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Comparison of two decontamination procedures, three culture media, and real time-PCR assay for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from artificially contaminated raw sausage*

*Vergleich von zwei Dekontaminationsverfahren, drei unterschiedlichen Kulturmedien und real time PCR-Verfahren zum Nachweis von Mycobacterium avium subsp. paratuberculosis (MAP) aus artifiziell kontaminierter Rohwurst**

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** Dedicated to Prof. Dr. Karsten Fehlhaber on the occasion of his 65th birthday*
Herrn Prof. Dr. Karsten Fehlhaber zum 65sten Geburtstag gewidmet

Summary

Mycobacterium avium subsp. *paratuberculosis* (MAP) has been suggested as a possible cause of Crohn's disease (CD) in humans, which is a chronic granulomatous ileocolitis that typically affects young adults. Milk and meat are considered source of MAP exposure to humans. Depending on the initial concentration, few MAP cells can survive pasteurization process. A systematic review of previous studies demonstrated that meat contaminated with MAP can be a possible source of MAP-exposure to humans. The objective of this study was to establish a reliable method to detect viable MAP cells in meat products. In the study three procedures for MAP isolation from artificially contaminated raw sausage were compared. The influence of different decontamination methods (N-Acetyl-L-Cystein-NaOH [NAC-NaOH]; Hexadecylpyridiniumchlorid [HPC]) on survival of MAP, as well as the effectiveness of the decontamination methods regarding the elimination of companion undesirable flora using different MAP-specific selective culture media was tested. The confirmation of MAP was carried out using an in-house developed TaqMan Real-Time PCR assay targeted to the specific MAP genome regions IS*Mav2* and F57. The results of six spiking experiments showed that the combination of HPC used for decontamination and Herrold's Egg Yolk Medium (HEYM) used as MAP-selective culture medium is the most sensitive method to detect MAP in raw sausages (detection limit of approximately 10^2 colony forming units (cfu)/g). In addition, the results of the study showed that the in-house developed TaqMan Real-Time PCR in combination with modified DNA extraction methods is a reliable method to detect MAP in raw sausages.

Keywords: Johne's disease, Crohn's disease, decontamination, culture, PCR

Zusammenfassung

Mycobacterium avium subsp. *paratuberculosis* (MAP) wird als mögliche Ursache von Morbus Crohn (MC) beim Menschen diskutiert, einer chronischen, granulomatösen Ileocolitis, die vorwiegend bei jungen Erwachsenen auftritt. Als Expositionsquellen des Menschen kommen u. a. Milch und Fleisch in Betracht. In Abhängigkeit von der Ausgangskonzentration können MAP-Zellen bei der Milchbearbeitung Pasteurisierungsverfahren überleben. Eine systematische Literaturrecherche belegt, dass auch mit MAP kontaminiertes Fleisch und Fleischerzeugnisse eine mögliche Expositionsquelle des Menschen sein könnten. Ziel der eigenen Untersuchungen war es, Grundlagen für eine zuverlässige Erfassung von lebensfähigen MAP-Zellen in Fleischerzeugnissen zu schaffen. Dazu wurden drei unterschiedliche Nachweisverfahren zur Isolierung von MAP aus artifiziell kontaminierten Rohwurstproben vergleichend untersucht. Es wurde sowohl der Einfluss von zwei üblichen

Dekontaminationsmitteln (N-Acetyl-L-Cystein-NaOH [NAC-NaOH]; Hexadecylpyridiniumchlorid [HPC]) auf die Überlebensfähigkeit von MAP, als auch die Effektivität der eingesetzten Dekontaminationsmethoden hinsichtlich der Reduzierung der Begleitflora geprüft. Gleichzeitig wurden verschiedene MAP-spezifische Selektivnährmedien berücksichtigt. Die Bestätigung von MAP erfolgte unter Verwendung eines institutseigenen TaqMan® Real-Time PCR Assays für die MAP-spezifischen Zielregionen IS*Mav2* und F*57*. Die Ergebnisse aus sechs Einmischversuchen lassen erkennen, dass die Kombination von HPC als Dekontaminationsmittel und Herrold's Egg Yolk-Schrägmedium (HEYM) als Selektivmedium die sensitivste Methode (Nachweisgrenze ca. 10^2 Kolonie bildende Einheiten (KbE)/g Rohwurst) zum Nachweis von MAP aus Rohwurst darstellt.

