diaphragms and 11.1 to 38.9% of masseters. A noticeable dependence of the probability of *MAP* positivity of faeces versus gastrointestinal tract (GIT) and of GIT and muscles was observed. Due to the changing behaviour of consumers, both of these muscles have started to be widely used in cuisine. Therefore, the results of this paper imply that the processing of cows with paratuberculosis in abattoirs without any precautions (restrictions) and the usage of meat for human consumption should be rethought.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is the causal agent of paratuberculosis (Johne's disease) which affects mainly cattle and other domestic and wild ruminants. Paratuberculosis as a chronic granulomatous enteritis targets the gastrointestinal tract (GIT) as the primary site of the infection. In animals with clinical paratuberculosis, the gut wall is corrugated and thickened, mucosa swollen and regional mesenteric lymph nodes enlarged (Manning and Collins, 2001). These pathological lesions markedly resemble the lesions of the human GIT in patients suffering from Crohn's disease. For this and other reasons, *MAP* has been tightly connected with the Crohn's disease for years (Behr and Kapur, 2008; Bull et al., 2003). The current understanding of Crohn's disease is that it is a multifactorial disease, which is triggered by many factors, such as genetic, immune, environmental and infectious components, including *MAP* (Economou and Pappas, 2008; Sibartie et al., 2010). For this reason, minimising consumer exposure through reducing *MAP* presence in food is desirable.

In animals infected with *MAP*, the agent is shed mainly through contaminated faeces (Collins et al., 1993; Cousins et al., 1999). Therefore, examination of faeces using culture or quantitative real time (qPCR) is commonly used for diagnosis of paratuberculosis although it is often complicated by intermittent shedding of *MAP* (Palmer et al., 2007). In slaughtered animals, culture-dependent or culture-independent examination of intestinal tissues is performed to confirm the presence of *MAP* or to assess the distribution of the agent in other tissues and organs (Ayele et al., 2004; Pavlik et al., 2000). Quantitative determination and subsequent comparison of *MAP* numbers in intestine and faeces are however, missing although it can provide additional information about the infectious status of animals.

Faeces containing *MAP* can contaminate the environment and water, which represent, together with milk, a source of contamination for other healthy animals (Raizman et al., 2004; Streeter et al., 1995). Milk is also the most common source of *MAP* for humans (Slana et al., 2009; Streeter et al., 1995). Generally, in milk as well as meat, the presence of *MAP* or any pathogen is either connected with the infectious process and dissemination of the agent into animal tissues and organs or by secondary contamination due to a contaminated environment (Eltholth et al., 2009). In animals with disseminated infection, *MAP* has been described not only in the GIT including mesenteric lymph nodes, but also in other organs, such as the liver, spleen, kidney, lung, heart or reproductive organs (Antognoli et al., 2008; Ayele et al., 2004; Mutharia et al., 2010; Pavlik et al., 2000), some of which can be used for human consumption. Lymph nodes containing *MAP* can also

* Corresponding author. Tel.: +420 5 3333 1601; fax: +420 5 4121 1229.
E-mail address: pavlik@vri.cz (I. Pavlik).

0168-1605/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2011.09.025

represent a risk as they drain muscles and in this way red meat can be contaminated (Antognoli et al., 2008). Apart from smooth muscles, *MAP* has been found in cattle diaphragm muscles (Alonso-Hearn et al., 2009), beef steaks (Mutharia et al., 2010) and sheep rump and forequarter (Reddacliff et al., 2010).

Cattle with paratuberculosis are ordinarily sent to the slaughterhouse and their meat and meat products are used for human consumption. It is known that *MAP* is able to survive pasteurisation temperatures (Grant et al., 2002) and therefore improperly heat-treated meat and meat products represent a risk for consumers. Therefore, the aim of this work was (i) to quantify and compare the number of *MAP* in faeces, intestine, adjacent mesenteric lymph nodes and meat samples — muscle of the diaphragm (*musculus diaphragma*) and muscle of the masseter (*musculus masseter*) of infected cows by culture and qPCR and (ii) according to these results, assess the probability of whether meat from infected animals can pose a risk for human consumption. To accomplish these aims, DNA isolation yield and limit of detection from tissues had to be optimised and evaluated for the purposes of qPCR quantification.

