Mycobacterium avium subsp. paratuberculosis prevalence studies in bulk tank raw milk and slaughtered healthy dairy cows in Switzerland using an F57 sequence-based real-time PCR assay

Bosshard, Corinne

Abstract: Im Rahmen dieser Arbeit wurde eine auf dem Zielgen MAP F57 basierende real-time PCR Methode angewendet, um die Prävalenz von M. avium subsp. paratuberculosis (MAP) in Bestandesrohmilchproben und in Proben, die von geschlachteten Milchkühen erhoben wurden, zu bestimmen. Die minimale Nachweisgrenze des verwendeten Vorgehens liegt im Bereich von 100-1000 MAP pro 10 ml Milch und bei 10-100 MAP pro 200 mg Kot.Von den 100 getesteten Bestandesmilchproben waren 3 Proben (3%) M. avium subsp. paratuberculosis DNA-positiv. Von den geschlachteten Milchkühen waren 8.9% (9/101) der Kotproben, 4.9% (5/101) der Mesenteriallymphknoten, 0.9% (1/101) der Ileumproben, 3.6% (3/84) der Einzeltiermilchproben und 2.9% (3/101) der Zwerchfellmuskulaturproben M. avium subsp. paratuberculosis positiv. Nur von einem Tier waren jedoch mehr als eine Probe positiv.Diese Arbeit liefert im Rahmen von zwei Feldstudien erstmals Prävalenzdaten zu M. avium subsp. paratuberculosis in der Schweiz mittels eines F57 basierten PCR Systems und zeigt gleichzeitig die Anwendbarkeit dieses RT-PCR Systems bei verschiedenen Probenmatrices auf. Das Vorkommen von M. avium subsp. paratuberculosis wurde auch mit diesem neuen approach sowohl in Bestandesrohmilchproben als auch in Proben von geschlachteten Milchkühen bestätigt. A light cycler-based real-time PCR assay that targets the F57 sequence was used to monitor the prevalence of M. avium subsp. paratuberculosis in Swiss bulk tank raw milk and in a population of healthy slaughtered dairy cattle.The assay's minimal detection limits were in the range of 100-1000 cells per 10 ml of milk and about 10- 100 cells per 200 mg of feces. Amongst the 100 bulk tank milk samples that were tested, we found 3 samples (3%) that were positive for M. avium subsp. paratuberculosis. Amongst the slaughtered healthy dairy cattle, 8.9% (9/101) of the cows were positive for M. avium subsp. paratuberculosis DNA in their fecal samples, 4.9% (5/101) in their mesenteric lymph nodes, 0.9% (1/101) in their ileum tissue, 3.6% (3/84) in their milk and 2.9% (3/101) were positive in their diaphragmatic muscles.This study reports the first set of prevalence data on M. avium subsp. paratuberculosis organisms in Switzerland that are based on a F57 sequence targeting PCR system. M. avium subsp. paratuberculosis presence in both bulk tank raw milk and in slaughtered healthy dairy cattle is confirmed. This finding may be of relevance to public health in view of milk and beef products as potential vehicles of M. avium subsp. paratuberculosis transmission to humans in the context of the unresolved potential link of these microorganisms to some cases of human Crohn's disease.

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CONTENTS

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In fulfillment of the doctoral thesis of Corinne Bosshard

1. Summary

A light cycler-based real-time PCR assay that targets the F57 sequence was used to monitor the prevalence of *M. avium* subsp. *paratuberculosis* in Swiss bulk tank raw milk and in a population of healthy slaughtered dairy cattle.

The assay's minimal detection limits were in the range of 100-1000 cells per 10 ml of milk and about 10-100 cells per 200 mg of feces. Amongst the 100 bulk tank milk samples that were tested, we found 3 samples (3%) that were positive for *M. avium* subsp. *paratuberculosis*. Amongst the slaughtered healthy dairy cattle, 8.9% (9/101) of the cows were positive for *M. avium* subsp. *paratuberculosis* DNA in their fecal samples, 4.9% (5/101) in their mesenteric lymph nodes, 0.9% (1/101) in their ileum tissue, 3.6% (3/84) in their milk and 2.9% (3/101) were positive in their diaphragmatic muscles.

This study reports the first set of prevalence data on *M. avium* subsp. *paratuberculosis* organisms in Switzerland that are based on a F57 sequence targeting PCR system. *M. avium* subsp. *paratuberculosis* presence in both bulk tank raw milk and in slaughtered healthy dairy cattle is confirmed. This finding may be of relevance to public health in view of milk and beef products as potential vehicles of *M. avium* subsp. *paratuberculosis* transmission to humans in the context of the unresolved potential link of these microorganisms to some cases of human Crohn's disease.

Keywords:
M. avium subsp. *paratuberculosis*, F57 sequence, real time-PCR, detection

2. Introduction

Mycobacterium avium subsp. *paratuberculosis* is the etiological agent for bovine paratuberculosis also known as Johne's disease. The infection of domestic food animals with MAP organisms is associated with significant economic losses to the livestock industry worldwide through subclinical effects and subsequent death of the affected animals. In addition to cattle and other domestic ruminants, infections with *M. avium* subsp. *paratuberculosis* have also been described in several other animal species (recent reviews in Harris and Barletta, 2001; Collins, 2003; Chacon et al., 2004).

The suggestions by some investigators of a possible link between human Crohn's disease and *M. avium* subsp. *paratuberculosis* has led to increased awareness of these microorganisms as far as public health is concerned (reviewed in Hermon-Taylor et al., 2000; Chacon et al., 2004; Greenstein and Collins, 2004). The current situation is such that the association between *M. avium* subsp. *paratuberculosis* and Crohn's disease can neither be proven or disproved. This is due to a lack of conclusive scientific evidence as well as the complex nature of Crohn's disease in humans, but the causal link hypothesis still remains plausible at present (see reviews in Anonymous, 2000; Gould, 2004; Grant, 2005).

In this context, it is prudent that control measures that are designed to minimize public exposure to *M. avium* subsp. *paratuberculosis* organisms are encouraged until the potential public health issues are resolved (Anonymous, 2000; Gould, 2004). More so in view of the fact that numerous avenues currently exist for potential human exposure to these microorganisms. *M. avium* subsp. *paratuberculosis* organisms infecting domestic food animals such as beef and dairy cattle can potentially be transmitted through consumption of contaminated milk or meat. Furthermore, the infected animals are also capable of shedding large amounts of *M. avium* subsp. *paratuberculosis* organisms in their feces into the environment, and these may subsequently contaminate natural water supplies.

