Prevalence of *Mycobacterium avium* Subspecies *paratuberculosis* in Swiss Raw Milk Cheeses Collected at the Retail Level

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ABSTRACT

A total of 143 raw milk cheese samples (soft cheese, $n = 9$; semihard cheese, $n = 133$; hard cheese, $n = 1$), collected at the retail level throughout Switzerland, were tested for *Mycobacterium avium* ssp. *paratuberculosis* (MAP) by immunomagnetic capture plus culture on 7H10-PANTA medium and in supplemented BACTEC 12B medium, as well as by an F57-based real-time PCR system. Furthermore, pH and water activity values were determined for each sample. Although no viable MAP cells could be cultured, 4.2% of the raw milk cheese samples tested positive with the F57-based real-time PCR system, providing evidence for the presence of MAP in the raw material. As long as the link between MAP and Crohn's disease in humans remains unclear, measures designed to minimize public exposure should also include a focus on milk products.

Key words: raw milk cheese, *Mycobacterium avium* ssp. *paratuberculosis*, culture, real-time polymerase chain reaction

INTRODUCTION

Mycobacterium avium ssp. *paratuberculosis* (MAP) is the etiological agent for ruminant paratuberculosis (Johne's disease) as well as being a specific pathogen in several other animal species (reviews in Harris and Barletta, 2001; Collins, 2003; Chacon et al., 2004). There have also been numerous reports suggesting a potential association between MAP and human Crohn's disease (for reviews, see Hermon-Taylor et al., 2000; Chacon et al., 2004). However, because of the complex nature of human Crohn's disease as well as conflicting experimental evidence, a definitive link between MAP and Crohn's disease can neither be confirmed nor discarded at present. The possible involvement of MAP in human disease obviously raises significant public

health concerns, and measures to minimize public exposure are encouraged. Several opportunities for human exposure to MAP do exist, and the primary focus has mainly been on dairy products. Milk may be contaminated directly within the udder or indirectly as a result of fecal contamination. The direct shedding of MAP organisms into milk is lower than that with fecal shedding. Less than 100 cfu/mL was documented in symptomatic MAP-infected cows and 2 to 8 cfu/50 mL was found in MAP-infected but asymptomatic cows (Sweeney et al., 1992; Giese and Ahrens, 2000). Fecal shedding, on the other hand, can exceed 10^8 cfu/g and may thus be a significant contributor of MAP contamination in raw milk (Cocito et al., 1994). A recently published study on bulk tank raw milk performed using an F57 sequence-based PCR system estimated an average prevalence of 3.0% MAP in Swiss bovine bulk tank raw milk samples (Bosshard et al., 2006).

The fate of any viable MAP organisms found in raw milk is still not yet fully understood. Several experimental studies have shown that viable MAP organisms can survive the current standard pasteurization processes when high numbers are present (for reviews, see Grant et al., 2002; Grant, 2006). For cheese, so far very limited data are available on the prevalence of MAP and on its fate during the ripening process. In this study we used cultivation and a recently described real-time PCR protocol that targets the F57 sequence to investigate the presence of MAP in retail raw milk cheese sourced from various regions of Switzerland.

MATERIALS AND METHODS

Sample Collection

The 143 raw milk cheese samples (soft cheese, $n = 9$; semihard cheese, $n = 133$; hard cheese, $n = 1$; all produced from cows' milk) analyzed in this study were all manufactured from Swiss milk and collected within a national sampling plan at the retail level throughout Switzerland during the period of March to June 2006. Each sample consisted of approximately 50 g of cheese. The samples were split into subsamples of approxi-

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Figure 1. Schematic representation of the Pathatrix magnetic capture system (Matrix MicroScience Ltd., Newmarket, UK) showing the location of the inline connector where anti-*Mycobacterium avium* ssp. *paratuberculosis* beads were added and the magnet where beads were captured from the sample during circulation for 20 min (used with permission of Matrix MicroScience Limited).

mately 25 g (for culture), 10 g (for real-time PCR), and 10 g [for pH and water activity (a_w) determinations] and stored in a cooler. For MAP culturing, 4 batches of cheese samples packed in insulated cooling boxes containing ice packs were transported by overnight courier from the University of Zurich to Queen's University Belfast.