Schlüsselwörter: Johne'sche Krankheit, Morbus Crohn, Dekontamination, Kultur, PCR

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of Johne's disease, a chronic granulomatous enteritis of domestic and wild ruminants. Cattle are commonly infected at early stages of life by ingestion of MAP contaminated colostrum, milk or contaminated feedstuff but clinical symptoms appear after approximately 2–5 years (Whitlock and Buergelt, 1996; Stabel, 1998). Clinically and subclinically infected animals shed MAP periodically in their faeces and milk (Sweeney et al. 1992). In advanced cases of Johne's disease 10^6–10^8 MAP cells per gram faeces are commonly seen (Whittington et al., 2000). MAP has been suggested as a possible cause of Crohn's disease (CD) in humans, which is a chronic granulomatous ileocolitis that typically affects young adults (Hermon-Taylor and Bull, 2002; Uzoigwe et al., 2007). However, although the association between MAP and CD seemed to be confirmed, causality still remains controversial (Mendoza et al., 2009). Human exposure to MAP could be via milk and meat products. Depending on the initial concentration of MAP in milk, few MAP cells can survey usual pasteurization process (Grant et al., 2005). MAP has been detected in raw and pasteurized milk and cheese on retail sale in different countries and in powdered infant milk in the Czech Republic (Grant et al., 2002; Ayele et al., 2005; Stephan et al., 2007; Botsaris et al., 2010; Hruska et al., 2011). A systematic review of previous studies showed that meat can also be contaminated with MAP (Eltholth et al., 2009). Meat may be contaminated with MAP by dissemination of the bacterium in the tissues of infected animals via the blood stream, localized foci of infection (lymph nodes that can be mixed into minced/ground beef) or by faecal contamination of the carcass (Grant, 2010). Previous studies reported an infection prevalence of MAP at the slaughterhouse of 16 % in culled dairy cattle in North America (McKenna et al., 2004), 34 % in culled cows in the US (Wells et al., 2009), and 16 % in Danish dairy cattle (Okura et al., 2011). In a previous study to investigate the extent of contamination of beef carcasses with MAP, swab samples from the surface of skinned and dressed carcasses were collected and MAP-DNA was detected by PCR from the surface of the latter (Meadus et al., 2008). Until now, there have been few investigations on MAP in meat. Negative results were obtained in the USA from 200 retail ground beef samples which were tested by PCR (Jaravata et al., 2007). MAP was recovered from prescapular and popliteal lymph nodes, which could be present in ground beef, but not from two skeletal muscles from several infected cows in the USA (Antognoli et al., 2008). Alonso-Hearn et al. (2009) reported for the first time the isolation of MAP from skeletal muscle (diaphragm samples) from 13 % of 47 dairy and beef cattle from Spain by culture. In this study, four of the six positive cattle had shown clinical symptoms of paratuberculosis. Whittington et al. (2010) evaluated the efficacy of cooking in destroying MAP in red meat. They tested the inactivation of MAP in a fluid homogenate of lamb skeletal muscle and showed that there is a very low risk of viable MAP cells in cooked red meat. This finding was confirmed by Mutharia et al. (2010) who showed that MAP may be present in meat from infected animals at low numbers, but that MAP is inactivated when meat is cooked to a well done condition. In general, there are no studies regarding the survival of MAP in meat products including raw sausages. It is not clear whether processing steps such as reduction of pH value, reduction of water activity, or different starter cultures could affect the survival of MAP in raw sausages.

Due to the necessary use of complex media and the long times required for cultivation, MAP is likely to be overgrown by other organisms naturally present in samples unless such organisms are inhibited or destroyed. Destruction or reduction of competing organisms is achieved by subjecting samples to decontamination treatments and/or supplementing media with antibiotics to which MAP are relatively resistant (National Advisory Committee on Microbiological Criteria for Foods, 2010; Whittington, 2010; Gill et al., 2011). In general, it has been stated that currently, neither cultural nor molecular methods are able to provide accurate quantitative information about the numbers of MAP present in a sample and lack of method standardization complicates comparison among studies (National Advisory Committee on Microbiological Criteria for Foods, 2010; Grant, 2010). The cultural methods could be considered a "reference" standard, but they are not ideal. Factors such as the adverse effect of chemical decontamination on the viability of some of the MAP present, overgrowth of cultures by other bacteria potentially masking the presence of MAP colonies, and declumping steps may affect the accuracy of detection and are expected to underestimate the true number of organisms present in any particular sample (National Advisory Committee on Microbiological Criteria for Foods, 2010; Grant, 2010). On the other hand, PCR is an excellent method for confirmation of cultures thought to be MAP. However, the sensitivity of molecular-based methods as applied to complex

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samples is limited by matrix and sampling effects which can result in false-negative results if internal controls are not used in the assay. DNA-based molecular methods are hampered by an inability to discriminate viable from non viable MAP (National Advisory Committee on Microbiological Criteria for Foods, 2010; Grant, 2010). In addition, limits of detection, sensitivity, and specificity vary with the targeted sequence and primer choice, matrix tested, and PCR format. The real-time PCR format eliminates the subjective interpretation of gels, providing an objective determination of the presence or absence of MAP DNA and theoretically can be adapted to produce quantitative estimates of MAP concentration. Incorporation of an internal amplification control to each reaction minimizes the likelihood of false-negative results in both conventional and real-time PCR formats (National Advisory Committee on Microbiological Criteria for Foods, 2010).