Studies performed in several countries have demonstrated evidence of raw milk contamination based on either culture of *M. avium* subsp. *paratuberculosis* or detection of organisms' DNA in milk samples (Grant et al., 2000; Grant et al., 2001; Corti and Stephan, 2002; Pillai and Jayarao, 2002). Meanwhile the fate of viable organisms found in milk remains unclear. Some researchers have shown that MAP organisms can occasionally survive the standard pasteurization protocols and some of the cheese production processes (Spahr and Schafroth, 2001; Grant et al., 2002b; Donaghy et al., 2004). Viable *M. avium* subsp. *paratuberculosis* organisms have been isolated in some instances in commercial pasteurized retail milk samples from the Czech republic, USA and United Kingdom (Grant et al., 2002a; Ayele et al., 2005; Ellingson et al., 2005). But there are also studies that have found that current pasteurization protocols might be sufficient in most cases to inactivate the *M. avium* subsp. *paratuberculosis* organisms in milk (Gao et al., 2002; O'Reilly et al., 2004; McDonald et al., 2005).

The role of bovine meat products as potential food vehicles for the transmission of *M. avium* subsp. *paratuberculosis* to humans has so far received less attention compared to milk. *M. avium* subsp. *paratuberculosis* infections have also been shown to be prevalent in beef cattle herds around the world using different methods (Thorne and Hardin, 1997; Boelaert et al., 2000; Gasteiner et al., 2000; Whittington et al., 2000; Hill et al., 2003). It has also been shown that in advanced stages of bovine paratuberculosis there is dissemination of the *M. avium* subsp. *paratuberculosis* infection from the gastrointestinal tract locations to various other organs (Hines et al., 1987). Meanwhile in some studies the prevalence of *M. avium* subsp. *paratuberculosis* in samples collected from slaughter cattle has also been reported. Based on early studies prevalence of *M. avium* subsp. *paratuberculosis* organisms ranging from 1.6% to 18% were detected in culled cattle sampled at abattoirs in different parts of the USA (Chiodini and van Kruiningen, 1986; Merkal et al., 1987). Mckenna et al. (2004) recently reported a 16.1% prevalence of *M. avium* subsp. *paratuberculosis* in a culled dairy cattle population sampled at slaughterhouses of Eastern Canada and Maine.

Therefore as long as the impact of *M. avium* subsp. *paratuberculosis* in public health remains uncertain, it is important that necessary precautionary measures aimed at reducing the presence of these organisms in human food products are encouraged. The routine screening of meat and milk products provides one way of monitoring for *M. avium* subsp. *paratuberculosis* contamination along the human food chain. However, such measures are often hampered by difficulties in detection of *M. avium* subsp. *paratuberculosis* organisms. The classical *M. avium* subsp. *paratuberculosis* detection methods based on culture or tissue biopsies are impractical in routine monitoring of food samples.

The use of PCR techniques to detect *M. avium* subsp. *paratuberculosis* DNA provides rapid alternatives for sensitive qualitative detection in food. Moreover the robustness provided by real-time PCR systems also allows the practical incorporation of such protocols in routine surveillance programs to monitor contamination along the human food chain. Along these lines, numerous PCR protocols have been designed and applied for the detection of *M. avium* subsp. *paratuberculosis* in food animal associated samples such as tissues, milk and feces, as well as for the analysis of water supplies (Gwozdz et al., 1997; Corti and Stephan, 2002; Whan et al., 2005). Most of these methods are based on the detection of the multicopy IS900 insertion elements, traditionally considered to be highly specific detection markers for *M. avium* subsp. *paratuberculosis* organisms until recently. This is because IS900-like sequences are present in other mycobacterium species and these can give false positive results in some of the available IS900 based PCR systems (Cousins et al., 1999; Englund et al., 2002; Tasara et al., 2005). This problem has prompted the development and evaluation of alternative PCR systems with improved specificity. Some of the developed methods have been designed to amplify IS900 sequence of *M. avium* subsp. *paratuberculosis* more specifically or target other genetic elements in the organism such as the F57, Hsp X, IS*Mav2* and *dnaA* sequences (Ellingson et al., 1998; Coetsier et al., 2000; Strommenger et al., 2001; Bull et al., 2003; Rodriguez-Lazaro et al., 2004).

We have recently described a real-time PCR protocol that targets the F57 sequence and demonstrated both its utility in the detection of *M. avium* subsp. *paratuberculosis* in spiked and naturally contaminated raw milk samples (Tasara and Stephan, 2005). The primary objectives of our present study were: i) optimization of the sample preparation and the F57 targeting real-time PCR protocols so that they could be applied to other types of bovine sample materials with an emphasis on *M. avium* subsp. *paratuberculosis* detection in tissue, fecal and raw milk samples ii) determine the prevalence of *M. avium* subsp. *paratuberculosis* in bulk tank raw milk delivered for processing at a cheese production plant iii) to perform a study designed to investigate the prevalence of *M. avium* subsp. *paratuberculosis* in samples collected from culled dairy cows sourced from various regions of Switzerland.

3. Materials and Methods

3.1. Sampling of the bulk tank raw milk

The raw milk analyzed in this study was sampled on three separate occasions during the months of August and September 2005. A total of 100 bulk tank raw milk samples were collected as follows: 10 samples were collected on the first visit, 56 samples were collected on the second visit, and 34 samples were collected on the third visit. For each sample, 30 ml were collected from individual farm raw milk bulk tank before they were pooled together in the cheese production factory's main bulk tank. The samples were immediately put in a cooler with ice packs and transported to the lab. Upon arrival the samples were divided into 10 ml aliquots and immediately frozen at -20°C until used for total genomic DNA template isolation as described below.

3.2. Slaughterhouse sample collection

During the period of February to April 2005, weekly visits were made every Tuesday to a slaughterhouse. This slaughterhouse processes cattle purchased from all over Switzerland. On each occasion 10-15 cows from different farms were randomly sampled from the group of cull dairy cows being processed on that particular day. The sampling focused on cows having had at least one lactation. This procedure was repeated until a total of 101 cull dairy cows had been sampled.

The cows were first identified using numbered ear tags in the pre-slaughter pens. At this point a milk sample was collected by hand milking from one of the quarters, except for 17 sampled cows, which were dry. Post slaughter the animal carcasses were tracked to the evisceration area using the tag numbers. At this stage a sample from each animal's mesenteric lymph nodes, ileum and feces were collected.

Samples of the jejunal lymph nodes and ileum (2 to 5 cm segment) were collected first, and in parallel on the carcass line, during routine meat inspection, the meat inspectors also collected samples of each animals' diaphragmatic muscle (roughly 2 cm by 2 cm). At the end a fecal sample (about 100-500 mg) was collected using a swab from the incised rectum or distal colon. A defined set of cutting instruments was used for collecting samples from each animal, except for the diaphragm samples which were collected by meat inspectors using their routine meat inspection equipment. The tissue samples were held in a cooler with ice packs and transported to the lab. The samples were immediately frozen at -20°C upon arrival until they were processed for DNA template isolation as described below.