Culture of MAP

Immunomagnetic capture using Pathatrix (Matrix MicroScience Ltd., Newmarket, UK), a patented magnetic capture system, was used in place of chemical decontamination prior to culture to recover MAP from the cheese samples. This protocol requires a preprogrammed workstation, generic consumables (tubing assembly, stoppered tubes, and elution pipettes) and, for the purposes of this study, paramagnetic beads coated with anti-MAP antibody (all supplied by Matrix MicroScience Ltd.). A general schematic overview of the operation of the Pathatrix system is shown in Figure 1. This system has recently been used for recovery of *Escherichia coli* O157:H7 from raw ground beef (Wu et al., 2004) and *Enterobacter sakazakii* from dried infant milk formula (Mullane et al., 2006).

Upon receipt in the laboratory at Queen's University Belfast, each preweighed cheese sample (20 to 25 g) was transferred to a Stomacher filter bag (Seward Medical, London, UK) and 225 mL of trisodium citrate buffer

[consisting of 0.5% sodium chloride (Sigma Aldrich Ltd., Poole, UK), 1% casitone (Difco, Detroit, MI), and 2% sodium citrate (Sigma)], prewarmed to room temperature ($\sim 20^\circ\text{C}$), were added. The cheese sample was homogenized for 2 min in a Stomacher 400 laboratory blender (Seward Medical). The filter component of the Stomacher bag was then removed and discarded, and the bag and contents were transferred to the Pathatrix workstation operating at 37°C . The Pathatrix tubing assembly was put in place and 50 μL of anti-MAP Pathatrix beads (product code: PM50) was then introduced into each sample via the inline connector. Each sample was circulated for 20 min at 37°C , during which time the complete sample homogenate circulated approximately twice per minute over the anti-MAP beads captured at an inline magnet. When the circulation period was complete, the captured beads (plus any MAP attached) were flushed into a stoppered tube by means of a pastette attached to the inline connector. The tubes were then transferred to a magnetic rack, where the beads were captured again for 10 min. Residual cheese homogenate was withdrawn with a pastette (taking care not to dislodge the captured beads) and discarded. Beads were washed once with 5 mL of PBS containing 0.05% Tween 20 (PBS-T20, Sigma Aldrich) by magnetic separation for 2 min before the PBS-T20 was withdrawn and discarded. Beads were finally resuspended in 800 μL of PBS-T20. The bead suspension was divided between 2 slopes of 7H10-PANTA medium (200 μL /slope) and one vial (400 μL /vial) of BACTEC 12B medium (Becton Dickinson UK Limited, Cowley, UK) supplemented with 0.5 mL of egg yolk emulsion (Difco), 2 $\mu\text{g}/\text{mL}$ of mycobactin J (Synbiotics Europe SAS, Lyon, France), and 100 μL of PANTA Plus antibiotic supplement (Becton Dickinson). The 7H10-PANTA medium consisted of Middlebrook 7H10 agar supplemented with 10% (vol/vol) Middlebrook OADC (oleic acid, albumin, dextrose, citrate) enrichment, 2% (vol/vol) reconstituted PANTA Plus antibiotic supplement (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin, Becton Dickinson), 0.5% glycerol, and 2 $\mu\text{g}/\text{mL}$ of mycobactin J. Both culture media were incubated at 37°C for up to 16 wk. For the first 60 cheese samples processed, a third culture medium, HEYM-VAN (Herrold's egg yolk medium supplemented with 2 $\mu\text{g}/\text{mL}$ of mycobactin J and the antibiotics vancomycin, amphotericin B, and nalidixic acid) was inoculated with the bead suspension. However, contamination rates on HEYM-VAN (i.e., overgrowth by non-acid-fast cheese bacteria nonspecifically bound to the beads after magnetic capture) proved to be very high, so its use was discontinued after batch 2. Slope cultures were examined periodically for evidence of acid-fast colonies. Vials of BACTEC were read every 2 wk on a BACTEC 460TB machine (John-