The objective of this study was to establish a reliable method to detect viable MAP cells in raw sausages.

TABLE 1: *Overview of the study design.*

Trial number	Raw sausage	Decontamination treatment	Culture media
1, 2, 3	"Teewurst"	Without decontaminant*	MGIT HEYM Middlebrook 7H10
		NAC-NaOH	MGIT HEYM Middlebrook 7H10
		HPC	MGIT HEYM Middlebrook 7H10
4, 5, 6	"Salami"	Without decontaminant*	MGIT HEYM Middlebrook 7H10
		NAC-NaOH	MGIT HEYM Middlebrook 7H10
		HPC	MGIT HEYM Middlebrook 7H10

* additionally investigated by the triplex real-time PCR assay

Materials and Methods

Preparation of inoculum

The *Mycobacterium avium* subsp. *paratuberculosis* (MAP) reference strain (DSMZ 441335/ATCC 19698) was used in this study. The inoculum used for spiking was prepared by inoculating 10 ml Middlebrook 7H9 broth (Difco Laboratories, Augsburg, Germany) containing 10 % OADC supplement (Becton-Dickinson, Heidelberg, Germany), 2 µg/ml mycobactin J (Allied Monitor, Fayette, USA), 0.05 % Tween 80 (Sigma-Aldrich, Schnelldorf, Germany) and 2.5 % glycerol with a colony of the reference strain of MAP obtained from a slant of Herrold's Egg Yolk Medium (HEYM, Becton-Dickinson). MAP reference strain were grown in a shaker incubator for 6–8 weeks at 37 °C. Before being used to spike raw sausages, broth cultures were centrifuged (2500 x *g,* 15 min) and the pellets resuspended in phosphate buffered saline (PBS, Na2HPO4, NaH2PO4, NaCl, H_2O; pH 7.4) and mixed to reduce clumping by vortexing (three times for 2 min) with five 2 mm sterile glass beads (VWR International, Darmstadt, Germany) to yield a suspension containing approximately 10^7 MAP-cfu/ml.

Preparation of spiked raw sausage

Raw sausages ("Teewurst" and "Salami") were obtained from a retail store. Two hundred grams raw sausage was homogenized under sterile condition with the Ultra-Turrax, and 18 g portions of the homogenate were placed into sterile tubes. An initial MAP concentration of approximately 10^7 cfu/ml was set using a photometer and a counting chamber. Ten-fold serial dilutions of initial suspension (10^5–10^3 cfu/ml) were prepared using PBS and 2 ml of each dilution was added to 18 g homogenate. The positive control used were "Teewurst" and "Salami" spiked with 10^7 cfu/ml MAP, the negative control "Teewurst" and "Salami" were spiked with equivalent volume of sterile PBS, respectively. The serial dilutions used for spiking were confirmed by PCR, culture on Middlebrook 7H10 agar, and counting chamber.

In this study, six trials (1 to 6) were carried out to determine the best combination of different decontamination methods and cultural media as well as die isolation of MAP-DNA for real time-PCR assay for raw sausages. Study design is shown in table 1.

MAP culture

The following decontamination and cultural methods were compared:

Method 1: Culture without decontamination. 10 g of spiked homogenate and 90 ml buffer solution were added in a stomacher bag with a filter and homogenized for 2 min. Then 100 µl were inoculated in duplicate into HEYM containing 2 µg/ml mycobactin J, into Mycobacteria Growth Indicator Tubes (MGIT, Becton Dickinson) containing 500 µl OADC supplement (Becton Dickinson), 9.42 µl mycobactin J (Allied Monitor) and 100 µl of an antibiotic mixture (polymyxin B at 2.000 U/ml, amphotericin B at 200 mg/ml, nalidixic acid at 800 mg/ml, trimethoprim at 200 mg/ml, azlocillin at 200 mg/ml, and polyoxyethylene stearate (PANTA, Becton Dickinson) and onto Middlebrook 7H10 agar plates. The Middlebrook 7H10 agar plates were prepared in house and contained (per liter) Middlebrook 7H10 agar basis (19.0 g, Becton Dickinson), glycerol (5.0 ml), the antibiotic mixture PANTA plus (21.0 ml, Becton Dickinson), a supplement (OADC enrichment, Becton Dickinson) and mycobactin J (2 µg/ml, Allied Monitor). The antibiotic mixture, the supplement and the mycobactin J were added after the medium was autoclaved. After using the spread plate technique plates were wrapped with Parafilm (Pechiney Plastic Packaging Company, Chicago, USA) to protect them from drying out during the long incubation period. Plates were incubated at 37°C for a minimum of 12 weeks. All media were checked weekly for mycobacterial growth and for contamination. The final evaluation was done after 12 weeks of incubation.

