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Short communication: Investigation into *Mycobacterium avium* ssp. *paratuberculosis* in pasteurized milk in Italy

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ABSTRACT

This study investigated the presence of viable *Mycobacterium avium* ssp. *paratuberculosis* (MAP) in pasteurized milk produced by Italian industrial dairy plants to verify the prediction of a previously performed risk assessment. The study analyzed 160 one-liter bottles of pasteurized milk from 2 dairy plants located in 2 different regions. Traditional cultural protocols were applied to 500 mL of pasteurized milk for each sample. The investigation focused also on the pasteurization parameters and data on the microbiological characteristics of raw milk (total bacterial count) and pasteurized milk (*Enterobacteriaceae* and *Listeria monocytogenes*). No sample was positive for MAP, the pasteurization parameters complied with European Union legislation, and the microbiological analysis of raw and pasteurized milk showed good microbiological quality. The results show that a 7-log (or >7) reduction could be a plausible value for commercial pasteurization. The combination of hygiene practices at farm level and commercial pasteurization yield very low or absent levels of MAP contamination in pasteurized milk, suggesting that pasteurized milk is not a significant source of human exposure to MAP in the dairies investigated.
Key words: *Mycobacterium avium* ssp. *paratuberculosis*, pasteurized milk

Short Communication

Mycobacterium avium ssp. *paratuberculosis* (**MAP**) is the causative agent of paratuberculosis, also called Johne's disease, primarily affecting the small intestine of ruminants. *Mycobacterium avium* ssp. *paratuberculosis* was considered to carry a zoonotic risk, on the basis of both clinical and gross lesion similarities between Johne's disease and human Crohn's disease and PCR evidence of MAP in the gut of Crohn's disease patients (Chiodini et al., 2012). Recent reports show that humans, like other species, are susceptible to MAP infection, and strongly support that MAP is the etiologic agent of a subset of patients with Crohn's disease and that it plays a role in the pathogenesis of other human diseases (Naser et al., 2014; Davis, 2015; Kuenstner et al., 2015). Presence of a high bio-load of MAP in domestic and wild ruminants indicates that animals represent a source of MAP infection to humans, directly or indirectly by consumption of animal products (Singh et al., 2014). Infection with MAP is widespread in cattle worldwide and its herd-level prevalence in dairy cattle has been reported to exceed 50% (Nielsen and Toft, 2009) even in Italy: the apparent prevalence of infected dairy cow herds was reported to range from 19.9 to 65% with an intraherd prevalence ranging from 2.8 to 5.9% (Lillini et al., 2005; Pozzato et al., 2011; Marchetti et al., 2013). In Italy, MAP infection was also reported in sheep flocks (73.7% of infected herds and 6.29% of infected animals) and in water buffaloes at lower prevalence (1.2%; Attili et al., 2011; Gamberale et al., 2014). Dairy cattle represent the largest population of MAP-infected animals and are the most likely source of direct or indirect exposure to humans (NACMCF, 2010) as milk may be contaminated by MAP through direct excretion or by fecal contamination during milking (Grant, 2005). Some investigations have also detected viable MAP in retail pasteurized milk (Grant et al., 2002; Ayele et al., 2005; Ellingson et al., 2005; Shankar et al., 2010; Carvalho et al., 2012); this may be due to postprocess contamination processing or survival through the process. Several thermal inactivation studies on MAP have shown conflicting results, but recent works have demonstrated that thermal processes achieve a 4- to 7-log reduction in the number of MAP cells during industrial-scale experiments (Pearce et al., 2001; Lund et al., 2002; Stabel and Lambertz, 2004; Grant et al., 2005; McDonald et al., 2005; Lynch et al.,

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2007; Rademaker et al., 2007). Thus, it appears that regardless of whether MAP is present in pasteurized milk, both the prevalence and the numbers of MAP in pasteurized products will be low (NACMCF, 2010).

