



J. Dairy Sci. 100:1–6
<https://doi.org/10.3168/jds.2016-11627>
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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 indicated 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.,

Received June 16, 2016.

Accepted September 11, 2016.

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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 are 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 $2,500 \times g$ 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 (American Type Culture Collection 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 (needle 26 G) several times and filtered through a sterile 5- μ m 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 amount 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

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%)

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

²Evaluated by streaking 100 μ L 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.

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

with each of the above suspensions of MAP and spiked milk was then aliquoted in 50-mL tubes and processed as previously described. Moreover, a negative control in duplicate (1 L of milk) was also processed in parallel with spiked samples. The value of LOD was estimated through logit link function (data from Table 1, column “mean cultural input”) as recommended in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE–World Organisation for Animal Health, 2012, 2014) using SPSS Statistics 20 (IBM, Armonk, NY).

As some studies have also pointed out the risk of under-pasteurization or postprocess contamination (Lund et al., 2002; Ellingson et al., 2005), we also investigated the pasteurization parameters and industry-generated data on the microbiological characteristics of raw milk (total bacterial count according to EC Regulation 853/2004; EC, 2004) and pasteurized milk (*Enterobacteriaceae* and *Listeria monocytogenes* according to EC Regulation 2073/2005; EC, 2005) collected in the 2 plants.

The LOD_{95%} of the method was 11.5 cfu per 500 mL of milk (ranging from 7.4 to 49.0 cfu per 500 mL according to logit analysis), corresponding to 0.023 cfu per mL of milk. Figure 1 and Table 1 show the results of the tests to assess the LOD value of the cultural assay.

None of the 160 samples showed the growth of MAP colonies during the 16 wk of incubation; we observed an overgrowth of contaminant microflora in 8 out of 160 samples.

The industry collected samples for total bacterial count at every milk load from each farm in plant A and 8 times a month in plant B; the weighted mean of the bacterial count of all the raw milk collected in plants A and B was 35.043/mL and 38.387/mL, respectively.

Thermal processing of milk was performed at 76°C for 17 s in plant A and at 74°C for 21 s in plant B.

A total of 626 and 889 bottles of pasteurized milk and a total of 313 and 339 bulk pasteurized milk samples were analyzed in 2015 for *Listeria monocytogenes*, *Enterobacteriaceae* count [analyzed according to Commission Regulation (EC) 2073/2005; EC, 2005], and phosphatase and peroxidase activity in plants A and B, respectively; results showed the absence of *L. monocytogenes* in all pasteurized milk samples analyzed, an *Enterobacteriaceae* count always <1 cfu/mL, the negativity of phosphatase activity, and the positivity of peroxidase activity.

The lack of MAP positivity in any of the 160 samples appears to contrast with previous investigations that isolated vital MAP in 1.6 to 2.8% of pasteurized milk samples (Grant et al., 2002; Ayele et al., 2005; Ellingson et al., 2005; Carvalho et al., 2012) up to a very high

proportion of positive pasteurized milk samples (67% observed in one investigation (Shankar et al., 2010). However, other investigations on pasteurized milk also found no MAP culture (Gao et al., 2002; O’Reilly et al., 2004; Méndez et al., 2006). Several explanations are possible for the different results of investigations into vital MAP in pasteurized milk and experimental tests assessing the efficacy of pasteurization to inactivate MAP in milk (Grant et al., 2002; Cerf et al., 2007). In brief, these differences concern (1) laboratory procedures, (2) postprocessing contamination, (3) under-pasteurization conditions, and (4) the number of MAP present in the raw milk before pasteurization.

Among the laboratory procedures, we paid attention to resuscitation and incubation for a sufficient period of time for the recovery of heat-injured MAP cells (Hammer et al., 2004) as the majority of isolates from pasteurized milk are obtained after 8 wk of incubation (Grant, 2004). Moreover, Grant hypothesized that false-negative results may occur in milk tested immediately after pasteurization because sub-lethally heat-injured MAP cells in milk may recover viability if given sufficient time (>48 h; Grant, 2004); longer resuscitation periods did not seem to significantly influence the results (Stabel and Lambertz, 2004).