3.3. Preparation of *M. avium* subsp. *paratuberculosis* control DNA template

M. avium subsp. *paratuberculosis* genomic DNA template used as a positive control in this study was purified directly from colonies of the MAP reference strain ATCC 6783 that had been grown on solid media (HEYM slants). Genomic DNA was isolated using the High Pure PCR template Preparation Kit following the procedures outlined in the kit protocol (Roche Molecular Diagnostics, Penzberg, Germany).

3.4. Preparation of genomic DNA templates from raw milk samples

Total genomic DNA template was isolated from 10 ml raw milk samples using the MagNA Pure LC instrument system (Roche Molecular Diagnostics, Penzberg, Germany).

Briefly, 100 μl of Triton X-100 were mixed with 10 ml of each raw milk sample. The mixtures were centrifuged for 30 minutes at 4,500 rpm and pellets were obtained. Most of the supernatant was discarded to leave about 0.5 ml in which the pellets were resuspended and transferred into eppendorf tubes.

A second centrifugation step was performed (10 min at 14,000 rpm), the rest of the supernatant removed, and the pellets either directly used for DNA isolation or stored at -20°C until processed.

To isolate genomic DNA, the milk pellets were mixed with 100 µl PBS and 300 µl of lysis buffer provided in the MagNA Pure LC DNA Isolation Kit I (Roche Molecular diagnostics). The mixtures were incubated for 15 minutes at 70°C with periodic mixing until the pellets were dissolved. The mixtures were transferred onto the lysing bead matrix in MagNA lyser tubes and the tubes were put into the MagNA Lyser instrument (Roche Molecular Diagnostics). A mechanical lysis step consisting of 60 sec at 6500 rpm followed by 60 sec on a cooling block held at 4°C was performed three times on the samples. After the last mechanical lysis step, the samples were cooled for about 5 minutes, and then a Proteinase K digestion step was done. This was performed by adding 100 µl Proteinase K solution provided in the kit (MagNA Pure LC DNA Isolation Kit I), and incubating the mixtures for 30 minutes at 70°C. Thereafter the samples were incubated for a further 15 minutes at 90°C, and then cooled to room temperature, and briefly centrifuged (14,000 rpm 30 seconds).

At this point, 400 µl of each sample lysate was transferred directly to the MagNA Pure LC instrument sample cartridge. Genomic DNA templates were then isolated from these samples using the automated MagNA Pure LC instrument and in accordance with the High Performance External Lysis protocol of the MagNA Pure LC DNA Isolation Kit I (Roche Molecular Diagnostics). The purified genomic DNA templates were eluted in 100 µl of elution buffer provided in the DNA isolation kit. The DNA yields were calculated based on optical density measurement at 260 nm using the Nanodrop and purity assessed by the templates' OD_{260}:OD_{280} ratios.

Thereafter samples were either directly analyzed by LC-PCR or stored at 4°C for later processing. The LC-PCR analysis was done in duplicate for each sample. In each case a 5-µl aliquot of the undiluted template was used per LC-PCR reaction. If inhibition was detected as judged by the lack of both IC template and F57 target amplification in the LC-PCR assay, then a template dilution (1:50) step was done, and the reaction repeated.

3.5. Preparation of genomic DNA templates from tissue samples

To prepare genomic DNA templates from bovine tissues (lymph nodes, ileum or diaphragm muscle), the MagNA Pure LC instrument was used and the High Performance External Lysis protocol of the MagNA pure LC Nucleic Acid Isolation Kit I was followed.

Briefly, about 50 mg of bovine tissue, 400 μl of lysis/binding buffer and 100 μl of proteinase K solution provided in the kit were mixed in an eppendorf tube, and the whole mixture incubated at 65°C for 1-2 hr (with periodic mixing) or overnight at 37°C. The digested tissue mixtures were transferred onto the lysing matrix (MagNA Lyser Green Beads) in MagNA lyser tubes, and a mechanical lysis performed as described above.

Thereafter 400 μl of the lysates were transferred directly to the MagNA lyser LC instrument sample cartridge. Genomic DNA templates were purified from the sample lysates using the MagNA pure LC instrument and following the High Performance External Lysis protocol of the MagNA Pure LC DNA Isolation Kit I as described above. The purified genomic DNA templates were eluted in 100 μl elution buffer provided in the kit, and DNA yield and purity assessed as described above, and the samples directly used for LC-PCR analysis or appropriately stored at 4°C until analyzed. For LC-PCR analysis, 5-μl aliquots of DNA template from each sample were amplified in duplicates.

3.6. Preparation of genomic DNA templates from fecal samples

To isolate genomic DNA from bovine fecal samples, we combined a mechanical lysis step with the High Pure template preparation Kit (Roche Diagnostics GmbH, Penzberg, Germany).

Approximately 100-200 mg of fecal sample collected on a swab were resuspended in 0.5 ml lysis buffer (20mM Tris-HCL (pH 8.0), 400 mM NaCl, 0.6% sodium dodecyl sulfate, 2 mM EDTA), and the homogenized mixture transferred onto the lysing matrix in the MagNA lyser tubes.

A mechanical lysis step consisting of 1 min at 6500 rpm in the MagNA lyser instrument was repeated three times. After the last step, 240 µl of each lysate were mixed with 60 µl of Proteinase K solution and 300 µl binding buffer provided in the High Pure Template preparation Kit. The mixtures were first incubated at 70°C for 30 min and then at 90°C for 10 minutes. The samples were cooled to room temperature, 150 µl isopropanol added and the samples mixed. The mixtures were applied onto the DNA binding columns, and processed as described in the kit protocol. The purified DNA templates were eluted in 100 µl of elution buffer supplied in the kit. Template concentration and purity were assessed as described above. For LC-PCR analysis, 5-µl aliquots of each template were used per reaction, and each sample was analyzed in duplicates.

3.7. *M. avium* subsp. *paratuberculosis* detection in clinical bovine paratuberculosis cases by LC-PCR analysis

To evaluate the ability of the different template preparation procedures combined with LC-PCR protocol in detection of natural infection, we analyzed samples of mesenteric lymph node, ileum, feces and milk collected from two clinical cases of Johne's disease. The samples were processed to obtain DNA templates from each type of sample material as outlined above. In parallel, sections from the ileum and mesenteric lymph nodes from these clinical cases were processed and examined using conventional histopathological protocols. Furthermore, a direct Ziehl Neelsen (ZN) staining was done with the fecal samples.

