Survival of *Mycobacterium avium* subsp. *hominissuis* in Homemade Smoked Pork Sausages

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ABSTRACT

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We assessed the survival of *Mycobacterium avium* subsp. *hominissuis* (*MAH*) in artificially contaminated homemade smoked sausages prepared from pork meat according to traditional recipes, the effect of storage of such sausages at –20°C for three months on *MAH* viability and to compare assessment of *MAH* viability/presence by culture and qPCR. Three isolates of *MAH* were inoculated into the sausage mixture at concentrations of 10^6 CFU per gram of meat and cold smoked at 40°C for 12 h or hot smoked at 70°C for 6 hours. *MAH* survived the cold smoking procedure without any significant decrease in viable *MAH* CFU counts; no viable *MAH* were detected in the hot smoked sausages. The storage of sausages at –20°C caused a decrease in viable *MAH* counts of about 1 to 3 $\log_{10}$. Absolute *MAH* counts determined by qPCR were not significantly reduced by the storage or smoking. The presence of viable *MAH* in sausages after the cold smoking should be considered as a risk for immunodeficient individuals and children.

Keywords: IS*1245*; pork meat; food safety; zoonosis; heat treatment

Mycobacterium avium subsp. *hominissuis* (*MAH*) belongs to the potentially pathogenic or non-tuberculous mycobacteria (NTM) that are known as causative agents of avian mycobacteriosis in humans and animals. In contrast to *M. avium* subsp. *avium* (*MAA*) *MAH* has been found to be non-virulent for birds, but causes lesions in lymph nodes and parenchymatous organs in domestic pigs (Pavlik *et al.* 2000, 2003, 2007; Mijs *et al.* 2002; Shitaye *et al.* 2006). *MAH* does not infect domestic pigs exclusively, other animals including cattle, wild boar or wild ruminants are common hosts (Machackova *et al.* 2003; Dvorska *et al.* 2004; Pavlik *et al.* 2005; Trcka *et al.* 2006; Moravkova *et al.* 2008). *MAH* infects humans, in which immunocompromised individuals and children are especially susceptible (Horsburgh *et al.* 1994; Bruijnesteijn van Coppenraet *et al.* 2008; Oloya *et al.* 2008). It has been described that *MAH* is capable of survival in the environment and multiplication outside the host organism (Kazda *et al.* 2009).

The main route of *MAH* infection for animals and humans is consumption of contaminated water, food and/or feed (Oloya *et al.* 2008; Kazda *et al.* 2009). The agent is then disseminated to the whole body infecting mainly organs associated with the immune response, e.g., lymph nodes (Pavlik *et al.* 2003; Shitaye *et al.* 2006; Slana *et al.* 2010). There has only been a single study

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dedicated to the presence of NTM in the environment of slaughterhouses and in meat (Shitaye *et al.* 2009). However, NTM other than *MAH* were detected. This notwithstanding, it can be expected that due to the ubiquitous presence of *MAH* in the environment and its proven presence in pigs, *MAH* can be present in the foodstuffs prepared from contaminated meat (Matlova *et al.* 2003; Pavlik *et al.* 2007; Krizova *et al.* 2010).

Detection and identification of *MAH* is mainly based on culture, which is followed by confirmation of the identity of mycobacterial colonies by biochemical or molecular methods (Springer *et al.* 1996; Moravkova *et al.* 2008). Molecular methods are now of great interest because they can distinguish members of the *M. avium* complex and *M. avium* subspecies according to the presence or absence of specific insertion elements in a very short time with high accuracy (Bartos *et al.* 2006; Moravkova *et al.* 2008). *MAH* is characterised by the presence of insertion sequence IS*1245*, which is present in its genome in 7 to 27 copies and absence of the IS*901* sequence (Guerrero *et al.* 1995; Dvorska *et al.* 2003).

