Detection of *Mycobacterium avium* Subsp. *paratuberculosis* in Pasteurized Milk Samples in Northeast of Iran by Culture, Direct Nested PCR and PCR Methods

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ABSTRACT: *Mycobacterium avium Subsp. paratuberculosis (MAP) is a gram-positive, small, acid-fast bacillus with high environmental resistance. In animals, especially ruminants, it leads to Paratuberculosis (PTB) or Johne's disease, which is chronic granulomatous enteritis. This bacterium as the main causative agent of Crohn's disease can be a serious threat to human health. This study aimed to detect MAP in pasteurized milk samples produced in Khorasan Razavi province, Iran, using Direct Nested PCR, PCR, and culture methods. In this study, 544 milk samples from Pasteurized Milk Production Companies were selected randomly during the 3-month period. DNA was extracted from milk fat after centrifugation. In order to identify the bacteria, Direct Nested PCR and PCR tests were applied using IS900 and f57, respectively. Furthermore, to detect viable MAP, positive samples resulted from Direct Nested PCR assays were cultured on Herrold's egg medium. For identification of mycobacterial isolates all colonies were processed by PCR based on f57. A total of 544 pasteurized milk samples were assayed, and Mycobacterium paratuberculosis was detected in 39% of them by IS900 Nested PCR, and only 4.9% of samples were positive in culture method. All the colonies were positive for the f57using PCR. The results of this study indirectly indicated a high level of contamination of pasteurized milk to Mycobacterium paratuberculosis which is due to the large number of affected animals in livestock farms in Khorasan Razavi province. However, in comparison with the other researches, the low percentage of viable bacteria in pasteurized milk can be due to changes in temperature and time in pasteurizing systems of milk production companies in Khorasan Razavi province, Northeast of Iran.*

KEYWORDS: *Mycobacterium avium; Paratuberculosis; IS900; Nested PCR; f57.*

INTRODUCTION

Mycobacterium avium Subsp. *paratuberculosis (MAP)* is a small bacillus-shaped, gram-positive, non-spore forming, and non-motile bacterium. Moreover, it is an aerobic and facultative intracellular bacterium. This bacterium

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is a slow-growing bacterial pathogen. Also, it is resistant to many types of disinfectants. The environmental resistance of this bacterium is so high that it is reported it has been separated even from chlorinated water-pipes. Moreover, by making biofilm on interior surfaces of pipes, this bacterium preserves its survival [1, 2]. In addition, in contrast to *Mycobacterium tuberculosis* which may show resistance to some anti-mycobacterium drugs (in the forms of MultiDrug Resistance (MDR), Extensively Drug Resistant (XDR) or Totally Drug Resistance (TDR)), *Mycobacterium paratuberculosis* is fundamentally resistant to all anti-mycobacterium drugs and using some antibiotics to remove it just leads to delaying clinical signs and symptoms [3]. This bacterium causes Johne's disease or Paratuberculosis in animals. This disease is a kind of chronic and progressive granulomatous enteritis, especially in ruminants. Clinical symptoms in animals include severe diarrhea and atrophy which is along with progressive anemia leading to death at the end [4]. Due to many similarities in terms of anatomy, pathologic lesion, disease symptoms, molecular diagnosis, and the separation of *MAP* from patients with Crohn's disease [5]. This bacterium is considered as the main candidate causing Crohn's disease in humans [1, 2]. *MAP* is separated from the infected blood, milk, and infected mono-nuclear cells of these cases [6]. This bacterium is transmitted to humans through infected livestock (meat and dairy products) and polluted water. *MAP* was detected in milk for the first time in 1935 when it was isolated from three out of four milk samples from clinically ill cows [7, 8]. It has been suggested that *MAP*-infected macrophages are present in lipid droplets on the cream layer of milk [9]. Some researchers stated that pasteurization via a standard method High Temperature Short Time (HTST) is only able to reduce the number of *MAP* logarithmically [10, 11]. Generally, to investigate the presence of *Mycobacterium* in suspicious samples, the molecular and cultural methods are utilized [12]. Molecular techniques are preferable to rapid detection of this fastidious, slow-growing organism [13, 14]. One of the molecular techniques is Nested PCR which is a method to increase the PCR specificity. It is the modification of polymerase chain reaction intended to reduce non-specific bindings in products due to the amplification of unexpected primer-binding sites [15]. The insertion element IS*900* is commonly chosen as a molecular target for identification of *MAP* [13, 16]. Other target elements such as f57, ISMav2 HspX and locus 255 can also be used for accurate detection [4, 17]. The targeted f57 sequence element is unique and is not known to exist in other bacterial species. Bacterial culture medium causes the detection of viable *Mycobacterium paratuberculosis* in pasteurized milk samples, but it is difficult to isolate and grow in culture [18, 19].