2. Materials and methods

2.1. Origin of the samples

Twenty-five cows with different clinical status of *MAP* infection originating from three different dairy herds with a known history of paratuberculosis were selected. In all herds, *MAP* infection was previously confirmed using quantitative real time PCR (qPCR) and culture. On the basis of number of *MAP* copies in faeces determined by qPCR, 25 examined animals were divided into five groups: non-infected — "negative", low shedders (10^2), moderate shedders (10^3), high shedders (10^4) and heavy shedders ($\geq 10^5$). The age of the cows ranged from 3 to 10 years. The cows were slaughtered during control programmes against paratuberculosis or for other health reasons. Samples of faeces, mesenteric lymph nodes, small intestine mucosa, diaphragm muscle and masseter were collected. After transportation (at 4 °C) to the laboratory, samples were divided and processed immediately or frozen at −70 °C until examination.

2.2. Isolation of DNA

DNA isolation from faeces was performed using a modified protocol of the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to Kralik et al. (2011). The method of DNA isolation from tissues was described previously (Slana et al., 2010) and was based on a modified protocol of the DNeasy Blood &Tissue kit (Qiagen). Briefly, 50 mg of tissue was lysed in the ATL buffer (DNeasy Blood & Tissue kit, Qiagen) and proteinase K (Sigma, St. Louis, MO, USA) at 56 °C (shaking at 1400 rpm). Homogenisation was performed using the MagNA Lyser instrument (Roche Molecular Diagnostic, Mannheim, Germany) and 0.1 mm zirconia silica beads (BioSpec, Bartlesville, OK, USA) at 6400 rpm for 60 s. After precipitation of DNA using ethanol, samples were loaded onto a column, washed with the provided Qiagen washing buffer and eluted into preheated TE buffer (pH 8.0; Amresco, Solon, OH, USA).

2.3. Determination of limit of detection (LOD) and DNA isolation yield from tissue

The DNA isolation yield and limit of detection in faeces were determined as described previously (Kralik et al., 2011; Slana et al., 2008). A *MAP* suspension, cattle strain CAPM 6381 (Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Czech Republic), grown in liquid Middlebrook 7H9 broth (DIFCO, Livonia, MI, USA) with Middlebrook AODC enrichment (DIFCO), 2 μg/ml Mycobactin J (Allied Monitor, Fayette, MO, USA) and antibiotics (penicillin G, chloramphenicol and amphotericin B; all from Sigma) at 37 °C for 6 weeks was washed and clumps were removed by 1 mm beads (Biospec). The final *MAP* suspension was 10-fold serially diluted and the number of *MAP* cells in each dilution was determined by *F57* qPCR assay. Eight dilutions in the range from 4×10^8 to 4×10^1 per spiking dose (20 μl) were used for the spiking of tissue samples.

Portions of 50 mg of cattle diaphragm muscle previously proven negative for the presence of *MAP* by IS*900* qPCR were spiked with 20 μl of diluted suspensions of *MAP* and subjected to DNA isolation (Slana et al., 2010). To allow statistical analysis of data DNA isolation from tissue samples spiked with identical *MAP* dilutions was performed six times. The percentage of DNA isolation efficiency was calculated as the quotient of *MAP* recovered after DNA isolation and theoretical input multiplied by 100. The median of mean values for all eight dilutions was further used as the recalculation coefficient to estimate the "true" number of *MAP* cells in unknown samples. The LOD for both assays was determined as the lowest theoretical number of *MAP* cells used for spiking of tissue that was possible to detect in all six replicates. For IS*900* qPCR assay the limit of detection was also calculated from *F57* values by dividing them by 15, which corresponds to the average number of IS*900* copies in the *MAP* genome (Bull et al., 2000; Slana et al., 2008).

2.4. Culture and qPCR

One gramme of each tissue or faecal sample was homogenised and decontaminated using hexadecylpyridinium chloride (Pavlik et al., 2000). Processed samples were inoculated onto three slants of Herrold's egg yolk medium (HEYM) with Mycobactin J (Allied Monitor) according to Whipple et al. (1991). Cultivation was carried out at 37 °C for up to 4 months. Samples were considered positive when one or more CFU appeared in one or more tubes.

Two independent qPCR assays targeting two *MAP*-specific elements: multicopy IS*900* element and single copy *F57* element (Slana et al., 2008) were applied. Both qPCR assays incorporated competitive internal amplification controls. Absolute quantification of *MAP* cells was performed according to the calibration curve derived from a 10-fold diluted plasmid standard with known copy number. The calibration curve was included in each qPCR run. According to the analytically determined yields from faeces (Kralik et al., 2011) and tissues (this study), the absolute number of *MAP* per 1 g of tissue and faeces was calculated. Each sample was examined in duplicate.

2.5. Statistical analysis

Statistical analysis was performed using the statistical software GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). P values lower than 0.05 were considered statistically significant.