Chemical decontamination may have some detrimental effect on the viability of MAP. Pearce (2004) reported that decontamination and the use of antibiotics in Herrold’s egg yolk medium + Mycobactin J

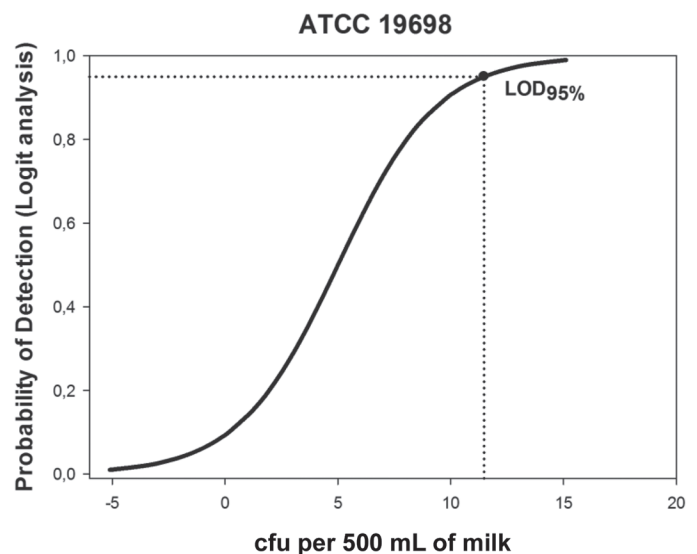


Figure 1. Graph of logit analysis for the determination of the limit of detection (LOD). Dashed lines indicate the LOD_{95%} of *Mycobacterium avium* ssp. *paratuberculosis* in 500 mL of milk, estimated as 11.5 cfu per 500 mL of milk (ranging from 7.4 to 49.0 cfu per 500 mL of milk; CI were not reported in the graph). ATCC = American Type Culture Collection.

do not have a negative effect on the survival of MAP during culturing; on the contrary, prolonged exposure to decontaminants influences their survival (Gao et al., 2005). In addition, viable MAP was recovered more frequently from pasteurized milk when antibiotics were omitted or used in reduced quantities (Grant, 2004). Our study tested milk 48 to 72 h after pasteurization, the incubation was prolonged up to 16 wk and the decontamination step was omitted to enhance the probability of isolating MAP. On the other hand, the addition of antibiotics to the medium was necessary to avoid the overgrowth of contaminant microflora.

Given that low numbers of MAP are likely to survive pasteurization, testing as large a volume of milk as possible should boost the chances of detecting surviving MAP. Grant (2004) reported that vital MAP in pasteurized milk could be isolated only in studies testing at least 50 mL of milk. Because of the expected low number of MAP predicted, we tested 500 mL of milk.

Detection of MAP in commercially pasteurized milk does not necessarily indicate that the organism survived pasteurization because under-pasteurization and postprocess contamination may occur (Grant, 2004). Regarding the possibility of under-pasteurization, we accessed processing and self-control data in the dairy plants investigated, finding temperature/time process parameters in line with the normal commercial practice of pasteurizers (usually set at >73.5 to 74.0°C to provide an additional margin of safety above the legal standard temperature, 71.5°C ; Pearce, 2004). Phosphatase activity and microbiological data on the process hygiene criteria (*Enterobacteriaceae*; $n = 5$, $c = 0$, where c is the number of sample units giving values over the critical limit; limit 10 cfu/mL), together with the absence of *L. monocytogenes* (European Union regulation 2073/2005; EC, 2005), confirmed these data, showing that both dairy plants applied correct pasteurization and appropriate postprocess hygiene procedures.

As the inactivation kinetics of unicellular organisms is a first-order reaction (Cerf et al., 2007), the number of MAP cells present before pasteurization is a key factor for potential survival of MAP after pasteurization (Grant et al., 2002). Sweeney et al. reported a contamination level from 2 to 8 cfu per 50 mL of milk for direct excretion (Sweeney et al., 1992) in symptomatic or asymptomatic cows, but raw milk can be contaminated with MAP through fecal contamination (Nauta and van der Giessen, 1998) and clinically infected cows can shed up to 10^{12} MAP cells per day in feces (Cocito et al., 1994). Studies on the presence of MAP in bulk tank milk showed that milk contamination is mainly related to the presence of clinical and subclinical high shedders (Sweeney et al., 1992; Boulais et al., 2011; Khol et al., 2013) and milking hygiene (Jayarao et al., 2004).