3.8. Preparation of the PCR internal amplification control (IC) template

The IC template was designed so that it could be amplified using the same primer set that also amplifies the *M. avium* subsp. *paratuberculosis* F57 sequence target, but complementary to the MAP f57-3'Fluoro and PuC19-5'Red705 probes for its detection.

Briefly, primers MAP f57 p1 and MAP f57-IC1 (see table 1) were used to amplify a 180 bp target region of *M. avium* subsp. *paratuberculosis* F57 sequence using the Expand Long Template PCR System (Roche) in accordance with the suppliers' protocol. The PCR product from this reaction was purified and reamplified using the primers MAP f57 p1 and MAP f57-IC2. This second amplification step resulted in a 257 bp PCR product identical to the *M. avium* subsp. *paratuberculosis* F57 sequence region targeted by the MAP f57 p1/p2 primer set, except that the 18 bp region that binds to the MAP f57-5'-Red 640 probe in the F57 sequence target is replaced by a 21 bp sequence derived from the puC19 plasmid. This was incorporated as part in the MAP f57 IC1 primer design (see underlined region in table 1). This region is complementary to the puC19-5'LC Red 705 probe and therefore the generated IC template can be amplified using MAP f57 p1/p2 primer set and detected using the MAP f57-3'Fluo/puC19-5'LC-Red 705 probe combination in the LC-Red 705 channel of the LC instrument. This IC template was gel purified, quantified and aliquots were prepared and frozen at -20°C until used.

3.9. LC-PCR assays

Real-time PCR was used to co-amplify and detect a 254 bp target region in the *M. avium* subsp. *paratuberculosis* F57 sequence (Tasara and Stephan, 2005) and a 257 bp IC template.

The reactions were performed in the Light Cycler 2.0 instrument (Roche Molecular Diagnostics) in a total reaction volume of 20 μl in glass capillary tubes. The optimal reaction mixture contained 1x concentration of LightCycler-Faststart DNA master plus^{TM} hybridization probes mix (Roche Molecular Diagnostics, Penzburg, Germany), 1000 nM of each primer (MAP f57p1, MAPf57p2), 200 nM of each LC probe (MAP f57-3'Fluo, MAP f57-5'LC-Red640, PuC19-5'LC-Red 705) and 20 copies of IC template. The amplification consisted of an initial preincubation step at 95°C for 10 minutes to activate the DNA polymerase, followed by 45 cycles of 95°C for 10s, 56°C for 20s and 72°C for 18s.

The fluorescence signals corresponding to F57 sequence target and the IC template amplification were monitored during the 56°C annealing step in the LC-Red 640 nm and LC-Red 705 detection channels of the light cycler 2.0 instrument, respectively.

3.10. Defining the LC-PCR assay's analytical detection limits

The lower detection limits of the *M. avium* subsp. *paratuberculosis* LP-PCR assay under the conditions used in this study were defined using DNA templates purified from *M. avium* subsp. *paratuberculosis* colonies, as well as total genomic DNA template purified from *M. avium* subsp. *paratuberculosis*-spiked bovine raw milk and fecal samples.

Spiked bovine raw milk samples containing approximately 1000, 100, 10 and 1 *M. avium* subsp. *paratuberculosis* cells per ml were prepared starting from a confirmed *M. avium* subsp. *paratuberculosis* negative stock raw milk sample as previously described (Tasara and Stephan, 2005). A *M. avium* subsp. *paratuberculosis* negative fecal stock sample was collected from a dairy cow resident in the University of Zurich Veterinary hospital. This cow had no history of paratuberculosis and the negative status of the stock fecal sample was confirmed to be free of *M. avium* subsp. *paratuberculosis* DNA by LC-PCR analysis prior to spiking experiments.

Tenfold serial dilutions of *M. avium* subsp. *paratuberculosis* cells were prepared starting from a stock suspension containing 10^8 cells per ml in PBS as previously described (Tasara and Stephan, 2005). Aliquots (100 μl) of dilutions containing from 10^4 to 10^1 cells per ml were added to 200 mg feces to yield duplicate samples with approximately 1000, 100, 10 and 1 cells.

DNA templates were separately prepared from the *M. avium* subsp. *paratuberculosis* colonies, spiked milk and fecal samples as already described above. Each template preparation run included a process negative control to monitor for potential cross contaminations.

Thereafter, 5-μl aliquots containing defined amounts of *M. avium* subsp. *paratuberculosis* genomic DNA or eluates of total genomic DNA purified from the spiked bovine milk and fecal samples were analyzed by LC-PCR assay. Standard steps in avoiding potential sample cross contamination and false positive results were taken through out this study. 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 use of filtered reaction tips.

4. Results

4.1. Optimization of the LC-PCR assay and evaluation of analytical sensitivity

As part of the current work, a new IC template was developed and incorporated into the LC-PCR assay. The IC template developed consists of a PCR generated 257 bp fragment that can be amplified with the same primer set as the *M. avium* subsp. *paratuberculosis* F57 sequence target. It consists of a 257 bp sequence, which is identical to the *M. avium* subsp. *paratuberculosis* F57 sequence except that the detection probe region in the IC template has been replaced by a PuC19 based 21 bp fragment complementary to the PuC19-5'-LC-Red 705 detection probe. This distinguishes the IC template from the *M. avium* subsp. *paratuberculosis* F57 sequence, allowing the separate detection in different channels on the LC-instrument during the assay, when both templates are amplified in the same reaction. We established by titration that approximately 20 copies of the IC template per reaction were sufficient to give a clear IC amplification signal without severely compromising the detection of low amounts of the *M. avium* subsp. *paratuberculosis* F57 sequence in the same reaction (data not shown). These conditions therefore allow a competitive advantage to the *M. avium* subsp. *paratuberculosis* F57 sequence target at low concentration as well as allowing the detection of low levels of sample associated inhibition.

As an example graphical plots from an optimized LC-PCR assay is presented in figure 1. This shows the co-amplification of the F57 sequence target from 1ng of *M. avium* subsp. *paratuberculosis* genomic DNA template and IC template in the same reaction and monitored in their respective detection channels on the LC instrument.

We then evaluated the analytical sensitivities of the LC-PCR assay system when applied to the raw milk and fecal samples. The assay's minimal analytical detection limits were evaluated using purified *M. avium* subsp. *paratuberculosis* genomic DNA as well as total genomic DNA templates purified from *M. avium* subsp. *paratuberculosis*-spiked raw milk and fecal samples, and the results are summarized in table 2.

