Production of *Mycobacterium Avium* subsp. *Paratuberculosis* detection Kit in dairy Products

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**Abstract**

*Mycobacterium avium* subsp. *Paratuberculosis* (MAP), the causative agent of Johne disease in ruminants, has been incriminated as the cause of Crohn disease in humans. Cattle with Johne’s disease can shed MAP in their milk, and MAP can survive under simulated commercial pasteurization conditions. In this study, we investigated the presence of MAP retail cheeses, raw and pasteurization milk from Khorasan Razavi by polymerase chain reaction (PCR). We found that 36.66% of raw milk samples reacted positive by PCR. PCR results for cheese sample was negative. 413 bp PCR product was cloned in pTZ57 R/T plasmid for positive control. These results suggest that the use of the specific PCR assay with the primer sets used in the study is a rapid, reliable, and accurate technique in comparison to traditional and conventional methods for the detection and diagnosis of MAP in milk samples.

**Keywords:** *Mycobacterium avium* subsp. *Paratuberculosis*, PCR, Detection kit, milk and food safety

**Introduction**

*Mycobacterium avium* subsp. *paratuberculosis* (MAP) which is a gram-positive, acid-fast bacillus, is the etiological agent of paratuberculosis, ordinarily recognized as Johne’s disease (JD), in cattle and other ruminants (Cocito et al., 1994). Common symptoms of this infection include diarrhea, reduced milk production, emaciation, and eventually death in afflicted animals, and infections leading to considerable economic losses for individual farms and the dairy industry as a whole. Annual losses in the United States alone are reported to be as high as $250 million (Ott et al., 1999). There has also been an association of MAP with Crohn’s disease in humans (Dalton et al., 2014). Johne’s and Crohn’s disease share some clinical characteristics: both are chronic granulomatous diseases of the gut, which influence nutrient absorption and commonly occurs in the young and it has been shown that the ingestion of a few organisms can create disease (Chiodini et al., 1984). Animals may shed MAP in their milk, even at the time of subclinical infection (Streeter et al., 1995; Ellingson et al., 2005), and human exposure to MAP through the medium of dairy products has emerged as an issue of concern, in spite of the fact that there is no deducible evidence supporting the association of this pathogen with Crohn’s disease (Hermon Taylor and Bull, 2002). The ability of MAP to withstand industrial or laboratory pasteurization has been evaluated in various investigations (Keswani and...
During the last decade, there has been substantial advancement in the study of the association between this microorganism and Crohn’s disease. The researchers have focused mainly on food products as the transmission pathway and MAP has been considered as a possible zoonotic microorganism (Naser et al., 2000). If the same is also true for Crohn’s disease, then the adequacy of milk pasteurization is of critical importance. Clinical similarities between Johne’s disease in cows and Crohn’s disease in humans have been detected, and both viable MAP and MAP genetic material have been identified in some patients diagnosed with Crohn’s disease (Chamberlin et al., 2001). Nevertheless, no cause and effect relationship between MAP and Crohn’s disease has been determined and no documentation establishing zoonotic transmission from a cow to a human has been recorded (Van Kruiningen, 1999). If there is a causal relationship between MAP and Crohn’s disease, investigations into possible vectors through which MAP is spread should present helpful information.

The established ‘gold standard’ testing procedure for Johne’s disease (MAP infection) is fecal culture. This culture method is time-consuming, demanding a lengthy incubation period of 8 to more than 16 weeks for bacterial recovery (Ellingson et al., 2005). In order to overcome these complications, several PCR tests have been developed to identify MAP (Kawaji et al., 2014). Although these assays suggest the advantages of sensitivity and speed, they cannot differentiate between viable and nonviable MAP cells.

The purpose of this study was to investigate the viability of the PCR assay as a diagnostic tool for the detection of MAP DNA in dairy products. Also, ISO900 gene was inserted in pTZ57R/T for positive control in the final detection kit.

**Material and methods**

**Sample Collection**

30 pints of milk and fecal samples were obtained from both the northern and central regions of Khorasan Razavi, Quchan and Mashhad (15 samples per state). Approximately 2 g of the fecal sample was transferred from the rectal sleeve into a labeled ointment container. About 15 mL of milk was collected from each of the four quarters of individual cows in milk and pooled to make one composite sample per cow. The herd size ranged from 100 to 340 cows, with an average herd size of 150 cows. The samples were transported to the laboratory on ice. The MAP was kindly provided by faculty of veterinary (Ferdowsi University of Mashhad, Iran) on Herrold’s egg yolk agar medium and was used as the positive control.

**DNA Extraction**

Pooled quarter milk (40 mL, including 10 mL from each quarter) was centrifuged at 10000 rpm for 30 min. The supernatant was discarded and the pellets were washed twice in PBS, pH 7.2. Then, resuspension was used for DNA extraction. Fecal samples were prepared by washing twice by PBS. DNA extraction was done according to Zhang et al. (2013). DNA extracts were stored at -20°C and re-centrifuged before use.

**IS900 PCR**

For further differentiation between M. avium and MAP, PCR targeting the insertion sequence IS900 (primer: IS900-1: 5’TGTTCGGGGCCGTCGTAG; IS900-2: 5’-CGTTCCAGCGCCGAAAGTAT), which is present only in MAP strains (9), was done. The IS900 PCR was performed in a total volume of 25µl and the reaction mixture had a final concentration of PCR buffer 10X (2.5µl), dNTP mixture (20mM) 2 µl, MgCl2 (50mM) 1.3 µl, primer (20 M of each) 2µl and 2 U of Taq DNA polymerase. DNA extract
containing approximately 100 ng of bacterial DNA was added. IS900 PCR amplification was performed in a thermocycler (T-Personal, Germany) with an initial denaturation at 94°C for 2 min, then 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 66°C for 1 min, and extension at 72°C for 1 min. The resulting PCR amplified products were electrophoresed in 2.5% agarose using a Tris borate EDTA buffer (Sigma, St Louis, MO) in gels at 150 V for 3 h. Gels were stained with Gel Red (1 mg/mL). DNA was visualized by UV transillumination (Uvidoc, England) and photographed with Adobe Photoshop CS6 (California, USA).