3. Results

3.1. Determination of LOD and DNA isolation yield from tissue

The comparison of *MAP* input and recovered numbers showed that the DNA isolation efficiency varied from 26.48 to 165.50%, with a median of 43.30% and a mean of 61.19% (Table 1). For the subsequent calculation of *MAP* numbers recovered from diaphragms and masseters, the median value was used. The LOD for the tissue samples was experimentally determined to be 4×10^4 and 4×10^3 *MAP* cells per 1 g of tissue for *F57* and IS*900* qPCR assays, respectively (Table 1). The LOD for the IS*900* qPCR assay calculated from *F57* qPCR values was determined to be 2.66×10^3, which is very close to the experimentally determined value.

Table 1
Evaluation of the efficiency of the total *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) DNA isolation procedure from tissue using the IS*900* and *F57* real time quantitative PCR (qPCR) assays.

Mean theoretical input of *MAP* cells by *F57* qPCR[a]	Experimental output of *MAP* cells by *F57* qPCR[b]			Experimental output of *MAP* cells by IS*900* qPCR[c]			Mean *MAP* DNA isolation efficiency (%)[d]
	Mean[e]	SD	Signal ratio[f]	Mean[e]	SD	Signal ratio[f]	
4.00×10^{8}	1.06×10^{8}	2.05×10^{7}	6/6	9.98×10^{7}	1.12×10^{7}	6/6	26.48
4.00×10^{7}	1.10×10^{7}	1.54×10^{6}	6/6	1.29×10^{7}	2.41×10^{6}	6/6	27.38
4.00×10^{6}	1.10×10^{6}	4.11×10^{5}	6/6	1.02×10^{6}	3.57×10^{5}	6/6	27.50
4.00×10^{5}	1.73×10^{5}	6.78×10^{4}	6/6	1.89×10^{5}	5.42×10^{4}	6/6	43.30
4.00×10^{4}	3.17×10^{4}	1.39×10^{4}	6/6	2.45×10^{4}	2.85×10^{3}	6/6	79.13
4.00×10^{3}	2.36×10^{3}	1.15×10^{1}	2/6	1.66×10^{3}	5.15×10^{2}	6/6	59.02
4.00×10^{2}	–	–	0/6	6.62×10^{2}	–	1/6	165.50
4.00×10^{1}	–	–	0/6	–	–	0/6	–

[a] Number of *MAP* cells used for the spiking of tissue.
[b] Number of *MAP* cells recovered after DNA isolation by *F57* qPCR.
[c] Number of *MAP* cells recovered after DNA isolation by IS*900* qPCR, the value was divided by 15 (average number of IS*900* copies in *MAP* genome) to obtain real number of *MAP* cells.
[d] Calculated as the quotient of the mean experimental and theoretical yield of *MAP* cells from IS*900* and *F57* qPCR added to and obtained from the DNA isolation multiplied by 100 (resulting values are in percentages).
[e] The mean values correspond to the absolute amount of *MAP* cells per gramme of tissue.
[f] Number of positive replicates/total number of replicates.

3.2. Culture and qPCR results

Twenty-five examined animals were split into five groups according to the number of *MAP* in their faeces (from 0 to 10^5). One group contained three animals in which the presence of *MAP* DNA or viable cells was not demonstrated in faeces either by qPCR or by culture. However, viable bacteria as well as their DNA were revealed in the mesenteric lymph nodes and intestine of all of them. In one animal from this group, 4.31×10^2 of *MAP* cells were even found in diaphragm muscle (Table 2).

As expected, highest positivity was observed in the intestine and mesenteric lymph nodes (Table 2). IS*900* qPCR showed positivity in 100 and 92.0% of intestine and mesenteric lymph node samples, respectively. Using *F57* qPCR, 88.0 and 72.0% of intestine and lymph node samples were positive, respectively. Viable *MAP* cells were shown to be present in 68% of both intestine and mesenteric lymph node samples. Apart from the GIT, *MAP* was found also in muscles. A relatively high number of *MAP* was detected in diaphragm muscles (68.0% using IS*900* qPCR and 40.0% using *F57* qPCR system) and in masseters (38.9% using IS*900* qPCR and 11.1% using *F57* qPCR).

Table 2
The presence of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in organs and tissues of cows in relation to the number of *MAP* copies in faeces determined by IS*900* and *F57* real time quantitative PCR (qPCR) and cultivation.

