Distribution of Non-Tuberculosis Mycobacteria in Environmental Samples from a Slaughterhouse and in Raw and Processed Meats

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Abstract

Shitaye J.E., Horváthová A., Bartošová L., Morávková M., Kaevska M., Donnelly N., Pavlík I. (2009): **Distribution of non-tuberculosis mycobacteria in environmental samples from a slaughterhouse and in raw and processed meats**. Czech J. Food Sci., **27**: 194–202.

The notification of all cases of diagnosed bovine tuberculosis is a statutory requirement, while the same is not true for other mycobacterial infections. Thus, the establishment of the true incidence of infection with non-tuberculous mycobacteria (NTM) is difficult. The aim of this study was to describe the incidence of NTM in environmental samples from a pig slaughterhouse and from raw and processed meat samples collected from supermarkets and butchers. Three species of mycobacteria (*M. chelonae, M. kansasii*, and *M. intermedium*) were detected in 8.0% of the environmental samples from a pig slaughterhouse and in 9.3% of raw and 7.7% of processed meat, respectively. The isolation of a single NTM species from these samples is a disturbing finding and means that raw meat may be a potential pathway for the transmission of NTM infections to humans.

Keywords: mycobacteria; meat; food safety; zoonosis

Mycobacteria species which are not members of the *Mycobacterium tuberculosis* complex (*MT*C) are generally considered to be non-tuberculous mycobacteria (NTM) and are also known as potentially pathogenic or atypical mycobacteria. However, NTM are capable of causing mycobacterial infections, especially when the individual is predisposed to these species under the influence of certain risk factors (Wallace 1994). The range of infections in humans caused by these NTM is broad and includes, e.g., pulmonary and cervical lymphadenitis caused by *M. avium* complex (*MAC*), *M. kansasii* and *M. intracellulare* (Swanson *et al*. 1998), skin infections caused by *M. marinum* and *M. haemophilum* and nosocomial infections brought about by *M. chelonae* and *M. fortuitum* species (Falkinham 2002). In animals NTM cause infections (Pavlik *et al*. 2000; Shitaye *et al*. 2006b) whose clinical manifestations differ from those of classical tuberculosis infections; their veterinary importance lies instead in a sensitisation to the tuberculin test and/or the yield of para-allergic results.

In most cases, the detection of tuberculosis/mycobacteriosis in infected pigs, cattle and other

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Partially supported by the Ministry of Agriculture of the Czech Republic, Projects No. MZE 0002716202 and NPV 1B53009, and "PathogenCombat" Grant No. FOOD-CT-2005-007081 (Brussels, EC).

animals is based on the observation of tuberculous lesions in parenchymatous organs and lymph nodes during the veterinary meat inspection in abattoirs. NTM species have been known to cause the formation of tuberculous lesions in the lymph nodes of pigs (Pavlik *et al*. 2005; Shitaye *et al*. 2006b); however, this may not occur as frequently as in the cases of *M. bovis* infection in cattle (Teklu *et al*. 2004; Shitaye *et al*. 2006a). Thus, it is entirely likely that animals that lack visible tuberculous lesions may be missed during the veterinary meat inspection, while, in actual fact, they are infected with NTM species.

The main ecological niche for NTM is the environment, where they have been commonly isolated from soil and water (Pavlik *et al*. 2000; Falkinham 2002), from feed and animal droppings (Dvorska *et al*. 2007; Pavlik *et al*. 2007), from non-vertebrates (Fischer *et al*. 2001) and small terrestrial animals (Fischer *et al*. 2000; Pavlik *et al*. 2007). Despite this, NTM species such as members of the *MAC*, *M. fortuitum* and *M. kansasii*, have also been isolated from raw milk samples (Dunn & Hodgson 1982) and *M. avium* subsp. *paratuberculosis* (*MAP*) from retail milk samples and powdered infant milk and retail cheese (Ayele *et al*. 2005; Hruska *et al*. 2005; Ikonomopoulos *et al*. 2005). Although the role of meat in the spread of tuberculosis had not yet been elucidated, Gutierrez Garcia (2006) recently invoked a historical perspective, emphasising meat as a vehicle for the zoonotic transmission of the causal agent of bovine tuberculosis and highlighting its importance and the implications for the health inspections of meat in abattoirs.