ston Laboratories Inc., Towson, MD). When a positive growth index reading (>50) was recorded, an aliquot of the BACTEC culture was removed and stained by the Ziehl-Neelsen method to determine the presence or absence of acid-fast cells. When acid-fast cells were observed in any culture medium, the suspect colony or BACTEC culture was subcultured onto Herrold's egg yolk medium containing 2 $\mu\text{g/mL}$ of mycobactin J (HEYM) to verify typical colony morphology and slow growth. If these properties were observed upon subculture, then isolates were finally confirmed as MAP by IS900 PCR (Moss et al., 1992).

Preparation of Genomic DNA Templates from Cheese Samples

Ten-gram cheese samples were homogenized with 30 mL of buffer (25% sodium citrate, 4% polyethylene glycol 8000) for 2 min in a Stomacher 400 laboratory blender (Seward Medical). Ten milliliters of the homogenate was centrifuged for 15 min at $1,500\times g$ and pellets were obtained. The supernatant was discarded and the pellets were resuspended in 500 μL of PBS and 100 μL was transferred into Eppendorf tubes. A second centrifugation step was performed (10 min at $13,000\times g$), and the rest of the supernatant was removed. Thereafter, 240 μL of lysis buffer, 60 μL of proteinase K solution, and 300 μL of binding buffer provided in the MagNA Pure LC DNA Isolation kit I (Roche Molecular Diagnostics, Penzberg, Germany) were added, and the entire mixture was incubated overnight at 37°C . The mixtures were transferred onto the lysing bead matrix in the MagNA Lyser tubes and the tubes were placed into the MagNA Lyser instrument (Roche Molecular Diagnostics). A mechanical lysis step consisting of 60 s at 6,500 rpm, followed by 60 s on a cooling block held at 4°C was performed 3 times on the samples. After the last mechanical lysis step, the samples were incubated for a further 15 min at 90°C and cooled to room temperature. Then 150 μL of isopropanol was added and the samples were mixed. The sample mixtures were transferred onto the DNA-binding columns of the High Pure PCR Template Preparation kit (Roche Diagnostics GmbH, Penzberg, Germany) and processed as outlined in the kit protocol. The DNA templates from the samples were eluted into a 100- μL volume of elution buffer supplied in the kit, prewarmed to 70°C . The DNA yields were calculated based on the optical density (OD) measurement at 260 nm with a nanodrop instrument (Nanodrop Technologies, Wilmington, DE), and purity was assessed by inspection of the sample $\text{OD}_{260}:\text{OD}_{280}$ ratios.

Real-Time PCR Assays

Real-time PCR was used to coamplify and detect a 254-bp target region in the MAP F57 sequence and a

257-bp internal control (IC) template. The reactions were performed in a LightCycler 2.0 instrument (Roche Molecular Diagnostics) in a total reaction volume of 20 μL in glass capillary tubes. The optimal reaction mixture contained $1\times$ concentration of LightCycler-Faststart DNA Master Plus hybridization probes mix (Roche Molecular Diagnostics), 1,000 nM of each primer (MAP f57p1, MAPf57p2; Tasara and Stephan, 2005), 200 nM of each LightCycler probe (MAP f57-3'Fluo, MAP f57-5'LC-Red640, PuC19-5'LC-Red 705; Bosshard et al., 2006), and 20 copies of IC template. The amplification consisted of an initial preincubation step at 95°C for 10 min to activate the DNA polymerase, followed by 45 cycles of 95°C for 10 s, 56°C for 20 s, and 72°C for 18 s. The fluorescence signals corresponding to F57 sequence target and IC template amplification were monitored during the 56°C annealing step in the LC-Red 640 nm and LC-Red 705 nm detection channels of the LightCycler 2.0 instrument, respectively.