On purified genomic *M. avium* subsp. *paratuberculosis* DNA a reproducible detection limit of about 35 fg DNA template was established. The analysis of total genomic DNA templates isolated from 10 ml samples of *M. avium* subsp. *paratuberculosis*- spiked raw milk showed that the assay had a detection limit in the range of 10-100 cells per ml. Similarly the evaluation of *M. avium* subsp. *paratuberculosis*-spiked bovine feces also demonstrated that the assay had a detection limit in the range of about 10-100 cells if they were contained within 200 mg of fecal sample material.

4.2. Application of the *M. avium* subsp. *paratuberculosis* detection protocols to samples from clinical cases of bovine paratuberculosis

In the next step, we evaluated the different sample preparation regimens and LC-PCR assay for their ability to detect *M. avium* subsp. *paratuberculosis* organisms in samples from clinical cases of bovine paratuberculosis.

To this end samples of mesenteric lymph nodes, ileum, milk and feces were collected from two dairy cows that have been presented with clinical signs of Johnes' disease. These samples were processed to isolate total genomic DNA templates and followed by their analysis in the LC-PCR assay. In parallel the fecal sample, mesenteric lymph node and ileum of the same animals were further examined by direct ZN straining or by a histological examination.

A summary of the results from both histological and LC-PCR analysis of these samples for *M. avium* subsp. *paratuberculosis* organisms' presence is shown in table 3. As an example the stained histological sections of the mesenteric lymph node from cow 2 is shown in figure 2 and the presence of *M. avium* subsp. *paratuberculosis* organisms in this animal's mesenteric lymph node tissues was confirmed.

This part of the study on samples from clinical cases of paratuberculosis confirmed that our present detection protocols could also successfully detect *M. avium* subsp. *paratuberculosis* organisms when present in naturally contaminated bovine samples of tissues, feces and raw milk.

The specificity of the *M. avium* subsp. *paratuberculosis* F57 amplicons detected in these clinical cases was further confirmed by their sequencing. The F57 sequence was amplified in the LC-PCR positive samples by conventional PCR, the amplicons were purified and their sequences determined. In all the cases the obtained amplicons were 100% identitical to the reference *M. avium* subsp. *paratuberculosis* F57 sequences deposited in the public data bases (data not shown).

4.3. Prevalence of *M. avium* subsp. *paratuberculosis* in bulk tank raw milk samples and slaughtered dairy cows

Integrating the optimal DNA template preparation protocols for the different types of sample material and LC- PCR based detection were next evaluated in a field study.

Amongst the 100 bulk tank milk samples that were tested, we found 3 samples (3%) that were positive for *M. avium* subsp. *paratuberculosis*. Meanwhile from a total of 488 samples collected from culled dairy cows, *M. avium* subsp. *paratuberculosis* was detected by LC-PCR in 21 samples from 20 different animals (one cow in this group tested positive in two samples, a fecal and a lymph node sample). This gave an overall prevalence of 19.8 % positive animals in the 101 culled dairy cows that were examined. In total, 8.9% (9/101) of the cows were positive for *M. avium* subsp. *paratuberculosis* DNA in their fecal samples, 4.9% (5/101) in their jejunal lymph nodes, 0.9% (1/101) in their ileum tissue and 3.6% (3/84) were positive in their milk. Meanwhile in 2.9% (3/101) of the culled cows, *M. avium* subsp. *paratuberculosis* organisms were detected in samples of their diaphragmatic muscles. It cannot be ascertained if MAP organisms found on diaphragm samples were due to a disseminated infection or simply a result of cross contamination in the slaughterhouse. But the presence of *M. avium* subsp. *paratuberculosis* in these diaphragmatic tissue samples was confirmed when the entire detection protocol was performed on two separate occasions in the positive samples.

As an additional confirmation step, the target *M. avium* subsp. *paratuberculosis* F57 sequence was re-amplified from a selection of the LC-PCR positive samples by conventional PCR, and the resulting amplicons were sequenced. In all cases the amplicons displayed a 100% identity with the reference *M. avium* subsp. *paratuberculosis* F57 sequences (data not shown).

The monthly proportion of *M. avium* subsp. *paratuberculosis* positive cows varied from 1.8% to 7.5% over this 3 months sampling period (see figure 3). The highest proportion of *M. avium* subsp. *paratuberculosis* contamination was detected in samples collected during the month of March. In this period 7.5 % (14/188) of the samples collected from the sampled cull cows were found to be *M. avium* subsp. *paratuberculosis* positive.

5. Discussion

M. avium subsp. *paratuberculosis* is a significant economic problem for both dairy and beef farming around the world. Cattle infection with these organisms leads to significant losses in productivity as well as Johne's disease. The affected animals also shed *M. avium* subsp. *paratuberculosis* organisms primarily through their feces into the environment, and directly in their milk. Moreover, the unresolved potential link of *M. avium* subsp. *paratuberculosis* organisms to Crohn's disease also raises public health concerns due to its possible food-borne transmissions to human subjects through dairy and beef products (Anonymous, 2000; Gould, 2004; Grant, 2005). Therefore it is important that reliable prevalence data is generated about these organisms in food animals and their products that are destined for human consumption in order to understand their potential roles in *M. avium* subsp. *paratuberculosis* transmission.

In the present study we have applied optimized sample preparation regimens and an diagnostic F57 sequence based real-time PCR system (Tasara and Stephan, 2005) to determine the prevalence of *M. avium* subsp. *paratuberculosis*. This protocol was selected because it targets a genetic marker with a well documented specificty for *M. avium* subsp. *paratuberculosis* based on several studies reported to date (Coetsier et al., 2000; Vansnick et al., 2004, Tasara et al., 2005). Moreover, the presence of the IC template in each reaction allows to monitor for sample associated inhibition.

A lot of focus has already been on raw milk as a potential vector for *M. avium* subsp. *paratuberculosis* transmission to humans. Along these lines and using IS900 based PCR approaches, studies performed in several countries have detected varying levels of *M. avium* subsp. *paratuberculosis* contamination in bulk tank raw milk as well as in commercial pasteurized retail milk (Corti and Stephan, 2002; Grant et al., 2002a; Pillai and Jayarao, 2002; Jayarao et al., 2004). Viable *M. avium* subsp. *paratuberculosis* organisms have also occasionally been isolated from samples of commercial pasteurized raw milk destined for human consumption (Grant et al., 2002a; Ayele et al., 2005; Ellingson et al., 2005).