Cow no (age in years)	Faeces			Intestine			Mesenteric lymph node			Diaphragm muscle			Masseter muscle		
	IS*900*	*F57*	C	IS*900*	*F57*	C	IS*900*	*F57*	C	IS*900*	*F57*	C	IS*900*	*F57*	C
1 (4)	0	0	0	3.38×10^{1}	0	0	3.91×10^{1}	0	0	0	0	0	0	0	0
2 (4)	0	0	0	5.62×10^{4}	3.19×10^{4}	+	6.89×10^{3}	2.45×10^{3}	+	0	0	0	0	0	0
3 (5)	0	0	0	1.29×10^{5}	4.74×10^{4}	+	8.94×10^{4}	7.41×10^{3}	+	4.31×10^{2}	0	0	0	0	0
4 (3)	1.91×10^{2}	0	0	7.75×10^{3}	5.17×10^{2}	0	0	0	0	7.84×10^{3}	1.03×10^{4}	0	Na	Na	Na
5 (3)	7.36×10^{2}	0	0	2.15×10^{4}	6.65×10^{3}	0	2.75×10^{2}	0	0	0	0	0	1.00×10^{3}	0	0
6 (4)	2.36×10^{2}	1.02×10^{3}	0	4.71×10^{4}	3.15×10^{4}	+	1.17×10^{7}	5.85×10^{6}	+	3.39×10^{3}	0	0	5.35×10^{2}	0	0
7 (4)	3.53×10^{2}	0	0	8.73×10^{4}	5.82×10^{3}	0	2.28×10^{5}	1.52×10^{4}	+	6.54×10^{2}	0	0	4.05×10^{2}	0	0
8 (5)	7.13×10^{2}	0	0	1.36×10^{5}	8.19×10^{4}	0	2.73×10^{2}	0	0	6.77×10^{3}	0	0	0	0	0
9 (5)	5.65×10^{2}	0	0	2.75×10^{2}	0	0	0	0	0	0	0	0	Na	Na	Na
10 (5)	9.41×10^{2}	0	0	7.75×10^{3}	5.79×10^{3}	+	1.00×10^{0}	1.92×10^{1}	0	1.41×10^{3}	2.50×10^{3}	0	0	0	0
11 (5)	5.47×10^{2}	0	0	4.67×10^{5}	1.34×10^{5}	+	6.23×10^{2}	1.95×10^{2}	0	0	0	0	Na	Na	Na
12 (6)	3.18×10^{2}	0	0	9.80×10^{4}	5.69×10^{4}	+	1.25×10^{4}	6.48×10^{3}	+	0	0	0	Na	Na	Na
13 (3)	2.07×10^{3}	0	0	1.66×10^{3}	0	+	2.27×10^{3}	0	+	1.04×10^{3}	0	0	9.87×10^{2}	0	0
14 (3)	1.47×10^{3}	0	0	6.82×10^{2}	4.60×10^{1}	0	1.24×10^{4}	8.27×10^{3}	+	1.10×10^{4}	5.93×10^{3}	0	1.37×10^{4}	8.46×10^{3}	0
15 (3)	1.76×10^{3}	0	0	3.59×10^{4}	2.39×10^{4}	0	8.26×10^{4}	5.50×10^{3}	+	1.74×10^{3}	0	0	4.95×10^{2}	0	0
16 (4)	6.04×10^{3}	0	0	2.17×10^{6}	1.45×10^{5}	+	6.39×10^{6}	5.06×10^{6}	+	1.93×10^{3}	2.14×10^{3}	0	1.41×10^{3}	2.50×10^{3}	0
17 (6)	6.28×10^{3}	4.97×10^{3}	0	8.99×10^{8}	5.46×10^{8}	+	1.49×10^{7}	8.46×10^{6}	+	1.41×10^{3}	2.50×10^{3}	0	0	0	0
18 (6)	3.82×10^{3}	2.07×10^{3}	0	6.35×10^{7}	3.93×10^{7}	+	2.04×10^{3}	0	0	0	0	0	0	0	0
19 (7)	2.63×10^{3}	4.45×10^{2}	0	6.54×10^{7}	3.20×10^{7}	+	5.93×10^{7}	3.63×10^{7}	+	0	0	0	0	0	0
20 (7)	8.26×10^{4}	5.99×10^{4}	+	1.69×10^{8}	8.14×10^{7}	+	4.12×10^{8}	1.59×10^{8}	+	1.93×10^{3}	1.28×10^{4}	0	0	0	0
21 (9)	1.00×10^{4}	8.87×10^{3}	+	4.14×10^{8}	1.87×10^{8}	+	6.02×10^{8}	3.65×10^{8}	+	1.92×10^{3}	1.29×10^{3}	0	0	0	0
22 (9)	4.31×10^{4}	3.72×10^{3}	+	8.32×10^{6}	2.34×10^{6}	+	1.50×10^{7}	3.03×10^{6}	+	5.79×10^{4}	1.44×10^{4}	0	Na	Na	Na
23 (10)	1.97×10^{4}	1.25×10^{4}	+	5.61×10^{9}	3.73×10^{9}	+	1.42×10^{9}	9.23×10^{8}	+	1.03×10^{3}	0	0	0	0	0
24 (5)	3.20×10^{5}	2.32×10^{4}	+	8.08×10^{3}	2.65×10^{4}	+	6.29×10^{3}	3.58×10^{4}	+	1.49×10^{4}	9.93×10^{3}	0	Na	Na	Na
25 (5)	9.63×10^{5}	3.40×10^{5}	+	3.09×10^{10}	1.23×10^{10}	+	4.69×10^{8}	1.21×10^{8}	+	4.64×10^{3}	3.09×10^{3}	+	Na	Na	Na
Total	25	25	25	25	25	25	25	25	25	25	25	25	18	18	18
Positive	22	10	6	25	22	17	23	18	17	17	10	1	7	2	0
%	88.0	40.0	24.0	100.0	88.0	68.0	92.0	72.0	68.0	68	40.0	4.0	38.9	11.1	0