The PCR analysis was done in duplicate for each sample. In each case, a 5- μL aliquot of the undiluted template was used per PCR reaction. If inhibition was detected, as judged by the lack of both IC template and F57 target amplification in the PCR assay, then a template dilution (1:50) step was done, and the reaction was repeated.

Controls. Standard steps were taken throughout this study to avoid potential sample cross-contamination and false-positive results. These included use of separate rooms for DNA extraction, PCR mixture preparation, and post-PCR analysis, as well as inclusion of DNA extraction process and PCR reaction-negative controls, and the use of filtered reaction tips.

pH and a_w Values

The final 10-g subsample of each cheese sample was used to determine the pH value with an Orion 420A pH meter (Hügli, Zurich, Switzerland) and a_w value using an a_w Center Novasina 203 instrument (Axair, Pfäffikon, Switzerland) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

With the real-time PCR-based method, we found 6 semihard cheese samples (4.2%) that were positive for MAP F57 (Table 1), providing evidence for the presence of MAP in the raw material. The crossing-point values ranged from 32 to 37, indicating a moderate to low level of contamination.

With the culture-based method, only in one of the 143 cheese samples was acid-fast staining bacteria observed (Table 2). However, no acid-fast colonies were subse-

Table 1. *Mycobacterium avium* ssp. *paratuberculosis* DNA-positive cheese samples

Number	Cheese type	pH	a _w value ¹
28	Semihard	5.99	0.975
63	Semihard	ND ²	ND
74	Semihard	5.62	0.968
80	Semihard	5.55	0.965
95	Semihard	6.11	0.957
109	Semihard	5.65	0.966

¹a_w = water activity.²ND = not done, not enough sample.

quently obtained by subculture of the suspect acid-fast colony from a HEYM-VAN slope, so the presence of viable MAP was not confirmed. All retail cheese samples were therefore negative for viable MAP. Immunomagnetic capture by the Pathatrix system, rather than chemical decontamination, was used prior to culture during this study. Previous studies have shown that only a small proportion of viable MAP present in a sample is recovered if chemical decontamination with hexadecylpyridinium chloride is used (26 to 28% recovery, Dundee et al., 2001; 16% recovery, Gao et al., 2005). In contrast, the Pathatrix system theoretically has the potential to recover all MAP (viable or dead) from 25 g of cheese without the need for harsh chemicals and with no detrimental effect on MAP viability, although 100% recovery of MAP from a sample by the Pathatrix system has not been confirmed. However, the minimum detection limit of the Pathatrix immunomagnetic capture method for MAP was determined by testing Cheddar cheese spiked with MAP at different levels, and it was estimated to be ~10³ MAP/25 g of cheese or 40 MAP/g of cheese, which, if we assume a 10-fold concentration of MAP from milk to cheese curd (Donaghy et al., 2004), would be equivalent to detecting MAP in cheese made from milk containing 4 MAP/mL.

Although bovine paratuberculosis is a notifiable disease within Switzerland, the true prevalence of MAP within the country's cattle herds remains unknown. Studies have already been done in view of the prevalence of MAP in Swiss dairy cattle herds and bovine bulk tank raw milk. A small-scale study comparing fecal culture and an IS900-based PCR system was used to analyze fecal samples from 310 dairy cattle located on 10 dairy farms in different regions of the country. The estimated prevalence of MAP infection in this dairy cattle population ranged from 6.5 to 10% (Bogli-Stuber et al., 2005). In a recently published study, 3% of 100 bovine bulk tank raw milk samples analyzed with F57 sequence-based PCR tested positive (Bosshard et al., 2006). Results from these 2 studies therefore indicate low levels of subclinical MAP infection within the Swiss dairy herds. The data from this study on raw milk cheese samples reconfirms the MAP contamination of Swiss bulk tank raw milk. The current prevalence of 4.2% in raw milk cheese samples is similar to the prevalence previously determined from a bulk tank raw milk survey with the F57-based real-time PCR system (Bosshard et al., 2006).