Although bovine paratuberculosis is a notifiable disease within Switzerland, the true prevalence of *M. avium* subsp. *paratuberculosis* within the country's cattle herds remains unknown. There have already been studies done in view of *M. avium* subsp. *paratuberculosis* prevalence in the 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 *M. avium* subsp. *paratuberculosis* infection in this dairy cattle population ranged from 6.5% to 10% (Bogli-Stuber et al., 2005). A countrywide large scale analysis of bulk tank raw milk performed using an IS900 based nested PCR system estimated an average prevalence of 19.7% *M. avium* subsp. *paratuberculosis* in Swiss bovine bulk tank raw milk samples (Corti and Stephan, 2002). Results from these two studies therefore probably indicate high levels of subclinical *M. avium* subsp. *paratuberculosis* infection within the Swiss dairy herds. However, their estimation of the true prevalence of *M. avium* subsp. *paratuberculosis* in the country's dairy herds and bulk tank milk still remains unclear in view of the specificity questions associated with IS900 based PCR systems (Tasara et al., 2005).

Thus in order to clarify some of these issues, it was necessary that the prevalence question in Swiss bulk tank raw milk be revisited using an assay systems with enhanced specificity for *M. avium* subsp. *paratuberculosis* organisms. In this regard our current study focused on a small scale evaluation of bulk tank raw milk samples collected from Swiss dairy herds using an F57 sequence based PCR system. Based on the previous study with an IS900 nested PCR system, the *M. avium* subsp. *paratuberculosis* prevalence in bulk tank raw milk samples from this region of the country ranges from 8.5% to 14.3% (Corti and Stephan, 2002). In our current study, we tested 100 bulk tank raw milk samples from different dairy farms in this region and found a 3% prevalence of *M. avium* subsp. *paratuberculosis*. This study therefore reconfirmed that *M. avium* subsp. *paratuberculosis* contamination is found in the bulk tank raw milk. However, the current prevalence of 3% was substantially lower than the previous prevalence determined from bulk tank raw milk survey done in this region of the country, using an IS900 based nested PCR system (Corti and Stephan, 2002).

Meanwhile the role of beef and associated beef products in view of *M. avium* subsp. *paratuberculosis* contamination or dissemination to human population has not yet been studied to the same extent as bovine milk. Although it has also been shown that Johne's disease is prevalent in some beef herds around the world, and therefore it is possible that a proportion of *M. avium* subsp. *paratuberculosis* organisms infecting beef cattle are also transmitted to the human population through beef and other associated products.

In particular it is known that *M. avium* subsp. *paratuberculosis* infections get disseminated in the later stages via the blood stream to various organs of the animal such as the lymph nodes, liver and muscle tissue (Hines et al., 1987; Ayele et al., 2004). Moreover in a number of countries, dairy cows that are culled due to Johne's disease are often sent for slaughter. For example meat from old dairy cows that is used to make ground beef for human consumption has been suggested as a potential source for *M. avium* subsp. *paratuberculosis* infection in the USA (Manning and Collins, 2001). Several studies to date have documented *M. avium* subsp. *paratuberculosis* infections in cattle destined for beef production. Choidini et al. (1986) found an 18% *M. avium* subsp. *paratuberculosis* prevalence in slaughtered cattle of New England. This study used culture to analyze samples collected from 100 slaughter cattle that were randomly selected at the abattoir (Chiodini and van Kruiningen, 1986). Merkal et al. (1987) determined using the culture method a *M. avium* subsp. *paratuberculosis* prevalence of 2.9% and 0.8% in culled healthy dairy and beef cattle, respectively. This was based on the analysis of 7540 samples of ileocecal lymph nodes from healthy slaughter cattle collected over a two year period (Merkal et al., 1987). In a study conducted in Ontario Canada, between 1986 and 1989, *M. avium* subsp. *paratuberculosis* organisms were isolated in 5.5% of the samples collected from 400 cull cows in this region (NcNab et al., 1991). Cetinkaya and co-workers used an IS900 based PCR system and culture to evaluate intestinal lymph nodes from 1553 healthy adult cattle slaughtered at abattoirs in South West England. They detected a *M. avium* subsp. *paratuberculosis* prevalence of 3.5% and 2.6% in these samples by PCR and culture respectively (Cetinkaya et al., 1996).

Rossiter and Henning (2001) sampled the gut (feces and ileocecal lymph nodes), liver and meat associated lymph nodes of thin market dairy and beef cows and analyzed them for *M. avium* subsp. *paratuberculosis* presence. They were able to detect *M. avium* subsp. *paratuberculosis* by culture in 0.7 % and 11% of beef and dairy cows, respectively, that were sampled at slaughterhouses in north eastern USA. Recently, Mckenna et al. (2004) reported a study that examined samples collected from 984 culled dairy cows older than 20 months selected by random sampling at abbattoirs in eastern Canada and Maine. They found using histology, bacteriological culture and PCR methods a 16.1% prevalence of *M. avium* subsp. *paratuberculosis* in the culled dairy cattle population.

In our present study we have investigated the prevalence of *M. avium* subsp. *paratuberculosis* contamination in samples collected from lactating culled dairy cows sourced from all over Switzerland. Our study used F57 sequence based PCR on tissue, feces and raw milk samples collected from 101 healthy dairy cows that came from different farms located in different regions throughout Switzerland. We found an overall *M. avium* subsp. *paratuberculosis* prevalence of 19.8% upon the examination of feces, jejunal lymph nodes, diaphragm and raw milk samples obtained from these slaughtered dairy cows. One previous study has reported on the prevalence of *M. avium* subsp. *paratuberculosis* in Swiss cattle at the slaughterhouse level. Seitert (2003) used an IS900 based PCR system, culture and cryomicroscopy to examine samples of lymph nodes and muscle tissues collected from 134 cows at slaughter. This study, however, focused on a population of 100 emaciated cows older than 24 months, and these were compared to a group of 34 healthy young cows less than 18 months old. These animals were randomly sampled at slaughterhouses around Switzerland during the period May to August 2002. He found an overall *M. avium* subsp. *paratuberculosis* prevalence of 11.2% (15/134) by PCR of the lymph node samples from these cattle. *M. avium* subsp. *paratuberculosis* organisms were isolated from 14 of the 15 PCR positive samples by culture. In that study, 12 % (12/100) of the emaciated cows sampled, and 8.8% (3/34) of the young healthy cows sampled were found to be *M. avium* subsp. *paratuberculosis* positive based on an IS900 PCR system.

The same study reported an even higher prevalence (15.7 %) of *M. avium* subsp. *paratuberculosis*, when the same lymph nodes samples were tested using a cryomicroscopy based detection method (Seitert, 2003).

Our study as well as the earlier study by Seitert (2003), both show that *M. avium* subsp. *paratuberculosis* organisms are detected in cull cattle that are destined for beef production. The prevalence of *M. avium* subsp. *paratuberculosis* in processed beef products destined for human consumption nor these organisms' abilities to survive the processing of beef for consumption such as cooking remain largely unexplored. However, the fact that *M. avium* subsp. *paratuberculosis* is detected in beef carcasses suggests that some *M. avium* subsp. *paratuberculosis* organisms may find their way into consumer beef and hence a possibility that they may be consumed at least in raw or under cooked beef products.