Method 2: Decontamination of raw sausages with NAC-NaOH. 3 g of spiked homogenate were placed in a sterile tube and 3 ml of N-acetyl-L-cysteine (NAC, Merck, Darmstadt, Germany) were added. After vortexting, the samples were shaken at room temperature for 20 min. After addition of 2 ml phosphate buffer, samples were centrifuged at 3.500 x *g* for 15 min. Pellets were resuspended in 1 ml sterile saline solution, after that 100 µl were inoculated duplicate into HEYM, MGIT, and onto Middlebrook 7H10 agar plates (see above).

Method 3: Decontamination of raw sausages with 0.75 % hexadecylpyridinium chloride (HPC). 3 g of spiked homogenate were placed in a sterile 50 ml tube and the tubes were filled up to 30 ml with 0.75 % (w/v) hexadecylpyridinium chloride (HPC, Merck, Darmstadt). After vortexting and five minutes sedimentation, sausage particles were removed. Tubes were placed on a shaker incubator for 30 min followed by a 24 hours sedimentation period at room temperature in the dark. After sedimentation samples were centrifuged at 900 x *g* for 30 min. Pellets were resuspended in 1 ml of sterile saline and then 100 µl were inoculated in duplicate into HEYM, MGIT and onto Middlebrook 7H10 agar plates (see above).

Extraction of MAP DNA

DNA extraction was carried out starting from 1 ml of spiked raw sausage samples in duplicate and from 1 ml of the tenfold serial dilutions of broth cultures. For this purpose the protocol for gram-positive bacteria of the Qiagen DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) was used. Briefly, after centrifugation at 7.500 x *g* for 10 min, the pellets were resuspended in 180 µl enzymatic lysis buffer for gram-positive bacteria, mixed thoroughly by vortexing and were transferred to 2 ml screw cap tubes containing ceramic beads (SiLibeads®, Lindner, Germany). Tubes were subjected to a mechanical cell homogenization and disruption step with the Fastprep Ribolyzer (Q-biogene, Heidelberg; Germany) to achieve efficient cell lysis. Samples were then incubated for 90 min at 37 °C with shaking in a thermomixer (Eppendorf, Hamburg, Germany). After addition of 25 µl proteinase K (Qiagen) and 200 µl buffer AL, samples were incubated for 90 min at 70 °C with shaking. Subsequently samples were boiled for 15 min at 95 °C in a water bath and half of the volume of absolute 96–100 % ethanol (Roth, Karlsruhe, Germany) was added. The whole content was put into a QiAmp spin column and centrifuged 1 min at 6.000 x *g*. Flow was discarded and the column was put in a new storage vessel. After addition of 500 µl of buffer AW1 samples were centrifuged for 1 min at 15.800 x *g*. Flow was discarded and the column was put in a new collection tube. After addition of 500 µl of buffer AW2 samples were centrifuged 3 min at 15.800 x *g*. Flow was discarded and the column was put in a new storage vessel and centrifuged for 1 min at 15.800 x *g*. For elution column was put into a 1.5 ml tube, 100 µl AE buffer was added and incubated for 1 min at room temperature. After centrifugation for 1 min at 15.800 x *g* eluted DNA was used for PCR.

Triplex real-time PCR assay

The DNA isolated from each sample before decontamination treatment was tested for the presence of MAP-DNA using a triplex real-time PCR assay for the MAP specific sequences F57 and IS*Mav*2 with an internal amplification control (IAC) according to Schönenbrücher et al. (2008). The sequences of the oligonucleotide primers and the PCR conditions were previously described by Schönenbrücher et al. (2008). Briefly, the 50 µL PCR mixture for the triplex real-time PCR assay using TaqMan probes contained 300 nM of the primers F57-F/F57-R, 200 nM of the primers IS*Mav2*-F/IS*Mav2*-R, 250 nM of the target probes (F57 and IS*Mav2*), 175 copies of the IAC, 25 µl of 2 x qPCR MasterMix Plus without uracil-*N*-glycosylase (UNG) (Eurogentec, Köln, Germany), and a 5 µl aliquot of the DNA sample. All oligonucleotide primers were synthesized by MWG Biotech (Ebersberg, Germany). The PCRs were performed in a 96-well plate format on the ABI Prism 7000 sequence detection system (Applied Biosystems, Darmstadt, Germany). Thermal cycling conditions comprised 2 min at 50 °C followed by hot-start DNA polymerase activation at 95 °C for 10 min, 50 cycles of denaturation at 95 °C for 15 seconds, annealing and extension at 60 °C for 1 min.