To the knowledge of the authors, the isolation of NTM from raw and processed meat products (salami and sausages) has not been adequately reported. With regard to their zoonotic potential, more data is needed on these NTM species. Thus, in this study, the identification of NTM in environmental samples collected from a pig slaughterhouse (in different seasons) and in raw and processed meat is described.

MATERIALS AND METHODS

Sample collection. A total of 112 environmental samples from one pig slaughterhouse were collected in each season of the year 2005 (Table 1). Eighty six raw meat samples (beef, pork, and chicken) and 52 processed and fermented meat products, heat treated meat products, and other sausages of unknown origin were collected from different supermarkets and butchers.

Sample storage. The environmental samples from the slaughterhouse were kept at +4°C for one day only. Both meat samples were kept at –20°C for no longer than three weeks before being examined.

Microscopic examination. Slide smears from the environmental samples from the slaughterhouse and the tissue impressions were stained according to the Ziehl-Neelsen (Z-N) technique for the presence of acid-fast bacilli (AFB). At least 200 fields of view were examined for each sample by light microscopy using a 1000× magnification under oil immersion (Olympus B17, Japan).

Culture examination of environmental and meat samples. Approximately 1 g of each sample was homogenised with a laboratory blender stomacher (Kleinfeld Labortechnik GmbH, Gehrden, Germany) and was subsequently decontaminated in 1N HCl for 15 minutes. The suspension was then neutralised with 2N NaOH until the colour changed to light purple. Two or three drops of phenolphthalein (2%) were used as a pH indicator.

Eighty microlitres of the suspension were inoculated onto two slants each of two solid media and one liquid medium (egg-based media according to the method of Stonebrink; Herrold's egg yolk medium and liquid serum medium according to Sula; Bioveta, Ivanovice na Hané, Czech Republic). The liquid medium contained bovine serum, enzymatic casein hydrolysate, glycerin, L-alanine, phosphate salts, magnesium sulphate, citric salts and malachite green (Matlova *et al*. 2005). The incubation of the cultures was carried out simultaneously at two temperatures (25°C and 37°C). The cultures were monitored after the first week of incubation to rule out the fast growing mycobacteria species and early contamination and then every other two weeks for two months.

Identification of mycobacterial isolates. All AFB positive isolates were tested by PCR according to the technique of Wilton and Cousins (1992), which detects the 16S rRNA gene of the members of the genus *Mycobacterium* and which distinguishes between *M. avium* and other atypical mycobacteria. The presence of 1030 bp fragments indicated that the organism came from the genus *Mycobacterium* while the presence of a 1030 bp fragment together with one 180 bp in size identified the organism as belonging to the *MAC*. In brief,

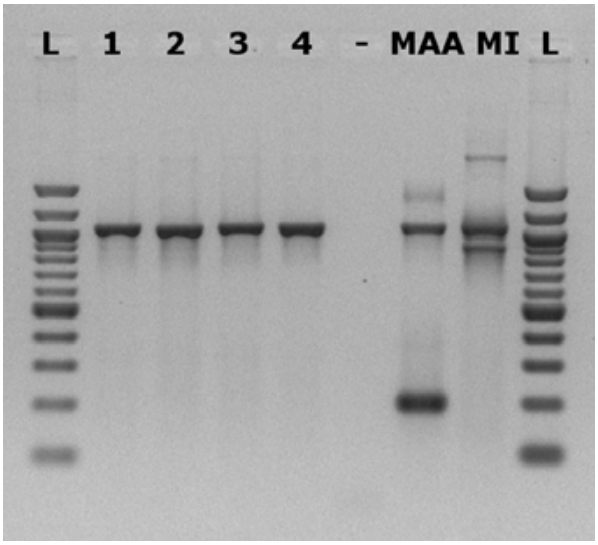
Table 1. Detection of mycobacteria in environmental samples from one pig slaughterhouse in different time intervals during 2005