C — cultivation.
Na — not available.

Viable bacteria were shown to be present in only one sample out of 25 diaphragms (4.0%); no viable *MAP* was isolated from masseters (Table 2).

3.3. Statistical analysis of the data

Binary logistic regression revealed no statistically significant difference between the age of the animals and the probability of their qPCR positivity in the five types of samples analysed. On the other hand, correlation and regression analysis revealed that the mean age of cows positive by qPCR in their faeces was higher than the mean age of negative animals for all groups of samples except masseters. When not considering masseters, the average age of positive animals ranged between 5.2 and 6.8; the average age of negative animals ranged between 4.0 and 5.0 years (data not shown). Statistical significance was demonstrated only for the *F57* values obtained from faeces and IS*900* values obtained from masseters ($P<0.01$ where P is two-tailed P-values for unpaired *t*-test with Welch's correction). The application of both statistical approaches to culture analysis did not reveal any statistically significant age dependence for all five types of samples.

With regard to the applicability of the data to predict the probability of tissue (meat) positivity based on the qPCR analysis of faecal samples, analysis of contingency tables was performed. Cows positive in faeces by IS*900* qPCR had a very high estimated probability of *MAP* presence in the GIT and adjacent lymph nodes. A similar finding was also observed for the values obtained by *F57* qPCR and culture. As for the presence of *MAP* in muscles (diaphragms and masseters) the highest probability of positivity was linked with the IS*900* qPCR analysis (77.3% in combined examination of diaphragms and masseters). A similar situation was also observed with the *F57* qPCR data, but the probability of *MAP* positivity was only 60.0%. The lowest probability was associated with the culture examination of muscles (Table 3).

4. Discussion

According to the common dogma, cows with paratuberculosis are thin, have poor body condition and suffer from diarrhoea. In recent years, this was rebutted by Antognoli et al. (2008) and Mckenna et al. (2004) who showed that there is no association between infection status and body condition status of cows as animals with disseminated *MAP* infection can be in good health condition and may not suffer from emaciation and diarrhoea. This was also confirmed by Alonso-Hearn et al. (2009) who demonstrated a massive *MAP* load in the gut of two cows, which did not show clinical signs of paratuberculosis. Currently, not only dairy and beef from subclinical animals with no signs of disease are allowed directly into the human food chain, but also animals with clinical symptoms of paratuberculosis are routinely sent to the abattoir and their meat is used for human consumption (Grant, 2005). Although carcasses undergo veterinary inspection at slaughterhouses, only tissues or organs with visible pathological lesions are condemned. However, such pathological lesions are only observed in animals in the progressive stage of infection (Manning and Collins, 2001).

None or only slightly visible and nonspecific lesions were observed during pathological examination in this study. However, dissemination into tissues and muscles was demonstrated using culture and culture-independent methods in all symptomatic as well as asymptomatic animals, including cows with only 10^2 *MAP* cells in faeces. The highest concentration of *MAP* was expectedly found in the GIT, which is in accordance with previous publications (Antognoli et al., 2008; Pavlik et al., 2000). Although peripheral lymph nodes are often cut off from carcasses and added into ground meat (Antognoli et al., 2008), mesenteric lymph nodes are discarded during the evisceration process and are not normally incorporated into ground beef (Arthur et al., 2008). In contrast, the gut is ordinarily used as a natural sausage casing in the production of homemade products, or by small manufactures and those who produce delicacy sausages. Considering the high concentration of *MAP* in the intestine, and the frequent finding of viable cells and the high positivity even in asymptomatic animals (Table 2), the use of tissues of the GIT for human consumption can represent a significant risk for consumers.

