

Goat milk as a non-invasive sample for confirmation of *Mycobacterium avium* subspecies *paratuberculosis* by IS900 PCR

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ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes Johne's disease (JD) in cattle, sheep, goats and other ruminants, and Crohn's disease in humans. MAPs are shed to external environment through feces and milk. The present study was aimed to evaluate the utility of milk as a non-invasive sample in stage II MAP infections in goats using IS900 polymerase chain reaction (PCR) and sequencing analysis. A total of 32 milk samples from lactating does were collected. Within these 32 milk samples, 15 were collected from pre-confirmed JD positive goats. By IS900 PCR, all the 15 (100%) known JD positive goat milk samples revealed the presence of MAP. However, no unknown goat was identified as MAP positive. The results of this study established the usefulness of milk as a non-invasive sample in screening and confirmation of stage II MAP infection in goats.

Keywords

Diagnostic test, Excretors, Goat milk, MAP

Received : 25 May 2014,
Accepted : 30 June 2014,

Revised: 30 June 2014,
Published online: 30 June 2014.

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causal agent of Johne's disease (JD), chronic infectious enteritis of domestic and wild ruminants, characterized by progressive weight loss and profuse diarrhea. The disease passes through four stages. In stage I, animal is infected but does not show any clinical signs. In stage II, the animal still does not show

any clinical signs but organism is excreted in feces, while stage III shows early signs of disease and many diagnostic tests can be used to detect the infected animals, and stage IV is the obvious clinical end stage of the infection (Magombedze et al., 2013).

The JD is, therefore, a spectral disease with variable bacteriological, immunological and pathological situations making diagnostic efficacy variable at different points of time during the course of disease (Barad et al., 2014). The disease has a worldwide distribution, and it is categorized by the Office International Des Epizooties as a list B disease, which is a serious economic or public health concern (OIE, 2004). In India, JD is considered as endemic in goat herds (Singh et al., 2008). In diseased goats, MAPs are known to multiply in the mucosa of intestines, and are frequently shed in feces and milk or colostrum.

The MAP could be isolated from milk or colostrum of clinically affected or apparently healthy animals (Grant et al, 2001; Pinedo et al., 2008). Singh and Vihan (2004) isolated MAP from milk sample of clinically infected goats. Thus, excretion of MAP in the milk suggested that milk may act as a source of infection to young kids or adult human who consume raw milk (Singh and Gopinath, 2011; Sartor, 2005).

Isolation of MAP by culturing takes about 4 months, and is difficult in some cases (Sergeant and Whittington, 2000). Moreover, only culturing is inadequate to distinguish MAP from other closely related mycobacterial species (Singh et al., 2008). Thus, sensitive biomolecular assay, like IS900 polymerase chain reaction (PCR) technique, could be used to

identify MAP in small ruminants within short time. The present study was aimed to evaluate goat milk as a non-invasive sample for the identification of stage II MAP infection.

MATERIALS AND METHODS

Study Population: Milk samples (n=32) were collected from lactating does in University Multispecies Farm, PGRIAS, and Madras Veterinary College Teaching Hospital, Chennai, India. Within these 32 milk samples, 15 were collected from previously confirmed JD positive goats. The JD confirmation work was done by Samuel Masilamoni Ronald (personal communication, June 2010) using acid fast staining and IS900 PCR of fecal samples. The remaining 17 samples were collected randomly, of which 7 samples were collected from the goats of the same flock having 15 JD positive goats.

Milk sampling: Milk samples (5-10 mL) were collected in sterile 10 mL centrifuge tubes from both quarters after discarding the initial 1-2 mL of milk during milking, and the samples were refrigerated at 4°C until analysis. Before collection of milk, the teats were thoroughly cleaned with warm water to avoid sample contamination from skin and feces.

DNA Extraction: The milk samples were centrifuged at 3,000 rpm for 15 min, and the supernatant, including the hardened fat cream layer, was aspirated and discarded. The resultant pellet was washed thrice in phosphate buffered saline (PBS, pH 7.3), and centrifuged at 1,000 rpm for 10 min. DNA was extracted from the pellet by using commercial kit (DNA extraction kit, Qiagen, Hilden, Germany). The final product was stored at -20°C for subsequent PCR.

IS900 PCR and gel-electrophoresis: IS900 PCR was performed as per the procedure described by Pillai and Jayarao (2002) using the primer set; forward: 5'-CCG CTA ATT GAG AGA TGC GAT TGG-3', reverse: 5'-AAT CAA CTC CAG CAG CAG CGC GGC CTC G-3', targeting a DNA amplicon of 229-bp. The amplified PCR products were electrophoresed in 1.5% agarose using sodium borate buffer (pH 8.2) (Fisher Scientific, India) with a constant voltage of 100v for 2h. The DNA fragments were stained with ethidium bromide (1 mg/mL) and were visualized using UV-trans illuminator. The size of the amplified product was compared by 100-bp DNA ladder (GeNei™, Bangalore, India).

Sequencing of PCR products and homology: A representative of 7 positive PCR products were gel

purified according to the manufacturer's instructions (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany). The purified PCR products were sequenced by DNA Sequence Analyser (Applied Biosystems, USA). The nucleotide sequences were analyzed to identify homology with other reference strain (GenBank accession HQ830160) by nucleotide BLAST search (BLAST®, NCBI, USA).