This means as long as the link between *M. avium* subsp. *paratuberculosis* and Crohn's disease in humans remain unclear, measures designed to minimize public exposure should also include a focus on beef products. Thus in this context, further studies are needed to evaluate the efficacy of beef processing procedures in inactivation or removal of *M. avium* subsp. *paratuberculosis* organisms found in culled cattle.

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7. Tables

Table 1. Amplification primers and LC probes[2] used in this study

Targets	Primer	Function	Sequence (5′-3′)
MAP F57 and IC	MAP f57p1 MAP f57p2	Forward primer Reverse primer	TTGGACGATCCGAATATG T AGTGGGAGGCGTACC A
MAP F57 and IC	MAP f57-3′Fluo	LC-anchor probe	CACGCAGGCATTCCAAGT
MAP 57	MAP f57-5′Red640	LC-MAP F57 detection probe	TGACCACCCTTCCCGTCG
IC template	puC19-5′Red705	LC-IC template detection probe	CAGGGTTTTCCCAGTCACGAC
MAP F57	MAP f57IC1	First step reverse primer used for IC generation	AGTGCTCTGGGGTCGTTCGGTGCGTCAGCGGC GGTCCAGTTCGCTGTCAT*GTCGTGACTGGGAAA ACCCTG*GGACTTGGAATGCCTGCGT
MAP 57	MAP F57IC2	Second step reverse primer used for IC generation	AGTGGGAGGCGTACCAGGGTCGGGGAAAACC GCGTG GCTGACGAGTGCTCTGGGGTCGTT C

[1] All oligonucleotides were synthesized by Microsynth (Balgach, Switzerland).

[2] Probes were selected using the LC Probe Design Software and synthesized by TIB-Molbiol (Berlin, Germany).

Table 2. Evaluation of the LC-PCR assay analytical sensitivity on *M. avium* subp. *paratuberculosis* DNA and total genomic DNA template purified from *M. avium* subsp. *paratuberculosis*-spiked raw milk and fecal sample[1]

Processed sample	LC-PCR detection result			
MAP colonies	Amount of MAP DNA (fg) used per LC-PCR reaction			
	140 fg	70 fg	35 fg	10 fg
Run A	4/4	4/4	4/4	2/4
Run B	4/4	4/4	4/4	1/4
Overall result	8/8	8/8	8/8	3/8
10 ml of MAP spiked raw milk	Amount of MAP cells spiked per ml of raw milk			
	1000	100	10	1
Run A	2/2	2/2	2/2	0/2
Run B	2/2	2/2	0/2	0/2
Overall result	4/4	4/4	2/4	0/4
200 mg of MAP spiked feces	Amount of MAP cells spiked in a 200 mg fecal sample			
	1000	100	10	1
Run A	4/4	4/4	2/4	0/4
Overall result	4/4	4/4	2/4	0/4

[1] In one or two (Run A and Run B) experimental runs, replicates of indicated amounts of purified *M. avium* subsp. *paratuberculosis* genomic DNA or 5-µl aliquots of the total genomic DNA templates purified from the *M. avium* subsp. *paratuberculosis*-spiked samples of raw milk and fecal as shown were analyzed using the LC-PCR assay following the protocols outlined in materials and methods. In each sample the amplification of both the *M. avium* subsp. *paratuberculosis* F57 sequence target and the IC were monitored in their respective detection channels of the LC instrument. In each case the number of positive results/total number of reactions per run is listed. The overall result lists the sums up the total number of positives/total number of reactions from all the runs. In all the reactions recorded here,appropriate amplification conditions were confirmed by monitoring for the IC template amplification.

Table 3. Results of *M. avium* subp. *paratuberculosis* detection by histology and LC-PCR analysis on ileum, mesenteric lymph nodes, raw milk and feces samples collected postmortem of dairy cows showing clinical signs of Johnes' disease[1]

sample	test	Case A	Case B
Ileum	Histology/ZN staining	positive	not done
	LC-PCR	positive	positive
Mesenteric lymph node	Histology/ZN staining	positive	positive
	LC-PCR	positive	positive
Milk	LC-PCR	positive	negative
Feces	Direct ZN staining	positive	positive
	LC-PCR	positive	positive

[1] Total genomic DNA templates were extracted and analyzed for *M. avium* subsp. *paratuberculosis* organisms' presence by LC-PCR analysis as outlined. In parallel sections of ileum and mesenteric lymph nodes from these clinical cases were also stained and examined by histology for *M. avium* subsp. *paratuberculosis* organisms and associated pathological changes.