Apart from faecal-positive animals, viable *MAP* cells were also found in non-shedding animals. Such a finding is in concordance with previous reports. Pavlik et al. (2000) detected *MAP* in the GIT of 25.6% cows whose faeces were negative. Heterogeneous distribution of *MAP* and insufficient sensitivity of the decontamination process were proposed as the main reasons for this observation. In our case, the explanation will probably lie in intermittent shedding since qPCR, although a highly sensitive technique was not able to detect *MAP*-specific sequences in faeces. Although we are aware that only a limited number of cows were analysed in this study, there was a noticeable dependence of probability of *MAP* positivity in faeces on GIT and of the GIT on muscles. No dependence among different matrices analysed was found in absolute numbers of *MAP* (*MAP* organisms or CFU). Therefore, due to the still unclear relationship between *MAP* and Crohn's disease, it is highly desirable to minimise the contamination of *MAP* in food. Age dependence did not show any apparent differences between negative and positive animals between the different methods and tissues examined. Positive animals were approx. 1 year older compared to negative cows. Antognoli et al. (2008) did not observe any significant differences between average ages of animals with disseminated infection compared with uninfected animals (5 ± 1.6 and 5.2 ± 2.2 years, respectively).

The examination of muscles used for human consumption did not reveal the presence of viable *MAP* cells in either cow skeletal muscle tissues (Antognoli et al., 2008) or in 200 retail ground beef (Jaravata et al., 2007). In contrast, Alonso-Hearn et al. (2009) demonstrated the presence of *MAP* in 13% of diaphragm muscles using culture, conventional PCR and IS*900* qPCR. Our examination of diaphragm samples revealed the presence of viable *MAP* cells in only one diaphragm sample originating from the cow with a high concentration of *MAP* in faeces. Conversely, *MAP* DNA was shown to be present in a significantly

Table 3
Estimation of the probability of a positive finding of *MAP* in tissues (in %) based on faecal positivity.

Method		Gastrointestinal tract			Muscles		
		Intestine	MLN	Intestine or MLN	Diaphragm	Masseter	Diaphragm or masseter
IS*900* qPCR	P[a]	100.0	90.9	100.0	72.7	46.7	77.3
	CI[b]	nc	70.8–98.9	nc	49.8–89.3	21.3–73.4	54.6–92.2
F57 qPCR	P[a]	100.0	90.0	100.0	60.0	0.0	60.0
	CI[b]	69.2–100	55.5–99.8	69.2–100	26.2–87.8	0.0–41.0	26.2–87.8
Culture	P[a]	100.0	100.0	100.0	16.7	0.0	16.7
	CI[b]	54.1–100	54.1–100	54.1–100	0.0–64.1	nc	0.0–64.1

MLN — mesenteric lymph node.
nc — on the basis of the data available it was not possible to calculate the confidence interval.
[a] Probability of *MAP* finding in respective matrix (%).
[b] 95% confidence interval.

high proportion of diaphragms (Table 2). In spite of the low number of viable cells isolated, the presence of *MAP* DNA in a high proportion of diaphragm samples can point to the presence of viable but non-culturable cells, a negative effect of decontamination on cells (Reddacliff et al., 2003) or the presence of antibiotics in culture media (Whittington, 2009).

Masseter muscle has long been regarded as an inferior type of meat; however, it has begun to be regarded as a delicacy food in high class restaurants. In this study, the presence of *MAP* DNA was first demonstrated in seven masseter muscles using IS*900* and two masseters using *F57* (Table 2). All positive muscle tissues originated from animals in which *MAP* was found in faeces and the gastrointestinal tract. Strict conditions of sample collection were followed and external contamination of muscles by *MAP* can be excluded. In contrast to the diaphragm where viable *MAP* cells were found in one sample, positive findings of *MAP* in masseters were shown only at the DNA level. However, due to the same reasons mentioned for diaphragms, the possible presence of viable *MAP* in masseter muscles cannot be excluded.

