

Full Length Research Paper

Detection of *Mycobacterium avium* subspecies *paratuberculosis* in pasteurized milk by IS900 PCR and culture method

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Accepted 31 January, 2012

Prevalence of *Mycobacterium avium paratuberculosis* in commercially pasteurized milk was studied. A total of 300 commercially pasteurized milk samples were purchased from various parts of Eastern-Azerbaijan province of Iran. Two 50 ml from each sample were centrifuged. DNA extraction was performed on one of the pellets and extracted DNA was evaluated for the presence of *Mycobacterium avium paratuberculosis* specific IS900 by PCR assay. In order to detect viable cells of the bacterium, the second related pellet was treated with 0.75% HPC and decontaminated samples cultured on two Herrold's egg yolk medium (supplemented with mycobactin J and amphotericin B, nalidixic acid, and vancomycin). Isolated colonies by culture method were confirmed by IS900-based PCR. Although *M. avium paratuberculosis* DNA was detected in 32 (10.7%) samples by PCR assay, viable bacterium was not isolated by culture method in any sample.

Key words: PCR, IS900, *Mycobacterium avium paratuberculosis*, pasteurized milk.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes chronic intestinal inflammation in dairy cattle eventually leading to Johne's disease (paratuberculosis) (Kralik et al., 2010). Johne's disease is prevalent in domestic animals worldwide and has significant impact on the global economy. In the United States, it is estimated that nearly 40% of US dairy herds are infected with MAP and its influence is estimated in a loss of \$1.5 billion to the agriculture industry every year (Harris and Barletta, 2001; Stabel, 1998).

It is believed that, calves become infected by MAP early by ingestion of the bacterium in infected milk, colostrum, or via farm environment, but clinical symptoms

appear after 2-5 years (Grant and Rees, 2010). Asymptomatic animals can shed MAP in feces and milk for up to 18 months prior to showing any clinical signs of infection. Although clinically infected animals can shed as many as 5×10^{12} MAP cells per day in feces and these cells can remain viable for several months in the environment (Grant et al., 2002b), the number of MAP in milk from subclinically and clinically infected cows was reported between 2 and 8 cfu per 50 ml (Lund et al., 2002). Milk could get contaminated with animal feces during the milking process. Therefore, the extent of contamination depends on hygienic practices prior and during milking (Dundee et al., 2001).

In spite of conflicting reports on the possible etiological role of *Mycobacterium* spp. in the development of inflammatory bowel (Crohn's) diseases (Akarsu et al., 2011), there is no definite evidence to prove or disprove the suggestion that MAP is associated with Crohn's

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disease (Donaghy et al., 2008). Considering the similarity of some clinical symptoms of Crohn's disease and those of paratuberculosis, MAP has been causing considerable concern to the dairy industry worldwide in recent years (Gill et al., 2011; Grant et al., 2002b).

MAP has been identified in many body locations, such as lymph nodes, spleen, liver and animal hide (Wells et al., 2009; Wu et al., 2007) as well as in semen (Sharifzadeh et al., 2010), drinking water (Gill et al., 2011), milk and milk products (Corti and Stephan, 2002; Djønne et al., 2003; Donaghy et al., 2004; Millar et al., 1996). With the progression of the disease, the animal begins shedding MAP in the feces (Herthnek et al., 2008). Therefore, milk could easily get contaminated with high numbers of MAP during teat preparation (Dundee et al., 2001).

In various studies, MAP has been detected and isolated from pasteurized milk (Ellingson et al., 2005; Grant et al., 2002b; Stabel et al., 1997) or survived after application of commercial pasteurization process on experimentally contaminated milks (Grant et al., 2002a; Lund et al., 2002). Essentially, there are two approaches to determine the effect of high-temperature, short-time (HTST) pasteurization on the viability of MAP in pasteurized milk. Firstly, laboratory scale pasteurization which has been carried out by laboratory scale equipments (i.e., 72 °C for 15 s) of raw cows' milk inoculated with high numbers of MAP (Grant et al., 1996; Grant et al., 2002a; Grant et al., 2005). Secondly, commercial scale testing of pasteurized cows' milk in order to determine whether viable MAP exists in retail pasteurized milk (Millar et al., 1996). Neither approach is without problems, given the slow growth of MAP (incubation times of up to 18 weeks at 37 °C) and the absence of an appropriate selective culture medium for this organism (Grant et al., 2002b). Although the culture detection of MAP in any matrix is considered the gold standard for MAP detection, cultivation techniques are not standardized and the ability of different laboratories to cultivate varies considerably (Slana et al., 2008). On the other hand, optimal sensitivity is difficult to achieve, as chemicals used for decontamination of the faster growing sample microflora also kill some of the MAP, or decrease their viability (Sergeant et al., 2002). Tackling the problem encountered with conventional methods, PCR assays have provided a better estimation of the occurrence of MAP in clinical, food, and environmental samples (Whittington et al., 2004).