Results

Culture without decontamination

Excepting experiment 6, all HEYM slopes showed contamination when the decontamination step was not carried out. Up to 15 different colony morphologies that had no similarity to mycobacteria were visible. Furthermore, there was a change in color of the HEYM slopes, which could be caused by reduction of egg yolk due to undesirable-flora. Almost all inoculated MGIT (88.9 %) showed no fluorescence meaning no mycobacterial growth. In some cases, the fluorescent compound of the MGIT tubes was not distinguishable due to the sedimented sausage matrix. On Middlebrook 7H10 agar, MAP colonies were countable but 25 % of the plates were contaminated. The detection limit was approximately log 3.3 cfu/g raw sausage (Fig. 1). Successful spiking of raw sausages with MAP was confirmed by the triplex real-time PCR assay (Tab. 2).

Decontamination of raw sausages with NAC-NaOH

Decontamination of raw sausages with NAC-NaOH and cultivation on HEYM and on Middlebrook 7H10 agar showed little contamination, which means that this decontamination solution provides good elimination of companion undesirable-flora. Decontamination with NAC-NaOH provided a shorter decontamination time (20 minutes) compared to decontamination with HPC (25 hours). The detection limit was approximately log 2.9 cfu/g raw sausage (Fig. 1). Almost all inoculated MGIT (98.7 %) showed no fluorescence which means no growth. As in the case of the non-decontaminated samples, it was not easy to accurately interpret the few MGIT tubes that showed fluorescence because of the difficulty to observe the fluorescent compound of the MGIT tubes caused by the sedimented sausage.

Decontamination of raw sausages with HPC

Samples decontaminated with HPC and inoculated onto HEYM slants used as MAP-selective culture medium showed no contamination. The detection limit was approximately log 2.3 cfu/g raw sausage (Fig. 1). There was no growth on Middlebrook 7H10 agar. All inoculated MGIT showed no fluorescence. The fluorescent compound of the MGIT tubes was difficult to interpret due to the sedimented sausage matrix.

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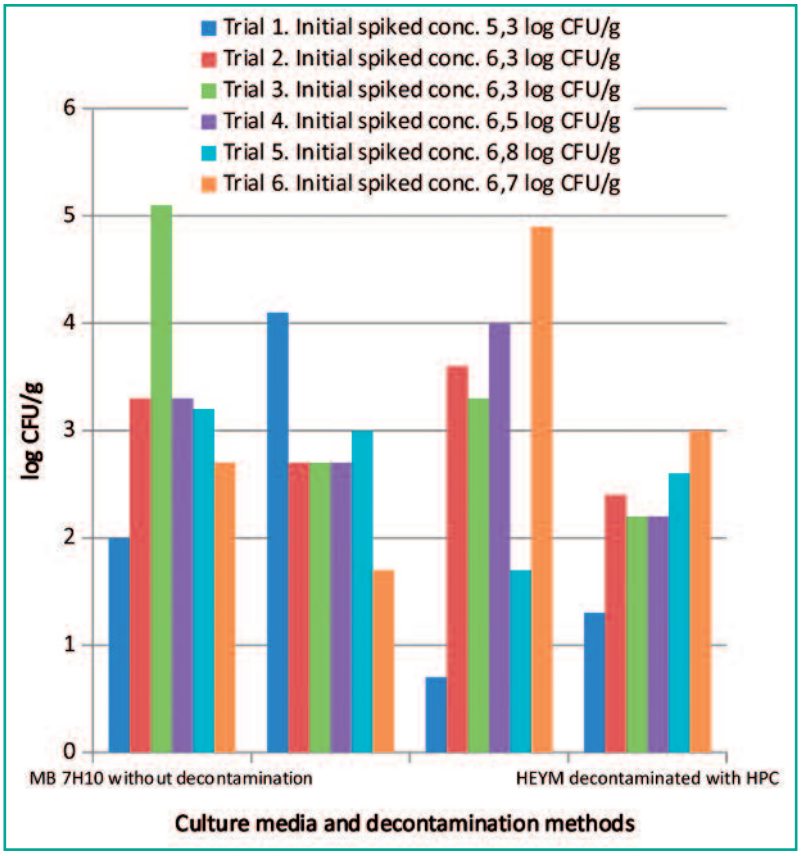

FIGURE 1: *Results from MAP artificially contaminated raw sausages inoculated onto Middlebrook 7H10 agar and Herrold s Egg Yolk Medium (HEYM) without decontamination treatment and previously decontaminated with HPC and NAC-NaOH. Results of decontamination treatment with NAC-NaOH and HPC subsequently cultured onto Middlebrook 7H10 agar as well as data from MGIT of all trials are not included. Initial spiked concentrations of MAP of Trial 1 to Trial 6 were 5.3, 6.3, 6.3, 6.5, 6.8, and 6.7 log cfu/g, respectively.*

Detection of MAP-DNA by Real-Time PCR

The presence of MAP in spiked raw sausages was successfully confirmed by the triplex real-time PCR assay (Tab. 2). All Ct-values correspond to MAP concentration in negative control and spiked samples. Both MAP-specific target regions were appropriate to determine MAP-Genes in the matrix raw sausage.