A role for *MAP* in Crohn's disease has neither been fully confirmed nor refuted. Taking into consideration the fact that cattle with paratuberculosis are ordinarily sent to abattoirs, the finding of *MAP* in all examined types of organs is significant from the point of view of food safety. Meat is still considered as only a minor source of infection compared in milk. However, our data suggest that the probability of *MAP* presence in meat is higher than was expected. With changes in consumer behaviour, diaphragm and masseter muscles are now widely used, mainly as a delicacy. For this reason, preliminary precautions, e.g., not using paratuberculosis cattle for human consumption and not consuming raw or low-heat treated meat should be followed with the aim of minimising the risk for consumers.

Acknowledgements

The work was supported by the Ministry of Agriculture, Czech Republic (Nos. MZe0002716202 and QH81065) and by the Ministry of Education, Youth and Sports (AdmireVet, CZ 1.05/2.1.00/01.0006; ED0006/01/01) of the Czech Republic.

References

Alonso-Hearn, M., Molina, E., Geijo, M., Vazquez, P., Sevilla, I., Garrido, J.M., Juste, R.A., 2009. Isolation of *Mycobacterium avium* subsp *paratuberculosis* from muscle tissue of naturally infected cattle. Foodborne Pathogens and Disease 6, 513–518.

Antognoli, M.C., Garry, F.B., Hirst, H.L., Lombard, J.E., Dennis, M.M., Gould, D.H., Salman, M.D., 2008. Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection in dairy cattle and its association with antemortem test results. Veterinary Microbiology 127, 300–308.

Arthur, T.M., Brichta-Harhay, D.M., Bosilevac, J.M., Guerini, M.N., Kalchayanand, N., Wells, J.E., Shackelford, S.D., Wheeler, T.L., Koohmaraie, M., 2008. Prevalence and characterization of *Salmonella* in bovine lymph nodes potentially destined for use in ground beef. Journal of Food Protection 71, 1685–1688.

Ayele, W.Y., Bartos, M., Svastova, P., Pavlik, I., 2004. Distribution of *Mycobacterium avium* subsp *paratuberculosis* in organs of naturally infected bull-calves and breeding bulls. Veterinary Microbiology 103, 209–217.

Behr, M.A., Kapur, V., 2008. The evidence for *Mycobacterium paratuberculosis* in Crohn's disease. Current Opinion in Gastroenterology 24, 17–21.

Bull, T.J., Hermon-Taylor, J., Pavlik, I., El Zaatari, F., Tizard, M., 2000. Characterization of IS*900* loci in *Mycobacterium avium* subsp *paratuberculosis* and development of multiplex PCR typing. Microbiology-SGM 146, 2185–2197.

Bull, T.J., Mcminn, E.J., Sidi-Boumedine, K., Skull, A., Durkin, D., Neild, P., Rhodes, G., Pickup, R., Hermon-Taylor, J., 2003. Detection and verification of *Mycobacterium avium* subsp. *paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. Journal of Clinical Microbiology 41, 2915–2923.

Collins, D.M., Stephens, D.M., Delisle, G.W., 1993. Comparison of polymerase chain-reaction tests and fecal culture for detecting *Mycobacterium paratuberculosis* in bovine feces. Veterinary Microbiology 36, 289–299.

Cousins, D.V., Whittington, R., Marsh, I., Masters, A., Evans, R.J., Kluver, P., 1999. Mycobacteria distinct from *Mycobacterium avium* subsp. *paratuberculosis* isolated from the faeces of ruminants possess IS*900*-like sequences detectable by IS*900* polymerase chain reaction: implications for diagnosis. Molecular and Cellular Probes 13, 431–442.

Economou, M., Pappas, G., 2008. New global map of Crohn's disease: genetic environmental and socioeconomic correlations. Inflammatory Bowel Diseases 14, 709–720.

Eltholth, M.M., Marsh, V.R., Van Winden, S., Guitian, F.J., 2009. Contamination of food products with *Mycobacterium avium paratuberculosis*: a systematic review. Journal of Applied Microbiology 107, 1061–1071.

Grant, I.R., 2005. Zoonotic potential of *Mycobacterium avium* ssp *paratuberculosis*: the current position. Journal of Applied Microbiology 98, 1282–1293.

Grant, I.R., Hitchings, E.I., McCartney, A., Ferguson, F., Rowe, M.T., 2002. Effect of commercial-scale high-temperature, short-time pasteurization on the viability of *Mycobacterium paratuberculosis* in naturally infected cows' milk. Applied and Environmental Microbiology 68, 602–607.

Jaravata, C.V., Smith, W.L., Rensen, G.J., Ruzante, J., Cullor, J.S., 2007. Survey of ground beef for the detection of *Mycobacterium avium paratuberculosis*. Foodborne Pathogens and Disease 4, 103–106.