Examined environmental samples	Season of the year									Mycobacterial species
	winter			spring		summer		autumn		
Samples origin	No.	pos.	CFU	pos.	CFU	pos.	CFU	pos.	CFU	
Curded blood mixed with waste water	4	0	0	0	0	0	0	1*	2	*M. intermedium*
Bristle residue from splinter	4	0	0	1*	20	0	0	0	0	*M.kansasii*
Bristle from the passage of splinter	4	0	0	1*	7	0	0	0	0	*M.* species
Meat scraps from circular saw	4	0	0	0	0	0	0	0	0	
Meat leftovers from traverse	4	0	0	0	0	0	0	0	0	
Meat leftovers from floor	4	0	0	0	0	0	0	0	0	
Meat leftovers from examination table	4	0	0	0	0	0	0	0	0	
Meat leftovers from chisel	4	0	0	0	0	0	0	0	0	
Tissue leftovers from the wall	4	0	0	0	0	0	0	0	0	
Tissue sediments from floor at washing site (carcass)	4	0	0	0	0	0	0	0	0	
Tissue scrapes from floor near inspection site	4	0	0	0	0	0	0	1*	5	*M.* species
Meat leftovers from laser needle for fat control	4	0	0	0	0	1*	3	0	0	*M. intermedium*
Meat leftovers from knife and sharpening steel	4	0	0	0	0	1*	10	0	0	*M.kansasii*
Meat residues from the apron	4	0	0	0	0	0	0	0	0	
Tissue leftovers from disinfectant container	4	0	0	1*	5	0	0	0	0	*M.kansasii*
Tissue leftovers under hunger	4	0	0	0	0	0	0	0	0	
Meat leftovers from carcass near sewage and chilling room	4	0	0	0	0	0	0	0	0	
Water from pipe in stunning cage	4	0	0	0	0	0	0	0	0	
Hot water (66.9°C) for cleaning	4	0	0	0	0	0	0	0	0	
Water used for washing half of pig carcass	4	0	0	0	0	0	0	0	0	
Condensed water near traverse rail	4	0	0	0	0	1*	3	0	0	*M.kansasii*
Condensed water from container of offal	4	0	0	0	0	0	0	0	0	
Condensed water from hook metals	4	0	0	0	0	0	0	0	0	
Disinfectant suspension	4	0	0	0	0	0	0	0	0	
Water for washing hands	4	0	0	0	0	0	0	0	0	
Water from sewage near chilling room	4	0	0	0	0	0	0	0	0	
Water for splash of carcass near chilling	4	0	0	0	0	0	0	0	0	
Condensed water from pipe line near the live weight balance	4	0	0	1*	3	0	0	0	0	*M.* species
Total examined samples	112	0		4*		3*		2*		

CFU = Colony forming units; pos. = positive sample; *M.*= *Mycobacterium*

*Non-tuberculosis mycobacteria species *M. kansasii, M. intermedium* and unidentified mycobacteria species were isolated neither by Geno-type *Mycobacterium* kits nor by biochemical methods

the PCR amplification reaction was made up to a total volume of 20 µl using the Taq PCR Master Mix Kit (QIAGEN, Germany) and two sets of primers (each 0.5 µM) MYCGEN-F (5'-AGA GTT TGA TCC TGG CTC AG-3'), MYCGEN-R (5'-TGC ACA CAG GCC ACA AGG GA-3'), MYCAV-R (5'-ACC AGA AGA CAT GCG TCT TG-3'), MYCINT-F (5'-CCT TTA GGC GCA TGT CTT TA-3'). Two µl of the isolated DNA or bacterial lysate were added to each PCR reaction, except in the case of the negative control. The PCR conditions were as follows: 94°C for 1 min; 30 cycles of 94°C for 1 min, 62°C for 2 min, 72°C for 1 min; and 72°C for 10 minutes. The mycobacterial isolates that were not classified as *M. avium* members were assessed by biochemical methods (pigment production, growth at different temperatures – 25°C, 37°C, and 42°C), nitrate reduction, catalase thermoresistance, Tween-80 hydrolysis, tellurite reduction and arylsulphatase activity (Wayne & Kubica 1986) and by the Geno-type *Mycobacterium* CM/AS kits (HAIN life Science, GmbH, Germany).