Another possibility for detecting and quantifying *MAH* directly in a sample is real time PCR (qPCR). There is a triplex qPCR assay available for the simultaneous detection of *MAA* and *MAH*. This test has been successfully applied for the detection of both agents in artificially infected pigs and in naturally infected hens (Kaevska *et al.* 2010; Kriz *et al.* 2010; Slana *et al.* 2010). The results of these studies showed that a specific triplex qPCR assay was more sensitive than culture and pathology.

During the last two decades, the consumer behaviour has changed and local meat products which are not properly heat-treated are consumed more and more. Particularly in rural areas, homemade sausages from pork meat from domestic pigs and wild boar are consumed not only during the winter time (traditional hunting and country style slaughter), but also during the holiday season. Due to these facts the aims of this study were: (*i*) to determine whether *MAH* can survive traditional rustic cooking procedures that are represented by the cold and hot smoking of sausages, (*ii*) to assess the effect of long term storage of smoked and non-smoked sausages at –20°C on the survival of *MAH*, and (*iii*) to use a previously published triplex qPCR for the detection and quantification of *MAH* regardless of viability and compare these counts with culture CFU before and at the end of the storage.

MATERIAL AND METHODS

Isolates. For the experimental cotamination of the sausage mixture three different *MAH* isolates were chosen: *MAH*-a originated from the submandibular lymph node of a domestic pig (Pavlik *et al.* 2007), *MAH*-b from naturally contaminated peat (Matlova *et al.* 2005) and *MAH*-c from potting soil purchased in a hobby market. A single colony of each isolate was inoculated into Middlebrook 7H9 liquid media (Difco, Livonia, USA) and was cultured at 37°C for one month. The number of *MAH* CFUs in the broth was determined by plating on Herrold's Egg Yolk Medium (HEYM) in triplicates.

Preparation of the mixture and sausage processing. Eight kilograms of ready-to-use sausage mixture was bought in a local butchery. It contained minced pork meat with approx. 40% fat content and flavour enhancers (salt with sodium nitrite, sucrose, spice mixture and dextrose). The mixture was split into four parts of 2 kg. The first one was used as a negative control for the whole experiment to prove that *MAH* was not present in the sausage mixture before the experimental inoculation. The remaining three parts were artificially contaminated with the *MAH* isolates. Fifty ml of the respective *MAH* suspension with the concentration 4×10^7 CFU/ml was added to each part to obtain the final concentration of each *MAH* isolate in the mixture at 10^6 CFU/g. Each mixture was precisely homogenised after the addition of the respective *MAH* isolate. Artificially contaminated and control mixtures were stuffed into a pork gut of a diameter of around 3 cm resulting in sausages with weights from 140 g to 150 g per single sausage. From each mixture, 15 sausages were prepared.

After overnight storage in a refrigerator, 5 sausages from each artificially contaminated mixtures and 5 sausages from control mixtures were subjected to cold smoking at 40°C for 12 h, 5 sausages were smoked at 70°C for 6 h and the remaining 5 ones were not smoked. From the total of 12 groups (smoked vs. non-smoked, artificially contaminated vs. control) one sausage was analysed from each group for the presence and viability of *MAH* by culture immediately after the smoking procedure. The remaining 4 sausages from each group were stored at –20°C.

Storage effect on the survival of MAH in the smoked and non-smoked sausages. In order to

determine the impact of long term storage of sausages at –20°C on the viability of *MAH*, sausages were stored in the freezer for 14, 30, 60 and 90 days. On each collection interval, a sausage from each group was sampled and then discarded to avoid possible reduction of *MAH* viability by repeated freezing and thawing.