In Iran, due to high occurrence of *M. avium* subsp. *paratuberculosis* in dairy cattle (Doosti and Moshkelani, 2010), determination of its occurrence in pasteurized milk is so important from public health viewpoint. Known contamination levels can be used by food safety organizations to establish national regulations for the control of foodborne pathogens. Therefore, the aim of this study was to determine for the first time, the prevalence of MAP in pasteurized milk in Eastern-Azerbaijan province (northern-west of Iran) by culture and PCR method.

MATERIALS AND METHODS

Sample collection and preparation

Twice a month, throughout a 10-month period (from March 2010 to January 2011), 300 samples of retail pasteurized milk (2.5% Fat) were collected randomly from 18 local dairy processing establishments throughout Eastern-Azerbaijan province of Iran. Two 50 ml of each milk sample were centrifuged at 4000 $\times g$ for 30 min. Cream layer and whey was carefully discarded (Grant et al., 2005). One of the pellets was used for DNA extraction and the second related pellet used for culture examination.

To evaluate the application of adequate time and temperature in pasteurization process, alkaline phosphatase test was performed on each sample by qualitative test kit (Lactognost, Italy). Moreover, information dealing with the intensity of heat treatment of the pasteurized milk samples test was recorded.

DNA extraction

Genomic DNA was extracted according to the protocol described by Djønne et al. (2003), with some modifications. In brief, first pellet of each sample was suspended in 1 ml lysis buffer containing 2 mM EDTA, 400 mM NaCl, 10 mM Tris-HCl pH adjusted at 8.0, 0.6% SDS (Sigma-Aldrich, St. Louis, MO, USA) and 20 mg proteinase K (Sigma-Aldrich, St. Louis, MO, USA). The bacteria were lysed by overnight treatment at 37°C and boiling for 15 min. DNA was extracted with 500 μ l of phenol chloroform isoamyl alcohol (25:24:1; pH 6.7) (All Merck, Darmstadt, Germany) and the tubes were centrifuged at 12,000 $\times g$ for 10 min. The aqueous phase was collected and transferred to a new tube and the nucleic acid was precipitated with cold (-20 °C) absolute ethanol. DNA was dissolved in 30 μ l of deionized (nuclease-free) water (Cinagen Inc., Tehran, Iran).

The extracted DNA was quantified using NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The A260/A280 absorbance ratio was used to determine undesired contaminations.

PCR protocol for detection of MAP

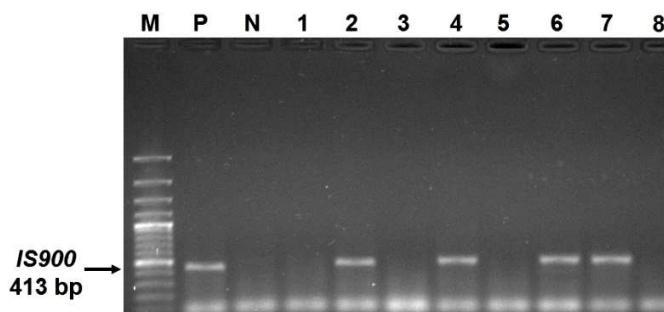
Detection of *IS900* (insertion sequence 900) in the lysate was done by PCR with primers P90+ (5'-GAA GGG TGT TCG GGG CCG TCG CTT AGG-3') and P91+ (5'-GGC GTT GAG GTC GAT CGC CCA CGT GAC-3') resulting in a 413 bp product as previously described by Millar et al. (1996).

The reaction mixture, with a total volume of 25 μ l, consisted of 12.5 μ l of 2X Master Mix (Cinagen Inc., Tehran, Iran), 60 pmol each of forward and reverse primers (Cinagen Inc., Tehran, Iran), 25 ng of template DNA (extracted from pellet of each pasteurized milk sample). Nuclease-free deionized water (Cinagen Inc., Tehran, Iran) was added to final volume of 25 μ l and subsequently overlaid with 5 μ l of light mineral oil (Cinagen Inc., Tehran, Iran). The MAP (DSM 44133, Braunschweig, Germany) was used as positive control. Reactions contained DNA-RNA free water (without DNA as template) was used as negative control.

The PCR was carried out under the following conditions: an initial denaturation at 94°C for 5 min and 40 cycles of 93°C for 1 min (denaturation), 58°C for 1 min (annealing) and 72°C for 3 min. Milk samples were tested with a simultaneous no-template PCR negative control and process controls (Millar et al., 1996). Extracted DNA (5 μ l) was loaded on 1% agarose gel (Invitrogen, California, USA), which contained ethidium bromide (1 μ g/ml) for DNA staining. A 100-bp ladder DNA (Fermentas, St. Leon-Rot, Germany) was used in the gel to determine the fragment size of the PCR product. For image acquisitions, G: Box™ gel documentation system (Syngene, Cambridge, UK) was used.

Table 1. Number and percentage of positive samples of MAP detected by PCR and culture method in pasteurized milk.