The aims of this study were to discover the public health-related contamination of pasteurized milk with *Mycobacterium avium* Subsp. *paratuberculosis* organisms of different areas in Khorasan Razavi province, Northeast of Iran.

EXPERIMENTAL SECTION

Sample collection

In this study, 544 samples of processed milk were randomly collected from milk production companies in Khorasan Razavi province during a period of 3 months. Then, the collected samples were transmitted to the laboratory in 50 mL falcons under aseptic conditions at 4 °C.

DNA extraction

Falcons containing 50 ml of milk were centrifuged at 3500 rpm for 20 minutes at 4° C. Then, the middle phase between pellet (below) and the milk fat (above) was separated and removed. The pellet and butter fat of milk were used for DNA extraction using phenol-chloroform protocol. A volume of 200 µL of Tris- EDTA (TE) was added to each sample. The sample was placed at 100 °C for 10 minutes. Then, 500 µL of lysis buffer (Tris-HCl 10mM, EDTA 2mM, NaCl 0.4M and Triton X-100 0.5%) (pH 8.0) and 7 µL of lysozyme enzyme (10 mg/ml) were added and incubated for about 1 hour at 37 °C. Then, a total of 20 µL of proteinase K (20 mg/ml stock) was added to the last-step suspension and the mixture was incubated for 2 hours at 65 °C. In the next step, in order to deactivate the proteinase, the samples were kept at 100°C for 10 minutes. To extract genomic DNA, the sample was combined one time with volumes of phenol: chloroform (24:1 v/v) followed by chloroform only, and the above phase was discarded after the centrifugation at 10000 g for 5 minutes. The aqueous phase was finally transferred in 2.5 volume of chilled ethanol and sodium acetate (0.3M final conc.) was added. Tubes were kept at -20°C overnight. After centrifugation, DNA sediment was washed with ethanol

Table 1: Primers used in Nested PCR and PCR.

Primers name		Sequence of primers (5-3)	Length (bp)	Size of PCR Products (bp)
f57	F	5'- GTCATTCAGAATCGCTGCAA-3'	20	248
	R	5'- ATGAAATGGGCGTCTACCAG -3'		
IS900 Outer Primer	Para1F	5'- TGA TCT GGA CAA TGA CGG TTA CGG A -3'	25	563
	Para4R	5'- CGC GGC ACG GCT CTT GTT- 3'	18	
IS900 Inner primer	Para2F	5'- GCC GCG CTG CTG GAG TTG A -3'	19	210
	Para3R	5'- AGC GTC TTT GGC GTC GGT CTT G -3'	22	

70% [20]. DNA purity was assessed on the basis of absorbance at 260–280 nm using the NanoDrop 1000 (Thermo Fisher Scientific Inc). A 260⁄ 280 ratio of 1/8–2/0 is indicative of high purity [21].

Nested PCR assay

Molecular techniques (Nested PCR and PCR) were used for identifying the sequences of IS*900* and f57, respectively. The following primers were extracted from published papers as well as National Center for Biotechnology Information (NCBI) [15] (Table 1). To perform Nested PCR, 5 µl of extracted DNA, 10 pmol of each outer primer PARA1F and PARA4R, and 13 µL of sterilized ultrapure water in a total volume of 20 µL were mixed on the specific PCR kit (Bioneer Corporation). In the reaction of the second step, instead of template DNA, the first product was used. Primers used in the second step included primers PARA2F, PARA 3R [15]. In this test, the strain of *M. avium subsp. paratuberculosis 316F* was used as positive control and the sterilized buffer was used as negative control.

Thermal cycle of Nested PCR reaction: in the first step, reaction was done by PARA1F and PARA4R, with initial denaturation at 95°C (4 minutes), denaturation at 94°C (60 seconds), annealing at 61.5°C (30 seconds), and extension at 72°C (60 seconds) for 20 cycles. The second step was done through the pairs of internal primers resulted from the first step (under the name of PARA3R and PARA2F). This step was done by the initial denaturation at 95°C (3 minutes), denaturation at 94°C (60 seconds), annealing at 58°C (30 seconds), and extension at 72°C (60 seconds), for 35 cycles.