Kralik, P., Slana, I., Kralova, A., Babak, V., Whitlock, R.H., Pavlik, I., 2011. Development of a predictive model for detection of *Mycobacterium avium* subsp. *paratuberculosis* in faeces by quantitative real time PCR. Veterinary Microbiology 149, 133–138.

Manning, E.J.B., Collins, M.T., 2001. *Mycobacterium avium* subsp. *paratuberculosis*: pathogen, pathogenesis and diagnosis. Revue Scientifique rt Technique-Office International Des Epizooties 20, 133–150.

Mckenna, S.L.B., Keefe, G.P., Barkema, H.W., McClure, J., VanLeeuwen, J.A., Hanna, P., Sockett, D.C., 2004. Cow-level prevalence of paratuberculosis in culled dairy cows in Atlantic Canada and Maine. Journal of Dairy Science 87, 3770–3777.

Mutharia, L.M., Klassen, M.D., Fairies, J., Barbut, S., Gill, C.O., 2010. *Mycobacterium avium* subsp. *paratuberculosis* in muscle, lymphatic and organ tissues from cows with advanced Johne's disease. International Journal of Food Microbiology 136, 340–344.

Palmer, M.V., Stabel, J.R., Waters, W.R., Bannantine, J.P., Miller, J.M., 2007. Experimental infection of white-tailed deer (*Odocoileus virginianus*) with *Mycobacterium avium* subsp. *paratuberculosis*. Journal of Wildlife Diseases 43, 597–608.

Pavlik, I., Matlova, L., Bartl, J., Svastova, P., Dvorska, L., Whitlock, R., 2000. Parallel faecal and organ *Mycobacterium avium* subsp *paratuberculosis* culture of different productivity types of cattle. Veterinary Microbiology 77, 309–324.

Raizman, E.A., Wells, S.J., Godden, S.M., Bey, R.F., Oakes, M.J., Bentley, D.C., Olsen, K.E., 2004. The distribution of *Mycobacterium avium* ssp *paratuberculosis* in the environment surrounding Minnesota dairy farms. Journal of Dairy Science 87, 2959–2966.

Reddacliff, L.A., Vadali, A., Whittington, R.J., 2003. The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. Veterinary Microbiology 95, 271–282.

Reddacliff, L.A., Marsh, I.B., Fell, S.A., Austin, S.L., Whittington, R.J., 2010. Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from muscle and peripheral lymph nodes using acid-pepsin digest prior to BACTEC culture. Veterinary Microbiology 145, 122–128.

Sibartie, S., Scully, P., Keohane, J., O'Neill, S., O'Mohony, J., O'Hanlon, D., Kirwan, W.O., O'Mahony, L., Shanahan, F., 2010. *Mycobacterium avium* subsp *paratuberculosis* (*MAP*) as a modifying factor in Crohn's disease. Inflammatory Bowel Diseases 16, 296–304.

Slana, I., Kralik, P., Kralova, A., Pavlik, I., 2008. On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS*900* and *F57* competitive real time quantitative PCR and culture examination. International Journal of Food Microbiology 128, 250–257.

Slana, I., Liapi, M., Moravkova, M., Kralova, A., Pavlik, I., 2009. *Mycobacterium avium* subsp. *paratuberculosis* in cow bulk tank milk in Cyprus detected by culture and quantitative IS*900* and *F57* real-time PCR. Preventive Veterinary Medicine 89, 223–226.

Slana, I., Kaevska, M., Kralik, P., Horvathova, A., Pavlik, I., 2010. Distribution of *Mycobacterium avium* subsp. *avium* and *M. a. hominissuis* in artificially infected pigs studied by culture and 1S*901* and IS*1245* quantitative real time PCR. Veterinary Microbiology 144, 437–443.

Streeter, R.N., Hoffsis, G.F., Bechnielsen, S., Shulaw, W.P., Rings, M., 1995. Isolation of *Mycobacterium-paratuberculosis* from colostrum and milk of subclinically infected cows. American Journal of Veterinary Research 56, 1322–1324.

Whipple, D.L., Callihan, D.R., Jarnagin, J.L., 1991. Cultivation of *Mycobacterium-paratuberculosis* from bovine fecal specimens and a suggested standardized procedure. Journal of Veterinary Diagnostic Investigation 3, 368–373.

Whittington, R.J., 2009. Factors affecting isolation and identification of *Mycobacterium avium* subsp. *paratuberculosis* from fecal and tissue samples in a liquid culture system. Journal of Clinical Microbiology 47, 614–622.