L – 100 bp ladder; 1–4 samples analysed (1030 bp); – Negative PCR control; MAA – *Mycobacterium avium* subsp. *avium* (positive control; 1030 bp, 180 bp); MI – *Mycobacterium intracellulare* (positive control; 1030 bp, 850 bp)

Figure 1. PCR results from the AFB positive isolates

RESULTS AND DISCUSSION

Part 1 Environmental samples from the pig slaughterhouse

In the environmental samples (collected in each season of the year) obtained from one pig slaughterhouse mycobacteria were detected by culture in 9 (8.0%) of 112 samples (Table 1). All of them were positive by the *Mycobacterium* genus specific PCR (Figure 1). Six of these mycobacterial species were identified as either *M. intermedium* or *M. kansasii*; three other mycobacterial species were also isolated which could not be identified either by Geno-type *Mycobacterium* kits or by biochemical methods. No mycobacteria were detected in the samples collected during the winter, while 4, 3 and 2 mycobacterial species were detected during the spring and summer and autumn seasons, respectively. No AFB organisms were directly detected microscopically in the environmental samples collected from the slaughterhouse.

Part 2 Meat samples

In the case of the raw meat samples examined, mycobacteria were detected by culture in 8 (9.3%) of 86 samples of chicken meat (n = 24), pork meat (n = 34), and beef meat (n = 28). All eight isolates were identified as *M. chelonae*; out of these, five and three isolates originated from unpacked and packed meats, respectively (Table 2). Mycobacteria were detected in only 4 (7.7%) of 52 of the processed meat samples of salami and sausages, which were freely stored (n = 26), packed (n = 6), or vacuum packed (n = 20). All isolates were identified as *M. chelonae* and were only detected in freely stored products (Table 2). At the microscopic level, AFBs were detected neither in raw meat nor in processed meat samples.

While the incidences of bovine tuberculosis have to be reported by law, the same is not true for other mycobacterial infections, a fact which hampers the collection of the data regarding the true incidence of infection with NTM species. NTM are transmitted to humans from the environment, including through the ingestion of food of animal and fish origins (Ristola *et al.* 1999; Mediel *et al.* 2000). Regardless of this fact, there is only a handful of old reports which studied the presence of NTM in food. NTM have been isolated from beef, pork, lamb (Tison *et al.* 1966), preserves and brine (Tison *et al.* 1966), oysters (Thomas & McDurmont 1975), fish such as Pacific salmon (Arakawa *et al.* 1986), snakehead (*Channa*

Table 2. Detection of mycobacteria in raw meat products from different animals and from fermented and heat treated meat products

Samples/storage status	Examined samples			Mycobacterial species
	No.	positive[1]	CFU[2]	
Raw meat products from different animals				
Chicken meat and internal organs				
Non-packed	8	2	10–20	*M. chelonae*
Packed	16	0	0	
Pork meat and internal organs				
Non-packed	22	2	8–40	*M. chelonae*
Packed	12	0	0	
Beef meat and internal organs				
Non-packed	10	1	6	*M. chelonae*
Packed	18	3	8–15	*M. chelonae*
Subtotal	86	8		
%	100	9.3		
Fermented and heat treated meat products				
Freely stored	26	4	2–20	*M. chelonae*
Packed	6	0	0	
Vacuum packed	20	0	0	
Subtotal	52	4		
%	100	7.7		
Total	138	12		
%	100	8.7		

[1] Negative after Ziehl-Neelsen staining but positive in culture examination; [2] Colony Forming Units

striatus; Chinabut *et al.* 1990), and raw fish for consumption (Ristola *et al.* 1999).