The Nested PCR production was loaded on 1% agarose gel in 0.5 X TBE buffer containing 0/0001 SYBR green and it was assessed through electrophoresis and finally U.V transilluminator in terms of the presence of the expected sequences. A DNA ladder (Thermo scientific GeneRuler) was used as the molecular weight marker (100bp plus).

Microbiological culture

Due to a large number of samples, all positive samples proved by Nested PCR test were selected. At first, milk samples decontaminated from other microorganisms for the successful recovery of *MAP*, by treating with 0.75% Hexadecylpyridinium chloride (HPC) while it has the least harm to *MAP*. Decontamination took 4 to 5 h. It was performed following the method of *Dundee L.* (2001) with some modifications [22].

To prepare the special selective media, a mixture of antibiotics including polymyxin B, Amphoterin B, nalidixic acid was added on Herrold's egg yolk medium (HEYM). Also, a conventional culture of *MAP* requires special media enriched with mycobactin J (an iron-chelating factor). Each sample was inoculated on two slopes, with and without mycobactin J [23]. Culture was prepared As previously described by Grant with some modifications [23]. The tubes were incubated at 37 °C for up to 4 months. During this time, the bacterial growth and conditions were examined. Also, the milk contaminated with *Mycobacterium avium* subsp. *paratuberculosis 316F* was included as positive control. The suspected colonies were identified by morphology and smear of developing colonies were stained with ZiehlNeelsen staining.

DNA extraction from culture

200µL of Tris- EDTA (TE) (pH 8.0) was added to the microtubes containing the colony of *Mycobacterium paratuberculosis*. The microtubes were incubated at 100°C for 20 - 30 min [20]. After that, 300 µL of lysis

buffer (Tris-HCl 10mM, EDTA 2mM, NaCl 0.4M and Triton X-100 0.5%) (pH 8.0) and 7 µL of lysozyme enzyme (10 mg/mL) were added and the microtubes were incubated again for 1 hour at temperature of 37°C. Then 20 µL of proteinase K (20 mg/mL stock) was added and the microtubes were incubated for 2 hours at 65°C. To deactivate the proteinase after incubation, the samples were kept at 100°C for 10 minutes. DNA extraction was carried on by adding a volume equal of phenol: chloroform (24:1 v/v) and then re-added a volume equal of chloroform only and after centrifuge, DNA sediment was finally obtained by adding 2.5 volume of chilled ethanol and sodium acetate (0.3M final conc.) to the resulting aqueous phase containing DNA after a day at -20°C. DNA sediment was washed with ethanol 70% [20].

PCR assay

PCR was done for each sample of f57 through the specific primers. A volume of 5µL of template DNA and 1µL of each primer (10 pmol) and 13 µl of sterilized ultrapure water in a total volume of 20 µl was added on the specific PCR kit (Bioneer Corporation). Thermal cycle of PCR reaction for f57 amplification was initial denaturation at 95°C (4 minutes), denaturation at 94°C (1 minute), annealing at 54°C (30 seconds), and extension at 72°C (1 minute), for 34 amplification cycles. PCR products were resolved by 1% agarose gel electrophoresis. Reactions were considered positive when they yielded products of 248 bp.

RESULTS AND DISCUSSION

The results showed that by using Nested PCR with specific primers for *MAP*, 217 samples out of 544 cases about 39% were detected as positive for contamination with the bacterium. In the direct detection of *MAP* from the milk sample, the inhibition factors in PCR are high. The Direct Nested PCR method was helped to increase recognition sensitivity of IS*900* sequence. The size of the PCR product with the outer primers (PARA1F and PARA4R) was about 560 bp, which was determined during testing on the positive control. The use of this outer primers alone in PCR assay was not sufficient to detect the *MAP* in pasteurized milk samples. So Direct Nested PCR method was used to increase the sensitivity of *MAP* detection. The first step of Nested PCR was performed with a pair of outer primers (PARA1F and PARA4R)

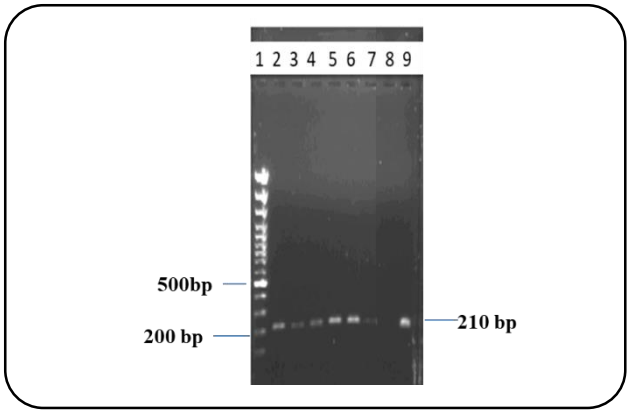

Fig. 1: Electrophoresis of Nested-PCR products obtained from milk samples. Lane 1: 100 bp plus DNA ladder (Thermo scientific GeneRuler); lane 2: positive control (the milk contaminated with Mycobacterium avium subsp. paratuberculosis 316F); lane 3-7, 9: positive samples; lane 8: negative control.