Determination of MAH viability in sausages by culture. For the culture, the inner section of the sausage was sampled, homogenised and decontaminated as described previously (Matlova *et al.* 2003). Briefly, 1 g of the sample was transferred to a stomacher bag and 7 ml of sterile PBS was added. The bag was sealed and the sample was mechanically homogenised in the stomacher at maximum speed for 2 minutes. The homogenised sample was centrifuged at 5000× g for 10 min and the supernatant was discarded. The pellet was resuspended in 4 ml of 1N HCl and decontaminated for 20 min at room temperature. HCl was neutralised by titration with 2N NaOH with phenolphthalein used as a pH indicator. The neutralised suspension was centrifuged at 5000× g for 10 min and the supernatant was discarded leaving approx. 1 ml in the tube. The pellet was resuspended in the rest of the supernatant. In order to determine the number of *MAH* CFU in each sample, the decontaminated suspension was further diluted 10^{-2} and 10^{-4} in PBS. One hundred microliters from undiluted and diluted *MAH* suspensions were precisely spread on three flasks with HEYM and cultured at 37°C for 2 months. Evaluation of the cultures was performed each week for the first month (in order to see possible contamination) and subsequently at the end of culture.

Identification of mycobacterial isolates by PCR. The identity of all isolates was confirmed by a conventional PCR method for the determination of *M. avium* subspecies (Moravkova *et al.* 2008).

Quantification of MAH directly in the sausages by triplex qPCR. A previously reported triplex qPCR assay amplifying insertion sequences IS*901* and IS*1245* for the simultaneous detection and quantification of *MAA* and *MAH* with an internal amplification control was used in order to determine the number of *MAH* in the non-smoked and smoked sausages regardless of viability (Slana *et al.* 2010). DNA from the sausages (including control ones) was isolated according to the protocol of DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) modified to improve the yield and purity of the DNA (Slana *et al.* 2010). The number of *MAH* in the isolated DNA was assessed according to the gradient of standard plasmids. The recalculation was done according to the assumption that a single *MAH* cell contains on average 15 copies of IS*1245* element. The number of *MAH* was then recalculated to 1 g of sausage and the experimentally determined efficiency of DNA isolation was taken into account (Slana *et al.* 2010). Isolated DNA was analysed by triplex qPCR in analytical triplicates. The *MAH* number in the sausages before storage and at the end of storage (after 90 days) was assessed.