Discussion

MAP is a fastidious slow-growing organism and very difficult culturable. Cultural techniques for the detection of MAP are still considered as "gold standard" by several authors (Collins et al., 2005; Harris and Barletta, 2001). For cultural isolation of this organism from several matrices such as milk, meat, tissue specimens, feaces, etc. a decontamination treatment should be performed in order to reduce the numbers of competing organisms (Stabel, 1997; Dundee et al., 2001; Alonso-Hearn et al., 2009). However, application of chemical decontamination could also reduce the numbers of viable MAP cells; particularly decontaminating treatments may hinder the cultivation of small numbers of MAP cells present in specimens (Stabel, 1997; Dundee et al., 2001; Grant et al., 2003). This study was carried out to compare the performance of different decontamination and cultural methods, as well as a triplex real-time PCR assay according to Schönenbrücher et al. (2008) for raw sausages. The percentage of recovery of spiked MAP cells, minimum detection limits and applicability of different chemical decontamination protocols, and culture media were evaluated.

Culture is the main method to detect MAP, but because of the companion undesirable flora in raw sausages a decontamination step was necessary. Based on the finding in the present study, it can be concluded that HEYM supported growth of MAP much faster and better than Middlebrook 7H10 agar based on the decontamination protocol including 0.75 % HPC, whereas in previous works HPC had been observed to own the least detrimental effect on growth of MAP (Whipple et al., 1991). Our results indicated that HEYM was a suitable medium for MAP culture from raw sausages. This finding has been cited in a previous report but for other matrices than raw sausages (Nielsen et al., 2004). The decontamination with NAC-NaOH and subsequently culturing onto HEYM as well as on Middlebrook 7H10 agar are suitable methods for the detection of MAP in raw sausages with a minimum detection limit of approximately log 2.9 cfu/g raw sausage. The solid media Middlebrook

TABLE 2: *Representative results of expressed as Ct[1]-values of triplex real-time PCR of MAP spiked into "Salami" sausages from trial 5.*

Sausage and treatment	MAP-specific sequence[2]	Negative control		MAP concentration (log cfu/g) 2.2 x 10^2		2.2 x 10^3		2.2 x 10^4		Positive control	
		A[3]	B[3]	A	B	A	B	A	B	A	B
Salami without decontamination	F57	Undet.	Undet	39.19	38.17	34.49	34.35	28.92	29.70	25.48	25.20
	ISMaV2	Undet.	Undet.	38.67	38.90	33.80	34.50	30.33	30.37	26.18	25.91
Salami before decontamination with NAC-NaOH	F57	Undet.	Undet.	Undet.	39.16	38.00	38.14	35.71	35.67	30,06	30.39
	ISMaV2	Undet.	Undet.	Undet.	Undet.	36.14	37.18	35.79	34.90	29.93	31.00
Salami before decontamination with HPC	F57	Undet.	Undet.	40.00	Undet.	Undet.	Undet.	35.57	35.65	31.00	30.77
	ISMaV2	Undet.	Undet.	30.91	Undet.	38.44	39.16	35.55	36.00	31.62	31.29

[1]) Cycle threshold (Ct) values. [2]) Real-Time PCR carried out according to Schönenbrücher et al. (2008). [3]) Samples were tested in duplicate A and B Undet.: Undetermined

7H10 did not support the development of visible colonies of MAP after HPC decontamination from any spiked samples from which the organism grew on HEYM. Isolation failures on Middlebrook 7H10 agar has been attributed to a carry-over of traces of HPC in the inoculum which may not be inactivated by lack of egg yolk supplement. It could be that remains of HPC inhibit all bacterial growth during the 12 weeks of incubation. Therefore a decontamination with HPC in combination with culture on Middlebrook 7H10 agar without egg yolk supplement is not advisable.

In conclusion, the results of six spiking experiments showed that the combination of HPC used as decontamination method, and the subsequently cultivation onto HEYM used as MAP-selective culture medium is the most sensitive method to detect MAP in raw sausages with a minimum detection limit of approximately log 2.3 cfu/g raw sausage (Fig. 1). This finding of this study suggests that when chemical decontamination is applied to raw sausage samples, culture will only be successful if MAP is present at level of 10^2 cells per g or above. In this study, the HPC decontamination procedure has been shown to be less detrimental to viable MAP cells than NAC-OH decontamination. The decontamination method involving treatment of milk and faeces with 0.75 % HPC was previously used by several studies (Sweeney et al, 1992; Dundee et al, 2001; Grant et al., 2003; Stabel, 1997).