for 20 cycles. These number of cycles were sufficient to increase the sensitivity of the insertion element IS*900* identification. Of course, with this number of amplification cycles, we didn't expect to observe 560 band on agarose gel. As a result, in the second step with inner primers (PARA2F and PARA3R) that the 35 cycles were carried out, the sharp band of 210 bp was seen on 1% agarose gel (Fig. 1).

During the culture, it was found that just 4.9% of the all pasteurized milk samples which were positive for *Mycobacterium paratuberculosis* at Nested PCR, showed positive at culture (Table 2).

All the colonies were positive for the sequence f57 using PCR (Fig. 3). Small, flat, yellowish white colonies specific for *MAP* were observed only in tubes containing medium with mycobactin J (Fig. 2). Regarding morphology and the time duration of culture, the effect of mycobactin J and PCR test based on f57 were confirmed the presence of *MAP*.

During the last 10 years, in America and Europe, *MAP* has been suspected as a zoonosis factor which can be transmitted to human through the animal.

With regard to the investigations, this transmission can be done through consuming meat, milk, and dairy products and can be considered as the main factor of Crohn's disease in humans [1, 16]. A recent research has shown that the lack of the suitable temperature for complete removal of this microorganism results in their survival in foodstuff [24]. Local research in countries such as Mexico has reported

Table 2: Results of M. avium Subsp. paratuberculosis detection on pasteurized milk samples.

Sample Type	No.	Type of test	Map-positive samples	
			No.	%
pasteurized milk samples	544	IS900 Direct Nested PCR	217	39
pasteurized milk samples	544	Culture	27	4.9
MAP colonies	27	f57-PCR	27	100

Fig. 2: Smooth colonies of MAP after 16 weeks of culture on a Herrold's media containing egg yolk slope in a sealed tube.

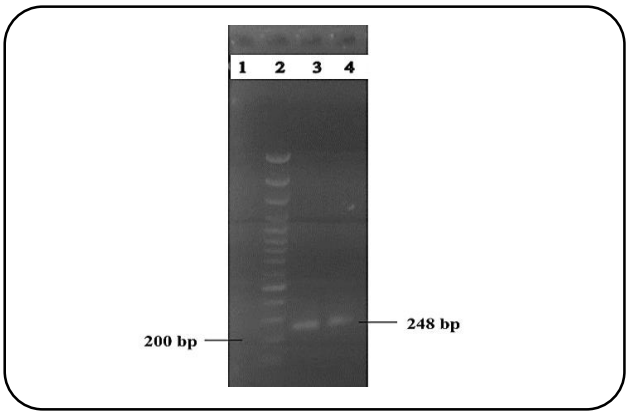

Fig. 3: Electrophoresis of PCR products obtained from colonies grown on Herrold's media which was confirmed as M. paratuberculosis. Lane 1: negative control, Lane 2: 100 bp plus DNA ladder (Thermo scientific GeneRuler); lane 3: positive control, Lane 4: positive sample.

that although *Paratuberculosis* was present as an endemic phenomenon, raw milk and the cheeses produced from non-pasteurized milk were still used traditionally. In the same vein, other research works reported the presence of *MAP* in pasteurized and other dairy products. Based on the research works done in Argentina, the presence of *MAP* in raw milk can be an important factor in infecting the pasteurized milk [25]. Many research works have focused on the effect of pasteurization temperature in removing *MAP*. The results have shown that this bacterium is naturally resistant to normal pasteurization temperature and there is just 1 $\log_{10}$ reduction (logarithmically) in the number of viable bacteria in milk during pasteurization [6, 18]. Research shows infection rate in cattle is high in Iran. Since these bacteria are naturally resistant to pasteurization temperature, they may be present in pasteurized milk. Moreover, due to its role in causing Crohn's disease in human, the investigation on the presence of *MAP* in milk and its products is of high importance.