RESULTS AND DISCUSSION

The shedding of MAP organisms into milk occurs by hematogenous or lymphatic spread (Slana et al., 2008). In the past decades, PCR based several methods for the detection, isolation and identification of MAP in raw milk were described; for example, 1.1% in the UK (Grant et al, 2001), 7.1% in Norway (Djønne et al., 2003), and 23% in Switzerland (Muehlherr et al., 2003).

Goat milk was reported to prevent replication of dengue virus by modulating the production of interleukin or by increasing the T cell function caused by the selenium (Se) present (Goldenberg, 2003). Thus, goat milk is recommended as a supportive measure for dengue affected humans (Mahendru et al., 2011). Also, goat milk is used for infants as in alternative foods (Basnet et al., 2010). In such situations, if demand for goat milk rises there could be a possibility of MAP ingestion through raw milk of infected goats, leading to the risk of Crohn's disease.

Streeter et al. (1995) recovered MAP as 22.2% from colostrum of clinically normal cows. Similarly, in our study, all the goats were clinically normal, and hence were considered as Stage II MAP infection. This stage II MAP infection may make the kids of the animal vulnerable for becoming infected. Further, this could lead to a vicious cycle of persistence and disease in the flock. Additionally, the organism might be consumed through raw goat milk as it is considered to have medicinal value (Mahendru et al., 2011).

The most often employed method of MAP detection from raw milk is culturing the organism, followed by direct PCR identification (Slana et al., 2008). In our study, 15 goat milk samples were amplified a product size of 229-bp, which were considered as positive by PCR (Figure 1). The PCR products, however, differed in intensity, which might be due to differences in concentration of MAP in the collected goat milk samples.

It was interesting that, out of 17 JD negative samples, 7 were collected from the same flock that contained the

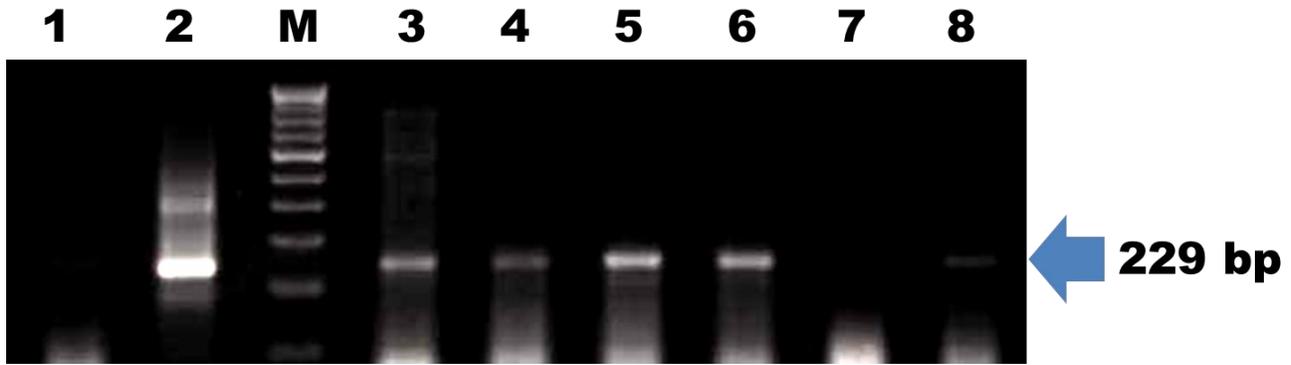


Figure 1: *Mycobacterium avium* subsp. *paratuberculosis* specific amplicons (229-bp) using IS900 specific primers in goat milk samples. M: 100 bp ladder; Lane 1: Negative control; Lane 2: Positive control; Lanes 3-6, 8: positive test samples; Lane 7: negative test sample.

15 JD positive goats, and no MAP DNA was detected. The remaining 10 negative samples were collected from apparently healthy goats having no signs of JD, and hence these could be termed as true negatives. Dimareli-Malli (2010) reported that shedding of MAP through milk was high in goats that had severe diarrhea. In our study, we did not observe any diarrhea in the 7 goats which were living along with the JD positive goats.

To determine whether the 229-bp fragment amplified by the IS900 PCR assay was specific for MAP, representative number PCR products (n=4) were sequenced and compared with that of known IS900 of MAP by BLAST analysis. The four nucleotide sequences were deposited in the GenBank with accession numbers of KJ882900 to KJ882903. On homology search, the sequenced isolates showed 99% similarities with IS900 gene of *Mycobacterium avium* subsp. *paratuberculosis* reference strain (GenBank accession HQ830160). In early stage, identification of MAP infection is difficult due to constraints of several methods in terms of sensitivity, specificity and inability to detect (Singh et al., 2010). In our study, utility of goat milk for the diagnosis of stage II MAP infection was confirmed.

CONCLUSIONS

The results of this study indicated that IS900 PCR assay can be used to detect MAP infection directly from milk samples within a short time (3-5 h). Thus, detection of MAP infection from milk by IS900 PCR is suggested as a valuable diagnostic or screening test of stage II MAP infection.

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