A previous investigation on MAP performed in the same dairy plants detected an apparent mean prevalence of 6.7% by ELISA in the animals of the farms delivering milk to the plants (Serraino et al., 2014a). This result is similar to the prevalence reported in other studies (Nielsen and Toft, 2009; Carter, 2012). However, we detected a very low proportion (0.9%) of MAP-contaminated raw milk samples by quantitative PCR targeting IS900 in comparison with data reported in other surveys reviewed in Okura et al. (2012) and we modeled a 95th percentile of MAP concentration in raw milk of 1.2 to 2.8 cfu/mL (Serraino et al., 2014b).

Data collected in the 2 dairy plants in the present study showed that plant A measured the total bacterial count of bulk tank milk at each load from each farm (weighted mean of the bacterial count during 2015 resulted in 35,000/mL), whereas plant B measured the total bacterial count 8 times a month in each farm (weighted mean of the bacterial count during 2015 resulted in 38.387/mL). European legislation (European Union Regulation 853/2004; EC, 2004) defines a maximum level of total bacterial count of 100,000/mL (geometric mean of 2 mo with at least 2 samples/month). The higher frequency of sampling indicates that the dairy industries put pressure on farmers in terms of raw milk hygiene parameters; the results of total bacterial count monitoring show results fully compliant with European Union legislation, indicating a good level of the milking procedure.

Another aspect to consider is the intensive dairy farming system in Italy, as in other countries: the mean number of lactations per cow reported by the Italian Farmers' Association is 2.4 (Andrighetto, 2011). This suggests that on average cows are culled when they are 4 to 5 yr old, thereby reducing the probability of clinical high shedders. Moreover, dilution during milk collection and other process steps usually applied before pasteurization could also play a role in reducing the MAP load (Hammer et al., 2004).

Mycobacterium avium ssp. *paratuberculosis* may be cultured from milk after HTST pasteurization if the organism is present in raw milk in sufficient numbers (Millar et al., 1996; Grant et al., 1996, 2002). Okura et al. (2013) reported that a MAP load at a concentration of 10^4 is plausible, but in extreme scenarios that require very poor hygienic conditions. In practice, the likely fecal contamination of raw milk can fluctuate with the level of hygiene during milking, but bulking in the farm or in the dairy company silos or both can dilute MAP spikes and 100 cfu/mL of MAP contamination at the silo level can be estimated as the worst case scenario (Pearce et al., 2004). Nauta and van der Giessen (1998) modeled a concentration of 5.4 cfu/L with the main contribution due to clinically affected animals. Accord-

ing to the same model, the exclusion of clinically affected animals from the production chain would reduce the estimated exposure by 99% to 0.06 cfu/L. Assuming these levels of contamination, a proper pasteurization process should completely kill MAP in milk (Stabel and Lambertz, 2004).

The risk assessment model previously developed (Serraino et al., 2014b) in the dairy plants predicted a proportion of 0.55 to 0.98% of liters of pasteurized milk having a MAP contamination >0 cfu/L considering a max 7-log reduction (range 4- to 7-log reduction) of MAP concentration due to pasteurization. Assuming a binomial distribution for positive/negative probability of detecting MAP-positive pasteurized milk and 95% sensitivity at LOD level of the applied method, the results of the present study (no positive milk out of 160 samples) indicate a maximal prevalence of about 1.3% of milk (95% confidence level) in line with the predicted estimation of the model. As noted by Hammer et al. (2006) and reported by the National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 2010), “despite all uncertainties regarding complete inactivation of (MAP) during heat treatment and possible reasons for survival, a reduction of at least 5 to 7 log₁₀ cycles could be demonstrated in whole milk, skim milk and cream. In the framework of the Codex Alimentarius for samples of pasteurized milk, this should be fully sufficient.”

In conclusion, our results show that a 7-log (or >7) reduction could be a plausible value for commercial milk pasteurization and that the combination of current hygienic farm practices and commercial pasteurization result in 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 investigated dairies.

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