Because the public health significance of low numbers of MAP is not known and there are public concerns about human exposure to MAP, we performed a large investigation in 569 Italian farms delivering milk to 3 industrial dairy plants (Marchetti et al., 2013; Serraino et al., 2014a,b). The apparent prevalence in the investigated dairy herds was 6.07% (range 0.00 to 22.73%; Serraino et al., 2014a). We used the findings to perform a quantitative risk assessment of MAP survival in pasteurized milk, estimating an overall percentage of 0.55 to 0.98 of pasteurized milk bottles having a MAP contamination >0 cfu/L and 0.04 to 0.11% of liters having a contamination >100 cfu/L (Serraino et al., 2014b). No data are available on the isolation of MAP in pasteurized milk to validate this estimation, so this study aimed to investigate the presence of viable MAP in pasteurized milk produced by 2 of the 3 Italian industrial dairy plants previously investigated and to verify the prediction of the previously performed risk assessment. The 2 dairy plants were located in 2 different regions and process 38.75 (plant A) and 89.29 (plant B) million liters of milk a year. We analyzed 160 one-liter bottles of pasteurized milk (80 bottles for each dairy plant, 10 bottles for 2 consecutive days in 4 samplings throughout the year to have a complete picture of milk consignments). Milk samples were collected in the period from November 2014 to November 2015 directly in the dairy plant at the end of the production line. Samples were transported on ice to the laboratory within 24 h of collection and analyzed the day after arrival at the laboratory, approximately 48 to 72 h after production.

Given the expected low contamination level, some changes to traditional cultural protocols were applied to enhance the test's sensitivity: 500 mL of pasteurized milk for each sample was aliquoted in 10 sterile plastic tubes (each containing 50 mL). The tubes were then centrifuged at 2,500 × *g* for 15 min at room temperature (21°C) and the supernatant was discharged, whereas the remaining pellets were suspended and aggregated in a single tube using PBS for a final volume of 3 mL. The suspensions were then streaked onto 6 Petri plates (diameter 9 cm) containing Herrold's egg yolk agar supplemented with mycobactin J (2 mg/L), nalidixic acid (50 mg/L), vancomycin (50 mg/L), and sodium pyruvate (4 g/L). Plates were incubated at 37°C for 16 wk and checked monthly for the presence of MAP colonies.

A MAP reference strain (ATCC 19698) was used to determine the limit of detection (**LOD**) of the method. *Mycobacterium avium* ssp. *paratuberculosis* suspensions were prepared according to Logar et al. (2012) and Plain et al. (2014). Briefly, colonies from solid cultures were harvested and suspended in PBS with glass beads (diameter approximately 5 mm), then vortexed for 45 s, and the optical density at 600 nm was adjusted to 0.7; the suspensions were forced through a syringe (26-G needle) several times and filtered through a sterile 5-μm filter. The suspension was examined in a Bürker chamber to count the number of MAP cells (expressed as MAP cells per 500 mL of pasteurized milk; see Table 1, "Mean theoretical input" column). The initial suspensions were serially diluted 10-fold in PBS using tubes containing glass beads (diameter approximately 5 mm), with vortexing for 20 s between dilution steps. One hundred microliters of each dilution was streaked in duplicate onto Petri plates (diameter 9 cm) of Herrold's egg yolk agar with Mycobactin J (2 mg/L) for the determination of MAP expressed as colony-forming units per 500 mL of milk.

To determine the LOD, 6 replicates of 500 mL of pasteurized milk containing concentrations of MAP cells ranging from 8.6×10^{-1} to 8.6×10^{1} cfu per 500 mL of milk (see Table 1, "Mean cultural input" column) were tested by cultural assay. Briefly, 3 L of milk was spiked with each of the above suspensions of MAP and spiked

Table 1. Limit of detection of the culture method used in the study (American Type Culture Collection 19698 strain)

Item	Mean theoretical input[1] (MAP cells per 500 mL)	Mean cultural input[2] (cfu per 500 mL)	Signal ratio[3]
Contaminated milk	2.1×10^{2}	8.6×10^{1}	6/6 (100%)
	2.1×10^{1}	8.6×10^{0}	5/6 (83.3%)
	2.1×10^{0}	8.6×10^{-1}	1/6 (16.7%)
Uncontaminated milk	0.0	0.0	0/2 (0.0%)

[1]Number of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) cells obtained by Bürker chamber count and used to spike 500 mL of pasteurized milk.

[2]Evaluated by streaking 100 μL of each dilution in Herrold's egg yolk agar supplemented with Mycobactin J (2 mg/L) plates in duplicate, reported as cfu per 500 mL of pasteurized milk.

[3]Number of positive replicates versus total number of replicates obtained with the cultural assay.