Result		No. of positive samples (%)
PCR	Culture	
+	-	32 (10.7)
-	+	0 (0)
+	+	0 (0)
-	-	268 (89.3)
Total		300 (100)

**Figure 1.** PCR amplification of 413 bp of *IS900* gene from pasteurized milk samples. M: 100-bp ladder; P: positive control; N: negative control; Lanes: 2, 4, 6 and 7 represent positive results; Lanes 1, 3, 5, 8 and 9 represent negative results.

Isolation and identification of MAP

The second subsequent pellet obtained from each pasteurized milk sample was resuspended in 15 ml of freshly prepared sterile 0.75% (wt/v) hexadecylpyridinium chloride (HPC) (Merck, Darmstadt, Germany), and kept at room temperature (approximately 25°C) for 5 h (Dundee et al., 2001; Grant et al., 2002a). The suspensions were centrifuged at 4000 \times g for 15 min, supernatants were discarded, and pellets were resuspended in 500 μ l of sterile phosphate-buffered saline containing 0.05% (wt/vol) Tween 20 (PBS-T) (Merck, Darmstadt, Germany) at pH 7.4. Suspensions were inoculated (250 μ l) on two slants of Herrold's egg yolk medium (HEYM) containing mycobactin J (2 μ g/ml) (Synbiotics Europe, Lyon, France) and supplemented with amphotericin B, nalidixic acid, and vancomycin (All Sigma-Aldrich, St. Louis, MO, USA) (Grant et al., 2005). Agar slants were incubated aerobically at 37°C for 16 weeks (Grant and Rees, 2010). Isolated colonies were confirmed by *IS900*-based PCR according to the above mentioned protocol.

RESULTS

Of the 300 pasteurized milk samples, MAP DNA was detected in 32 (10.7%) samples by *IS900*-based PCR assay, but despite considerable effort, authors were unable to provide conclusive evidence that any of the MAP bacteria detected by PCR assay, were present in a viable form (Table 1 and Figure 1).

Information recorded at the time of collection of pasteurized milk samples indicated that, all of the

pasteurized milk samples had received a heat treatment of 74 to 78°C for 15 s. Moreover, phosphatase test results were negative in all samples, which revealed that, there was no indication of ineffective pasteurization.

DISCUSSION

Various investigations have been carried out to determine the inactivation of MAP in pasteurized milk (Grant et al., 1996; 2002b; 2005; Lund et al., 2002; Pearce et al., 2001; Stabel et al., 1997). The overall findings suggest that, MAP is not completely inactivated by pasteurization of milk at 72°C for 15 s, the minimum heat treatment required for milk pasteurization by European Commission legislation. Therefore, findings achieved by different studies in 1998 led the United Kingdom dairy industry to adopt an increased holding time for commercial milk pasteurization (25 s rather than 15 s at 72°C) in order to increase the lethality of the pasteurization treatment (Grant et al., 2002b).

In a national survey in the United Kingdom, a total of 567 commercially pasteurized milk samples were tested for the presence of MAP. DNA specific for MAP has been detected in 67 (11.7%) of pasteurized milk samples by immunomagnetic PCR. Confirmed MAP isolates have been cultured from 10 (1.8%) of commercially pasteurized milk samples. These findings confirm that MAP has the potential to survive commercial HTST pasteurization processes, even on occasion, treatments involving the extended 25-s holding time (Grant et al., 2002b). During another survey by Millar et al. (1996), 312 samples of retail pasteurized cows' milk were examined with PCR assay for the presence of MAP. Despite MAP DNA was detected in 22 (7.05%) of pasteurized milk samples, authors were unable to distinguish between viable and dead MAP, which was in accordance with the findings of our study as no sample was culture positive for MAP.

In this survey, it has been indicated that MAP can be detected more rapidly and with better sensitivity through the use of PCR method. Accordingly, PCR-based method indicated a higher prevalence of MAP than culture method (Table 1). These findings were in accordance with the prevalence difference found by Donaghy et al. (2008) and Ellingson et al. (2005).

O'Doherty et al. (2002) examined 310 bulk raw milk and 86 pasteurized milk samples for MAP detection by two culture methods. The samples were cultured using BACTEC 12B and HEYM following decontamination with HPC. According to the findings of the study, all 396 samples tested negative for culture of MAP.

The results obtained from this study demonstrate a relatively high occurrence (10.7%) of MAP in pasteurized milk. In addition, regarding the possible etiological role of MAP in the development of Crohn's diseases, it is considered a serious public health concern. It seems that, high occurrence of MAP was in accordance with hand-

milking method in dairy farms. In other words, in regions in which dairy herds have been bred in small traditional farms rather than industrial ones and been milked by hand rather than by milking machine, the occurrence of MAP was higher. Thus, there is necessity to maintain strict adherence to proper sanitary procedures during milking and milk handling. Moreover, due to efficiency of HTST pasteurization on MAP, It is reemphasizes to perform the sufficient duration and intensity of heat treatment to inactivate the bacterium.

ACKNOWLEDGEMENT

Funding for this study was provided by Vice Chancellor in Research Affairs of Islamic Azad University of Tabriz.

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