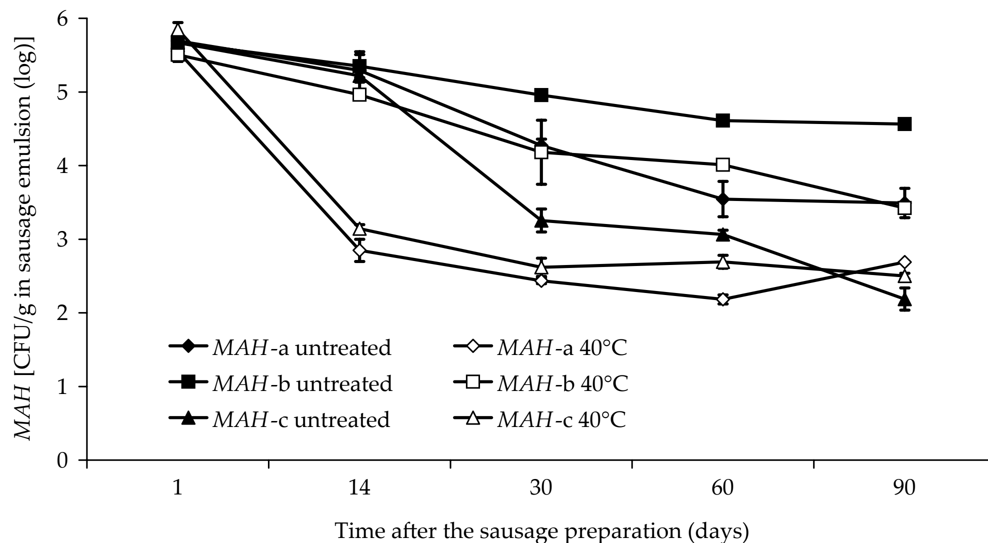
RESULTS AND DISCUSSION

Survival of *MAH* in sausages after smoking

Control sausages (not contaminated with *MAH*) were culture negative. All three isolates of *MAH* used in this study survived cold smoking at 40°C for 12 h (Figure 1). There was almost no change in *MAH* CFU numbers compared to the non-smoked sausages. The main risk associated with consumption of meat products contaminated with NTM (including *MAH*) is that they are generally more thermoresistant than the other mycobacteria (compared to *M. bovis*; Merkal & Whipple 1980; Pavlas 1997). The viability of *MAH* in contaminated Wiener sausages was not reduced even in the presence of a high concentration of sodium nitrite and smoked at 50°C for 30 min (Merkal *et al.* 1979). This shows that even higher temperature, but for shorter time does not reduce *MAH* viability in smoked sausages.

The efficient temperature that is lethal for *MAH* in Wiener sausages was determined to be 68.3°C for a moment (Merkal *et al.* 1979). As it would be expected, there was no growth of *MAH* observed after hot smoking at 70°C for 6 hours.

There is no information about the absolute numbers of *MAH* in the meat products. The initial number of *MAH* used for inoculation of mixtures in this study was chosen with regard to the study where it was found that pigs fed with the peat and reared on the organic farms can have heavy loads of *MAH* in various organs (Matlova *et al.* 2005). In further studies, different inoculum sizes should be tested in order to determine whether it has any impact on the survival of *MAH* during the smoking.



Black symbols represent the non-smoked sausages artificially contaminated with *MAH* whereas empty symbols represent sausages smoked at 40°C for 12 h. Error bars represent standard deviations of mean obtained from three culture media. *MAH*-a = isolate from submandibular lymph node of domestic pig; *MAH*-b = isolate from naturally contaminated peat; *MAH*-c = isolate from potting

Figure 1. Influence of sausage storage at –20°C on the *Mycobacterium avium* subsp. *hominissuis* (*MAH*) viability in time

Effect of sausage storage at –20°C on the viability of *MAH*

During the storage of sausages contaminated with different isolates of *MAH* at –20°C a reduction of CFU numbers was observed (Figure 1). During the storage, no growth of *MAH* was observed in hot smoked sausages. After 14 days of storage, there was a slight reduction in CFU numbers in non-smoked sausages. In contrast, cold smoked sausages contaminated with *MAH*-a and *MAH*-c isolates showed an almost 3 $\log_{10}$ reduction compared to Day 1. In cold smoked sausages contaminated with *MAH*-b, the reduction was not so dramatic and was comparable with the non-smoked samples.

After a month of storage in a freezer, the number of CFU in non-smoked sausages contaminated with the isolate *MAH*-c dramatically decreased by 2 $\log_{10}$. Over the next two months of storage there was an additional 1 $\log_{10}$ reduction. Such a trend was not observed in sausages contaminated with the other two *MAH* isolates where the overall reduction in viable *MAH* CFU counts was only 2 $\log_{10}$ and 1 $\log_{10}$ for sausages contaminated with *MAH*-a and *MAH*-b, respectively (Figure 1).

In cold smoked sausages no further significant reduction in CFU counts was observed for sausages contaminated with *MAH*-a and *MAH*-c and the final number of viable *MAH* after 90 days of storage remained similar as for that determined after 14 days. An exception to this was cold smoked sausages contaminated with *MAH*-b, where the CFU reduction continued and ended at 2 $\log_{10}$ after 90 days of storage (Figure 1).

The survival of tuberculous and NTM in smoked and dried sausages has been previously investigated (Savov 1975). In dried meat products *M. avium* of a non-defined serotype was detectable by culture even after 180 days of storage at 6°C. In smoked sausages viable mycobacteria were isolated until day 170. It can be concluded that although there were differences in CFU numbers among the *MAH* isolates and the smoked and non-smoked sausages, the general trend of a decrease in *MAH* CFU counts over the time was observed for all *MAH* isolates.