Cloning of IS900 gene

In order to design, the positive control and protect the PCR product, the target genes were inserted into T/A vector. Accordingly, PCR products were purified by QIA Quick PCR purification kit (QIAGEN, USA) according to the manufacturer’s instructions and cloned to T-vector pTZ57 R/T using a TA-Cloning Kit (Thermo, USA). In brief, a 10 μl ligation mixture of TA-vector and amplicon was incubated overnight at 8°C and 3 l of the mixture was then heat-shock transformed into bacterial cells of Escherichia coli DH5α, which were used for propagation of plasmid constructs. Mini-scale isolation of plasmid DNA kit was used for the preparation of recombinant plasmid for cloning confirmation (Thermo, USA). Blue-white screening and colony PCR were done for detection of recombinant plasmid.

Results and Discussion

The DNA concentration (μg/ml) derived from milk was determined spectrophotometrically. The DNA yield was calculated 120 μg/ml. Figure (1A and B) shows electrophoresis of raw milk and fecal samples extracted DNA bands on 0.8% agarose gel, respectively.

Researchers were able to use different methods in order to identify several microbial agents such as Brucella (Klevezas et al., 1995; Rijpens et al., 1996), Listeria monocytogenes (Herman et al., 1995), Yersinia enterocolitica (Ramesh et al., 2002), and Streptococcus agalactiae (Meiri-Bendek et al., 2002) species in low-volume samples of milk. Numerous efforts have also been made for direct identification of Mycobacterium paratuberculosis (Corti and Stephan, 2002; Jayaro et al., 2004; Nebbia et al., 2005). After studying different methods for DNA extraction of microbial agents from cow milk, Romero and Lopez-guni (1999) came to the conclusion that the use of cell dissociation buffer containing EDTA, Tris and NaCl, proteinase K, and incubation at high temperature are necessary for effective DNA extraction of microbial agents from milk.

The identification of MAP strains can be verified by the presence of a specific insertion element IS900 and cultivation on mycobactin J. The primers P1-IS900 and P2-IS900 applied on MAP produce a specific amplicon of 413 bp length (Fig.-2). To eliminate the presence of such non-specificity, we have optimized the condition of amplification by changing the annealing temperature in the extension from 60 to 70°C. The increase of annealing temperature to 65°C led to
elimination of the IS900 amplicon, and simultaneously resulted in decreased sensitivity of the amplification and false negative results in amplification of the control. The decrease of annealing temperature led to an increase in the number of non-specific fragments. Similar effects were observed when concentrations of MgCl₂ in the amplification mix were changed from 1.5 mM to 2.5mM. The PCR product, a single DNA band of approximately 413bp, was detected in 11/30 MAP strains (36.6%) isolated from raw milk samples (Fig.-3). But, the PCR product electrophoresis results for fecal samples showed 13/30 MAP strain contamination. Colony PCR analysis showed that IS900 gene was inserted correctly in T/A vector. The recombinant plasmid extraction and dilute 1/100 with H₂O was used main fragment in PCR (Fig.-4).

Although PCR has proven remarkably beneficial for detecting MAP, DNA extraction of high quality is one of its limiting factors. Therefore, given the higher level of MAP bacteria present in feces than in milk, this sample is suggested to be used to identify infected cattle. A study has shown that when the concentration of Johne’s disease causative agent in milk samples is less than 10 CFU/ml, confirming its presence by PCR can be variable, which is mainly due to the loss of many of these organisms in the cream during centrifugation of the sample. As a result, for a comprehensive and thorough study of milk samples, cream needs to be included in the tests as well (Pillai and Jayaro, 2002). In a study performed on the milk of 211 cows from herds in which there was a disease history, it was found that only 9 of the samples (4% of the cases) were positive in milk cultures. However, when the same samples were examined through PCR using IS900 sequence analysis of the bacterial genome of Johne’s disease causative agent, the presence of Johne’s disease causative agent in milk was proved in samples of 69 cows (33% of the cases) (Pillai and Jayaro, 2002).

Studies generally demonstrate that microbial culture cannot determine the real rate of milk contamination by MAP. On the other hand, prolonging of the incubation period for the observation of the bacteria colonies often leads to an increase in the...
probability of in vitro secondary infection and even drying and ineffectiveness of the culture medium before obtaining the necessary results, which may affect the actual results.

**Conclusion**

To sum up, it should be noted that one of the main problems in the detection of infection as a result of *Mycobacterium tuberculosis* in cattle is the lack of a complete test or a true gold standard for this purpose. Currently, bacterial culture is referred to as the gold standard, but unfortunately, this old and relatively valuable method cannot detect all the infected animals in a population and has a comparative value only when we aim to determine the cattle which shed the bacterium. Nevertheless, given the significant advantages of PCR such as high speed, accuracy, and specificity and also its capability to be used on a broad scale for detection of the overall contamination level in a herd or in a region, this method could be introduced as a new procedure which can be considered as the gold standard approach.

**References**


Kawaji, S., Nagata, R. and Mori, Y. 2014. Detection and confirmation of *Mycobacterium avium* subsp. paratuberculosis in direct quantitative PCR positive fecal samples by the manual fluorescent MGIT culture system.


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