MGIT tubes have been reported as a successful culture medium of MAP from animal and human organ and tissue specimens, as well as from milk samples (Grant et al., 2003; De Lisle et al., 1999; Naser, 2000; Mutharia et al., 2010). In our study, MGIT tubes were manually controlled for detection of fluorescence using an ultraviolet transilluminator at weekly intervals during incubation period. Spiking experiments showed that in almost all cases fluorescence was not visible, which means that MAP cells in spiked raw sausages were not detectable. Because of that, we would discourage the use of MGIT as a suitable culture medium for MAP in this matrix. False negative or positive fluorescence signals of MGIT tube could lead to inconvenient in diagnostic culture of MAP and subsequently to the need of additional species identification that create extra laboratory work. This could be cost prohibitive in most laboratories. The DNA extraction with subsequent the triplex real-time PCR assay is sufficiently sensitive in comparison to culture approximately 10^2 cfu/g spiked raw sausages and results are provided within a single day. Although further validation is necessary on a higher number of samples from the matrix meat, this assay could improve the determination of MAP in meat samples.

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References

Alonso-Hearn M, Molina E, Geijo M, Vazquez P, Sevilla I, Garrido JM, Juste RA (2009): Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from muscle tissue of naturally infected cattle. Foodborne Pathog Dis 6: 513–518.

Antognoli MC, Garry FB, Hirst HL, Lombard JE, Dennis MM, Gould DH, Salman MD (2008): Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection in dairy cattle and its association with antemortem test results. Vet Microbiol 127: 300–308.

Ayele WY, Svastova P, Roubal P, Bartos M, Pavlik I (2005): *Mycobacterium avium* subspecies *paratuberculosis* cultured from locally and commercially pasteurized cow's milk in the Czech Republic. Appl Environ Microbiol 71: 1210–1214.

Botsaris G, Slana I, Liapi M, Dodd C, Economides C, Rees C, Pavlik I (2010): Rapid detection methods for viable *Mycobacterium avium* subspecies *paratuberculosis* in milk and cheese. Int J Food Microbiol 141: 87–90.

Collins MT, Wells SJ, Petrini KR, Collins JE, Schultz RD, Whitlock RH (2005): Evaluation of five antibody detection tests for diagnosis of bovine paratuberculosis. Clin Diagn Lab Immunol. 12: 685–692.

De Lisle GW, Yates GF, Cavaignac S, Collins DM (1999): Evaluation of the MGIT system for culturing *Mycobacterium avium* subsp. *paratuberculosis* and characterization of strains by Polymerase Chain Reaction tests. In Proceedings of the 6th International Colloquium on Paratuberculosis ed. Manning, E.J.B. and Collins, M.T. p. 511. Rehoboth, MA: International Association for Paratuberculosis.

Dundee L, Grant IR, Ball HJ, Rowe MT (2001): Comparative evaluation of four decontamination protocols for the isolation of *Mycobacterium avium* subsp. *paratuberculosis* from milk. Lett Appl Microbiol. 33: 173–177.

Eltholth MM, Marsh VR, van Winden S, Guitian FJ (2009): Contamination of food products with *Mycobacterium avium paratuberculosis:* a systematic review. J Appl Microbiol 107: 1061–1071.

Gill, CO, Saucier L, Meadus WJ (2011): *Mycobacterium avium* subsp. *paratuberculosis* in Dairy Products, Meat, and Drinking Water. J Food Prot 74: 480–499

Grant IR, Ball HJ, Rowe MT (2002): Incidence of *Mycobacterium paratuberculosis* in bulk raw and commercially pasteurized cows' milk from approved dairy processing establishments in the United Kingdom. Appl Environ Microbiol 68: 2428–2435.

Grant IR, Kirk RB, Hitchings E, Rowe MT (2003): Comparative evaluation of the MGIT and BACTEC culture systems for the recovery of *Mycobacterium avium* subsp. *paratuberculosis* from milk. J Appl Microbiol. 95: 196–201.

Grant IR, Williams AG, Rowe MT, Muir DD (2005): Efficacy of various pasteurization time-temperature conditions in combination with homogenization on inactivation of *Mycobacterium avium* subsp. *paratuberculosis* in milk. Appl Environ Microbiol 71: 2853–2861.

Grant, IR (2010): *Mycobacterium avium* subsp. *paratuberculosis* in Animal-derived Foods and the environment. In: Behr, M.A., Collins, D.M. (Eds.), Paratuberculosis: organism, disease, control. CAB International, Oxfordshire, pp. 29–39.

Harris NB, Barletta RG (2001): *Mycobacterium avium* subsp. *paratuberculosis* in Veterinary Medicine. Clin Microbiol Rev. 14: 489–512.

Hermon-Taylor J, Bull T (2002): Crohn's disease caused by *Mycobacterium avium* subspecies *paratuberculosis:* a public health tragedy whose resolution is long overdue. J Med Microbiol 51: 3–6.

Hruska K, Slana I, Kralik P, Pavlik I (2011): *Mycobacterium avium* subsp. *paratuberculosis* in powdered infant milk: F57 competitive real time PCR. Vet Med (Praha) 56: 226–230.

Jaravata CV, Smith WL, Rensen GJ, Ruzante J, Cullor JS (2007): Survey of ground beef for the detection of *Mycobacterium avium paratuberculosis.* Foodborne Pathog Dis 4: 103–106.