Quantification of *MAH* in sausages by triplex qPCR

Samples of control and *MAH* contaminated sausages before storage at –20°C and after 90 days of storage were analysed. No *MAH* was detected in control non-contaminated samples confirming the results of culture. The absolute numbers

Table 1. Numbers of *Mycobacterium avium* subsp. *hominissuis* in artificially contaminated smoked and non-smoked sausages before and at the end of storage as determined by triplex quantitative PCR

Isolate	Smoking conditions	Before storage		After 90 days of storage	
		mean[a]	SD	mean	SD
MAH-a	non-smoked	1.07E+08	6.04E+07	5.63E+08	1.99E+08
	40°C/12 h	4.25E+08	3.28E+08	5.38E+08	1.58E+08
	70°C/6 h	3.10E+08	3.94E+08	5.28E+08	2.63E+08
MAH-b	non-smoked	2.08E+08	2.22E+08	3.79E+08	2.74E+08
	40°C/12 h	5.52E+08	2.75E+08	6.15E+08	2.37E+08
	70°C/6 h	5.88E+08	2.41E+08	1.07E+09	1.88E+08
MAH-c	non-smoked	1.21E+08	1.40E+08	2.41E+08	2.42E+08
	40°C/12 h	6.06E+08	3.42E+08	9.89E+08	1.27E+08
	70°C/6 h	5.16E+08	3.47E+08	1.49E+09	9.68E+08

MAH = *Mycobacterium avium* subsp. *hominissuis;* SD = standard deviation of mean; *MAH*-a = isolate from submandibular lymph node of domestic pig; *MAH*-b = isolate from naturally contaminated peat; *MAH*-c = isolate from potting soil purchased in a hobby market

[a]Mean absolute number of *MAH* cells per one g of the sausage calculated from the three analytical replicates.

of *MAH* without consideration of viability were stable for the whole course of the experiment and no significant changes were observed even in hot smoked sausages as well as in the cold smoked and non-smoked ones (Table 1). The overall absolute numbers of *MAH* CFU from smoked and non-smoked sausages was more than 2 $\log_{10}$ lower than absolute numbers of *MAH* obtained by a triplex qPCR assay. Similar results were observed with *MAA* and *MAH* when tissue samples from artificially infected pigs were tested by culture and triplex qPCR simultaneously (Slana *et al.* 2010) and in *M. a. paratuberculosis* when a sample is regarded as culture positive when it contains more than 10^3 of *M. a. paratuberculosis* cells as determined by qPCR (unpublished observation). What should be considered is the fact that triplex qPCR revealed that the absolute number of *MAH* cells in the samples did not change during the smoking and storage; the role of dead but intact mycobacterial cells (otherwise the DNA would be degraded in such a complex matrix as the meat is) in foodstuff should not be neglected.

Foodborne zoonoses originating from pork meat also now include mycobacteria. Statistical analysis of 440 articles about foodborne zoonoses reveals that although the presence of non-specified mycobacteria in meat is rather rare, the severity scores for mycobacterial infection is very high and comparable with infections with *Listeria monocytogenes* or *Clostridium botulinum*. Cases of mycobacterial infections originating from pork meat are frequently associated with systemic infections, hospitalization and even with death (Fosse *et al.* 2008).

CONCLUSIONS

In conclusion, from the food safety point of view traditional rustic cold smoking of sausages and their consumption could represent a potential risk for consumer health. Although the smoking itself should have antibacterial effects and was applied for 12 h, the viability of *MAH* was not reduced. Storage of the sausages in a freezer showed a negative impact on *MAH* viability in the sausages, but the reduction in viability was not complete. On the other hand, hot smoking of at least 70°C proved to be sufficient for the efficient eradication of viable *MAH* in sausages. Comparison between qPCR and MAH isolation suggested that molecular methods may be useless for evaluating the decontamination procedures because when it was used there were no changes in cells' number. With regard to human health, there is no information about the possible influence of intact dead mycobacterial cells on the immune system of immunocompromised individuals or children.

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