8. Figures

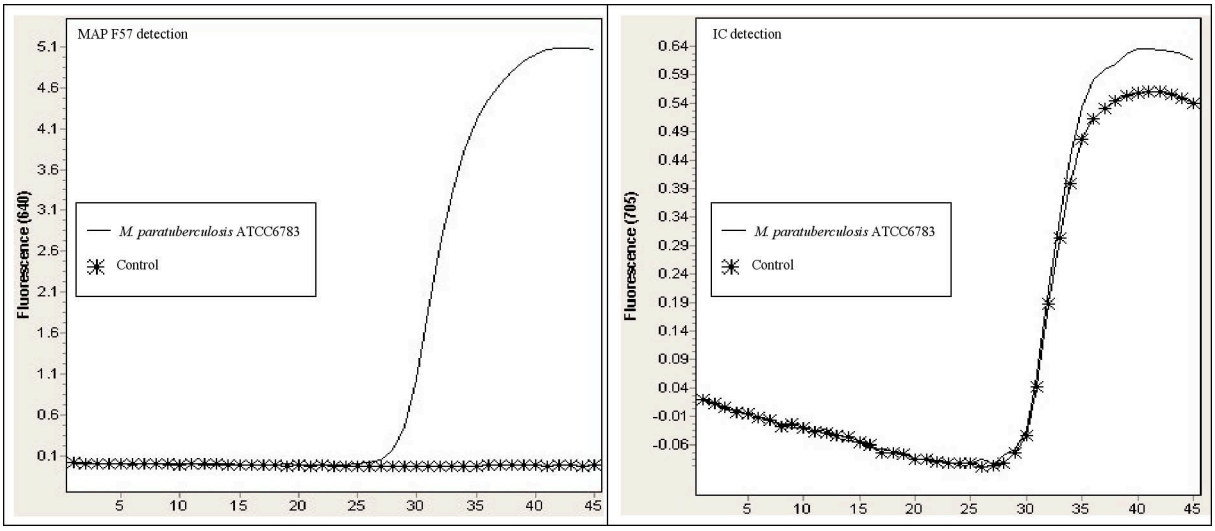
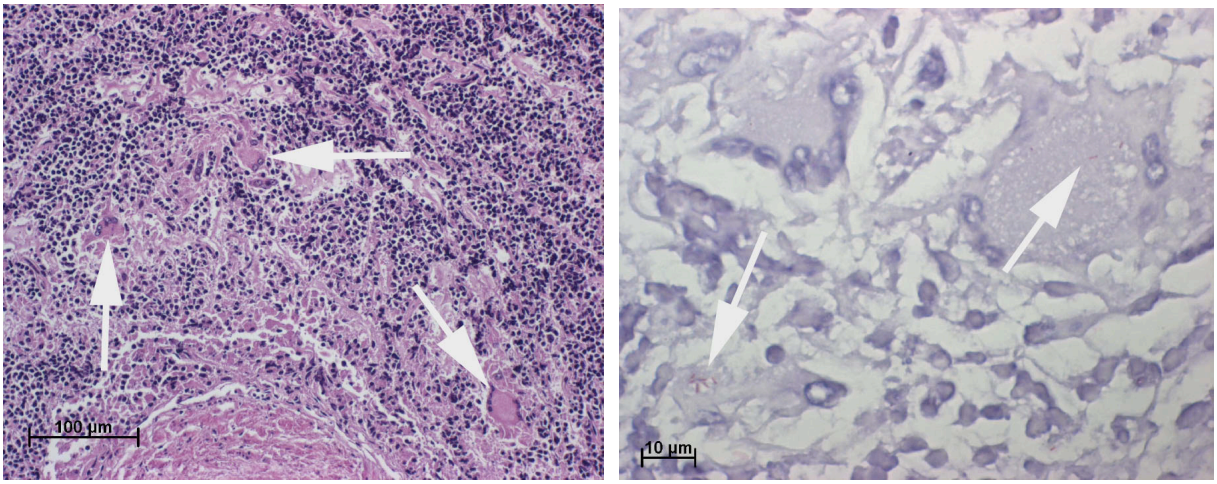


FIG 1. An example showing the co-amplification and detection of *M. avium* subsp. *paratuberculosis* F57 sequence and IC by LC-PCR assay under optimized conditions: In each reaction 20 copies of IC template and either 5 μl (1 ng) of purified *M. avium* subsp. *paratuberculosis* ATCC6783 genomic DNA template or 5 μl of water (control) were amplified as outlined in methods and materials.

A) The specific amplification of the F57 sequence from *M. avium* subsp. *paratuberculosis* genomic DNA template monitored in the LC-Red 640 detection channel.

B) The amplification of IC template in both reactions as monitored in LC-Red 705 channel, confirming that the PCR reactions were successful in both cases



A **B**

FIG 2. Histopathological examination of a mesenteric lymph node from a cow with clinical signs of Johne's disease.

A) Mesenteric lymph node showing chronic granulomatous lymphadenitis with giant cells (white arrowheads) in the parenchyma (HE stain).

B) Ziehl Neelsen staining of this mesenteric lymph showing giant cells with some acid-fast stained intracellular *M. avium* subsp. *paratuberculosis* organisms (highlighted by the white arrows).

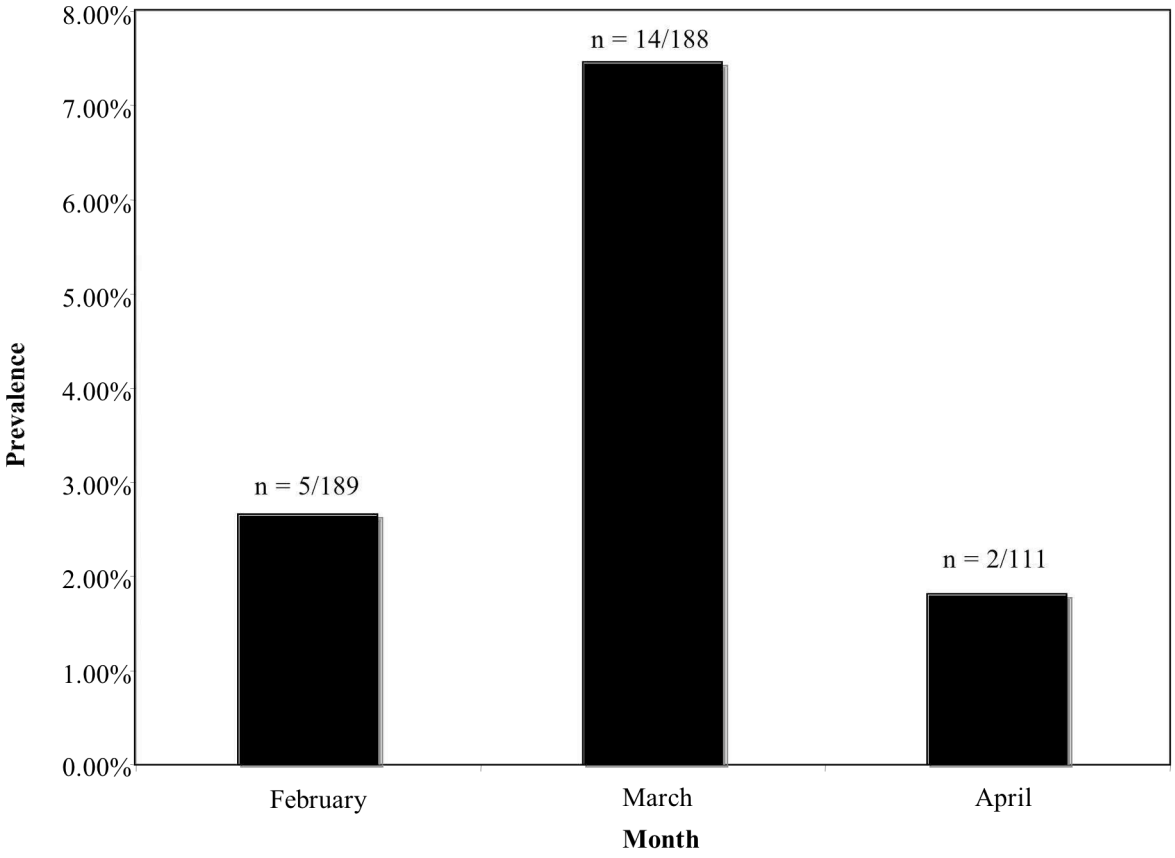


FIG 3. The prevalence of *M. avium* subsp. *paratuberculosis* detection in samples from culled dairy cows by month of sampling.

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