In the first part of this study we have focused on the mycobacterial contamination of the environment of a pig slaughterhouse, since this environment could subsequently serve as a source of mycobacterial contamination of meat or meat products. A direct link may exist a between the infection of animals with NTM species while they were in farm stables and the detection (8.0%) of these same NTM in the remnants of tissues, abattoir utensils, and in other samples from the slaughterhouse. The tap water used for washing the carcasses in the slaughterhouse may also be a source of contamination (Pavlik *et al.* 2000). In addition, animals themselves contaminated with faeces or other contaminants may mechanically transport the mycobacteria on their body and may in turn contaminate the premises during the slaughtering procedures. It has also been documented that some mycobacterial species termed in the literature as "aquatic mycobacteria" (e.g. *M. kansasii, M. chelonae, M. flavescens, M. fortuitum, M. gordonae*) can survive and multiply in biofilms, which are formed on the inner surfaces of tubes of water distribution systems in the process plants (Schulze Röbbecke & Fischeder 1989). Mycobacteria biofilms may be released from the inner surfaces and contaminate instruments, raw materials, products, and the working environment.

Although the environmental samples from the slaughterhouse were collected over different seasons of the year, the influence of seasonal patterns on the detection of mycobacterial isolates in the slaughterhouse was not found to be significant. De-

spite this, no mycobacteria were isolated during the winter season (Table 1). These findings may be related to the health status of pigs that were slaughtered in the respective period of time and to the actual level of contamination with NTM agents. Otherwise, mycobacteria can survive under environmental conditions that are intolerable for most other bacterial genera, including temperatures below 0°C over the course of years (Iivanainen *et al*. 1995).

In the second part of our study, we investigated the mycobacterial contamination of raw or processed meat. The isolation of NTM species from raw or processed meat (salami and sausage) samples from beef, pork, and also chicken is generally a rare event (Yajko *et al*. 1995; Ristola *et al*. 1999). The isolation of a single NTM species from a meat sample is a serious finding because the contaminated meat can possibly play an important role in the transmission of the mycobacterial infection, primarily to immunocompromised humans. For instance, an outbreak of *M. fortuitum* on a human immunodeficiency virus ward was, through molecular typing, strongly linked to an ice machine present on the ward (Gebo *et al*. 2002). Ristola *et al.* (1999) documented a very similar genetic profile between *M. avium* complex members and *M. genavense* from raw eating fish and the isolates from *HIV*-positive patients in the Helsinki area.

M. chelonae, detected in our study in all meat samples or their products, is not a classical causal agent of generalised infection in immunocompromised humans. In most cases it has been documented as a cutaneous infection or an infection after surgery. Regardless of that, this species should also be taken into account now. New cases of generalised mycobacterial infections following the consumption of food contaminated with "generally" NTM in immunocompromised patients have increased. An *M. chelonae* infection was, for instance, described in an 8-year-old girl who had a visibly enlarged submandibular lymph node and a reddish, spongy swelling of the gingiva in the upper right canine region. Culturing of the gingival tissue resulted in the isolation of *M. chelonae* (Pedersen & Reibel 1989).

Mediel *et al*. (2000) described the isolation of different NTM species (*M. peregrinum, M. gordonae* and *M. chelonae*) from gutted and frozen fish in a whole sale market which supplied shops for retail sale and human consumption. In a similar scenario, in the current study of NTM species *M. chelonae* was detected in 9.3% of 86 raw meat samples which had been destined for human consumption (Table 2). In contradiction to this study, however, Yajko *et al.* (1995) found that mycobacteria were almost entirely absent from the 100 meat samples which they examined. The likely explanation for the discrepancy may be associated with the difference in the health status of animals and in the epidemiological circumstances of the mycobacterial infection on the respective farms (intensity of contamination of farms), including the opportunities of surface contamination of meat in slaughterhouses and/or during transportation.