McKenna SLB, Keefe GP, Barkema HW, McClure J, Vanleeuwen JA, Hanna P, Sockett DC (2004): Cow-level prevalence of

paratuberculosis in culled dairy cows in Atlantic Canada and Maine. J Dairy Sci 87: 3770–3777.

Meadus WJ, Gill CO, Duff P, Badoni M, Saucier L (2008): Prevalence on beef carcasses of *Mycobacterium avium* subsp. *paratuberculosis* DNA. Int J Food Microbiol 124: 291–294.

Mendoza JL, Lana R, Díaz-Rubio M (2009): *Mycobacterium avium* subspecies *paratuberculosis* and its relationship with Crohn's disease. World J Gastroenterol 15: 417–422.

Mutharia LM, Klassen MD, Fairles J, Barbut S, Gill CO (2010): *Mycobacterium avium* subsp. *paratuberculosis* in muscle, lymphatic and organ tissues from cows with advanced Johne's disease. Int J Food Microbiol 136: 340–344.

Naser S, Shafran I, Schwartz D (2000): Isolation of *Mycobacterium avium* subsp. *paratuberculosis* from breast milk of Crohn's disease patients. Am J Gastroenterol 95: 1094–1095.

National Advisory Committee on Microbiological Criteria for Foods (2010): Assessment of food as a source of exposure to *Mycobacterium avium* subspecies *paratuberculosis* (MAP). J Food Prot 73: 1357–1397.

Nielsen SS, Kolmos B, Christoffersen AB (2004): Comparison of contamination and growth of *Mycobacterium avium* subsp. *paratuberculosis* on two different media. J Appl Microbiol. 96: 149–153.

Okura H, Toft N, Pozzato N, Tondo A, Nielsen SS (2011): Apparent Prevalence of Beef Carcasses Contaminated with *Mycobacterium avium* subsp. *paratuberculosis* Sampled from Danish Slaughter Cattle. Vet Med Int 2011: Article ID 152687, 7 pages, doi:10.4061/2011/152687.

Schönenbrücher H, Abdulmawjood A, Failing K, Bülte M (2008): New triplex real-time PCR assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine feces. Appl Environ Microbiol 74: 2751–2758.

Stabel JR (1997): An improved method for cultivation of *Mycobacterium paratuberculosis* from bovine fecal samples and comparison to three other methods. J Vet Diagn Invest. 9: 375–380.

Stabel JR (1998): Johne's disease: a hidden threat. J Dairy Sci 81: 283–288.

Stephan R, Schumacher S, Tasara T, Grant IR (2007): Prevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Swiss raw milk cheeses collected at the retail level. J Dairy Sci 90: 3590–3595.

Sweeney RW, Whitlock RH, Rosenberger AE (1992): *Mycobacterium paratuberculosis* cultured from milk and supramammary lymph nodes of infected asymptomatic cows. J Clin Microbiol 30: 166–171.

Uzoigwe JC, Khaitsa ML, Gibbs PS (2007): Epidemiological evidence for *Mycobacterium avium* subspecies *paratuberculosis* as a cause of Crohn's disease. Epidemiol Infect 135: 1057–1068.

Wells JE, Bosilevac JM, Kalchayanand N, Arthur TM, Shackelford SD, Wheeler TL, Koohmaraie M (2009): Prevalence of *Mycobacterium avium* subsp. *paratuberculosis* in ileocecal lymph nodes and on hides and carcasses from cull cows and fed cattle at commercial beef processing plants in the United States. J Food Prot 72: 1457–1462.

Whipple DL, Callihan DR, Jarnagin JL (1991): Cultivation of *Mycobacterium paratuberculosis* from bovine fecal specimens and a suggested standardized procedure. J Vet Diagn Invest. 3: 368–373.

Whitlock RH, Buergelt C (1996): Preclinical and clinical manifestations of paratuberculosis (including pathology). Vet Clin North Am Food Anim Pract 12: 345–356.

Whittington RJ, Hope AF, Marshall DJ, Taragel CA, Marsh I (2000): Molecular epidemiology of *Mycobacterium avium* subsp. *paratuberculosis:* IS900 restriction fragment length polymorphism and IS1311 polymorphism analyses of isolates from animals and a human in Australia. J Clin Microbiol 38: 3240–3248.

Whittington RJ, Waldron A, Warne D (2010): Thermal inactivation profiles of *Mycobacterium avium* subsp. *paratuberculosis* in lamb skeletal muscle homogenate fluid. Int J Food Microbiol 137: 32–39.

Whittington, RJ (2010): Cultivation of *Mycobacterium avium* subsp. *paratuberculosis.* In: Behr, M.A., Collins, D.M. (Eds.), Paratuberculosis: Organism, Disease, Control. CAB International, Oxfordshire, pp. 244–266.

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