There are a variety of methods for detecting *MAP* through which milk and its products can be examined [26, 27]. Using specific culture media is the golden standard to detect and identify the presence of *MAP*. This medium is of a high sensitivity level and can lead to the detection of viable bacteria [28]. However, nowadays, because of the long duration of bacterial reproduction and for accurate and fast detection of these bacteria, some molecular methods based on PCR such as directed Nested PCR were used. Also, Enzyme-Linked ImmunoSorbent Assay (ELISA) is used as one serologic method which is fast and cheap. However, due to late appearance of some antibodies in the blood, the utilization of this method did not work in the early stages of infection [29].

Because of high numbers of milk samples, in the present study, the Directed Nested PCR method was used as it is of high sensitivity in detection of *MAP*. In this test, identification of the IS*900* sequence was evaluated through selective pair primers, (PARA1F, PARA4R) and (PARA 2F, PARA 3R). This method can examine the presence of *Mycobacterium paratuberculosis* (both alive and dead). In addition, other studies were conducted based on primers P90 5' (GAAGGGTGTTCGGGGCCGTC) and P91 (GAGGTCGATCGCCCACGTGAC) and related to IS*900* sequence [25].

There is also another study that demonstrate a relatively high occurrence of *MAP* in pasteurized milk

samples in Tabriz- Iran [30]. As in recent years, IS*900* region was recognized in a number of mycobacteria. To be sure about the results, the f57 specific sequence, in spite of its less sensitivity, was used for molecular detection of *Mycobacterium paratuberculosis*. It is because of the fact that it is of high features and characteristics [17]. In order to separate and detect the active and viable *Mycobacterium paratuberculosis*, some samples traced by Direct Nested PCR method were cultured on Herrold's egg yolk medium. Generally, as the time for duplication and doubling for these bacteria is about 22-24 hours, so its colonies in the first culture were observable after 8-16 weeks. In the present study, it was possible to observe and count the developed colonies from 16th week on.

Comparing the results of our research with other countries such as Czech Republic, infection rate of pasteurized milk with *MAP* detected by PCR and culture methods were 31.7 and 3.6, respectively [31, 32]. In India, *Mycobacterium paratuberculosis* infection detected by PCR method was 67% in pasteurized milk [28]. In Canada, 15% of 710 cases of pasteurized milk were reported as positive. However, all cultured samples were negative [30]. In Britain, 1.7% of pasteurized milk kept at 72 °C (within 15-25 seconds) included viable *Mycobacterium paratuberculosis* [10]. Although the sensitivity of the culture method is less than PCR and especially Nested PCR, a few numbers of positively reported cases obtained from culture method compared with the molecular method can show the levels of live bacteria which are active and can cause disease. According to the results of this study, during the culture, only 4.9% of samples had a live and active *MAP* which differed significantly from the results obtained from Nested PCR. This can be because of the abnormal situation of pasteurization in terms of both time and place conditions which are used in some production companies in Iran because of high microbial load. In fact, sometimes, due to Receive raw milk with a higher microbial load, the temperature and time are used to higher than standard pasteurization limit.

In other studies, in Australia have shown that the temperature and holing time of milk pasteurization have strong effects on reduction of a logarithmic number of *Mycobacterium paratuberculosis* [33]. Although a low percentage of *MAP* in pasteurized milk is desired, higher temperature or longer time like sterile milk can destroy main parts of vitamins and it can cause changes in the chemical structure of the milk which are important and effective on the quality of milk.

CONCLUSIONS

The outcomes of the current study obviously showed that there is a high prevalence of Johne's disease among dairy cattle in the investigated area. Since Mycobacterium paratuberculosis is secreted in the milk of the livestock, this result was obtained from the evaluation of the presence of MAP in pasteurized milk that was detectable by Nested PCR and PCR. Using the culture method, we found that Mycobacterium paratuberculosis was existed alive in some milk samples due to logarithmic reduction in M. paratuberculosis organisms during milk pasteurization and the inherent resistance of bacteria to heat. Also, dairy manufacturing should be worried around the quality of milk because of high temperature and holding time in pasteurization may not protect main parts of vitamins and other factors in milk, as well as adequate pasteurization, suggested. It is concluded that doing some preventive and hygienic measures in animal farms as well as in the transmission of raw milk can be effective in reducing microbial load including MAP and in the physicochemical quality of raw and pasteurized milk. In the continuation of this study, it is suggested that in order to determine the dominant strains, genotyping can be done for the strains isolated from raw and pasteurized milk samples and then the vaccine or diagnostic kits can be made so that it can be effective in treating and controlling the disease.

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