Heat inactivation of *in vitro* grown mycobacteria has been reported in meat products (Merkal & Whipple 1980). However, we succeeded in isolating a similar mycobacterial species from both raw meat and heat treated samples irrespective of the level of the heat regimen. Similar reports regarding the isolation of mycobacterial species, such as *M. avium* subsp. *paratuberculosis*, from a processed food substance, e.g. retail milk (Ayele *et al.* 2005), where they can still be cultivated after undergoing pasteurisation, may indicate an increased capacity in NTM species to withstand heat as compared with pathogenic species, like *M. bovis* (Merkal & Whipple 1980; Cerf & Griffiths 2000). Hence, from the epidemiological point of view, this finding implies that humans, in particular immunocompromised persons, may be at a potential risk of zoonotic infections from the meat destined for consumption which is contaminated with NTM species and is not cooked well enough.

Unlike with the infection with *M. tuberculosis*, there is no evidence of the human to human spread of the NTM species (Horsburgh 1991); instead, all NTM species are environmental organisms which can easily gain access to clinical samples.

The heterocyclic amine 2-amino-1-methyl-6-phenylimidazopyridine, which is derived from cooked meat, has been shown to increase colonic epithelial apoptosis in rats, suggesting a potential disease mechanism (Hirose *et al.* 1998). According to Shoda *et al*. (1996), an increased animal protein intake was the strongest independent factor contributing to the development of Crohn's disease. Recently, Abubakar *et al*. (2007) have claimed that the consumption of tinned meat was associated with an increased risk of disease and it is also possible that *MAP* can be transmitted via meat. From the perspective of the food safety, therefore, the detection of mycobacterial isolates in processed

meat samples (salami and sausages) should be considered a serious development and attention should be focused on establishing a well designed heating process and maintaining stringent sanitation measures and high hygienic standards.

The zoonotic importance of the NTM species causing infections in humans has been adequately documented (Falkinham 2002). Lending further credence to our hypothesis, Gutierrez Garcia (2006), looking at the issue in a historical perspective, highlighted meat as a vehicle for the zoonotic transmission of bovine tuberculosis, underlined its importance and looked at the implications for the health inspection of meat in abattoirs.

With the exception of the *MAC* members, however, in animal species, the specific features and/or characteristics of mycobacterial infections caused by other NTM species have not yet been the focus of sufficient study.

We assumed that foods of meat origin, whether they have been heated, packed and frozen, or have undergone no treatment at all, can be contaminated with NTM. In all samples tested, we isolated *M. chelonae*. Although this species is primarily the causal agent of cutaneous infections after injury or surgery, the possibility that, if consumed, this organism can serve as an opportunistic causal agent of mycobacteriosis cannot be ruled out. However, such instances have not yet been described. At potential risk are largely old people and immunocompromised patients, groups in which mycobacterial infections caused by saprophytic mycobacteria have increased in recent years. The potential significance of this organism increases when one considers that populations are aging and that the number of people infected with *HIV* are increasing.

CONCLUSIONS

Upon the examination of a slaughterhouse environment, we have found that mycobacteria may be present in condensed water or on utensils. Although we detected different mycobacterial species in the slaughterhouse and in the meat samples studied, we do not rule out the possibility that the environment of the slaughterhouse itself could be the source of mycobacterial contamination because only one slaughterhouse was examined. Other studies are necessary to determine all possible routes for mycobacterial contamination of meat or meat products during transport or processing.

Acknowledgements. The authors wish to thank Ms. Eva Slezaková and Ms. Dana Svobodová for their competent technical assistance, MVDr. Alena Lorencová, Ph.D., for her valuable comments (the Veterinary Research Institute in Brno) and Miss Catherine Murdoch from the University of Aberdeen in Scotland (UK) for grammatical corrections.

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Received for publication July 8, 2008
Accepted after corrections June 2, 2009

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