The presence and viability of MAP in cheese has been investigated so far in a very limited number of studies. Clark et al. (2006) performed a study on 98 retail cheese curd samples purchased in Wisconsin and Minnesota. Although no viable MAP were cultured from the retail cheese curd, which was produced from pasteurized milk, 5% of the samples were PCR-positive with an IS900 and a *hspX*-based PCR system. In a second study on the detection of MAP in cheeses, Ikononopoulos et al. (2005) reported, for Feta cheese (Greece), a prevalence of 50 and 4.7% for an IS900-based PCR and a culture-based method, respectively. In the same study, a prevalence of 11.9% (IS900-based PCR) and 2.4% (culture-based method) was described for hard, semihard, and soft cheese collected from the Czech Republic.

Table 2. Culture results for 143 Swiss raw milk cheese samples

Culture status	Culture medium, no. of cultures (%)		
	BACTEC 12B + PANTA	7H10-PANTA	HEYM-VAN
Microbial growth observed	15/143 ¹ (10.5%)	57/143 ² (39.9%)	27/60 ³ (45.0%)
Acid-fast cells observed after Ziehl-Neelsen staining	0/15	0/57	1/27 ⁴
Presence of viable MAP confirmed	0	0	0

¹None of the BACTEC cultures exhibiting growth was from an F57 PCR-positive cheese sample.²Three of the 7H10-PANTA cultures exhibiting growth were from F57 PCR-positive cheese samples 28, 63, and 95.³Two of the HEYM-VAN cultures exhibiting growth were from F57 PCR-positive cheese samples 28 and 63.⁴The single suspect acid-fast colony obtained did not grow on the subculture despite several attempts, and IS900 PCR was not done because the subculture was unsuccessful.

In our study, as well as in the 2 studies mentioned above, significant differences were obtained between the prevalence results of the culture and the PCR-based methods. There are several potential explanations:

1. Clark et al. (2006) tested cheese curd produced from pasteurized milk and concluded that viable MAP may not have survived the pasteurization process.
2. In the same study, the authors also showed that, whereas MAP-positive control cheese samples spiked with ATCC 19698, ATCC 43545, and ATCC 43544 were positive by both culture and PCR, other positive controls spiked with ATCC 43015 were positive only by PCR and not by culture.
3. Commonly used starter cultures and typical non-starter microflora present during the manufacture and ripening of cheese (Spahr and Schafröth 2001; Donaghy et al., 2003) as well as sample decontamination procedures (Dundee et al., 2001; Gao et al., 2005) may also hamper the cultural detection of low numbers of MAP.
4. Bachmann and Spahr (1995) showed that a variety of potentially pathogenic bacteria are inactivated during the ripening process of hard and semihard cheeses made from raw milk. Three studies on the survival of MAP in model laboratory-produced cheeses have shown that certain changes in physicochemical characteristics during ripening and the time of ripening also influence the fate of MAP in different cheese types (Sung and Collins, 2000; Spahr and Schafröth, 2001; Donaghy et al., 2004). The most important factors responsible for the inactivation of MAP in cheese were the temperature applied during cheese manufacture, the pH value during cheese ripening, and the salt concentration, which can be indirectly measured by the a_w value.

CONCLUSIONS

No viable MAP were found in the retail cheese tested in our study. Nevertheless, the fact that MAP genetic elements were detected in raw milk cheese samples suggests that some of these organisms may find their way to the consumers. This means that as long as the link between MAP and Crohn's disease in humans remain unclear, measures designed to minimize public exposure to MAP via food should also include a focus on milk products.

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