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Original Article

IS1311 PCR-RFLP molecular epidemiological approach for genotyping of *Mycobacterium avium* subspecies *paratuberculosis* in sheep and goat milk

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Abstract

Background: Johne's disease, a chronic wasting diarrheal illness, affecting ruminants, poses diagnostic challenges due to the prolonged incubation period of the agent. **Aims:** The present study aimed to assess the efficacy of the IS900 PCR protocol, for detecting the causative agent and identifying predominant *Mycobacterium avium paratuberculosis* (MAP) genotypes circulating among small ruminants in the Punjab (India) through the application of IS1311 PCR-RFLP using milk samples. **Methods:** A total of 205 milk samples, comprising 102 from goats and 103 from sheep, were tested for MAP DNA through IS900 PCR. Samples positive in IS900 PCR assay were subjected to IS1311 PCR assay, using M56 and M119 primers, followed by digestion of the products using *Hinf I* and *Mse I* restriction endonuclease enzymes. **Results:** Of the 205 samples, 37 (16 sheep and 21 goats) tested positive. The IS1311 RFLP analysis of all positive samples exhibited a restriction pattern (67, 218, and 323 bp) corresponding to the Bison type MAP Strain. **Conclusion:** Identification of a single predominant RFLP type (Bison) in both sheep and goats underscores the potential interspecies transmission and endemic co-circulation of Bison type MAP. These findings emphasize the need for further studies at regional and national levels, offering valuable insights for the development of comprehensive, monitoring and surveillance programs.

Key words: Goat, IS1311 PCR-RFLP, Johne's disease, Sheep

Introduction

Johne's disease (JD), also known as paratuberculosis, is a chronic granulomatous infectious enteritis affecting animals, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP) (Harris and Barletta, 2001). The organism is acid-fast, aerobic, slow-growing, Gram-positive, and requires exogenous mycobactin for *in vitro* growth (Thorel *et al*., 1990). The disease manifests as intermittent or persistent chronic diarrhea, weight loss and generalized unthriftiness in animals (Lybeck *et al*., 2011). It is prevalent worldwide with a broad host range, including cattle, goats, sheep, and other domestic and wild ruminants (Khan *et al*., 2010). JD has also been reported in non-ruminant wildlife such as wild rabbits, foxes, weasels, rodents, and feral cats (Beard *et al*., 2001; Hutchings *et al*., 2010). While the incidence of clinical disease is generally low, economic losses are substantial due to reduced milk production, body weight losses, increased chances of mastitis and infertility, shedding of wool, increased treatment costs, and culling (Gonda *et al*., 2007; Richardson and More, 2009).

Diagnosis of JD relies on clinical signs, identification or isolation of MAP from suspected animals and measurement of host immune response against MAP. Acid-fast staining for identification of MAP from fecal samples, while commonly used, exhibits low sensitivity and specificity (Laga *et al*., 2014). Fecal culture (gold standard), takes up to four months for visible colonies (Whipple *et al*., 1991), with Herrold's Egg Yolk Medium being a widely employed medium for this purpose (Manning and Collins, 2001). The organism's fastidious and slow growth makes culture time-consuming, especially with challenging ovine fecal samples and the

contamination of culture by bacterial and fungal agents (Dimareli-Malli and Sarris, 2001; Nielsen *et al*., 2004).

Cell-mediated immunity tests aid in early disease detection. *In-vitro* interferon Gamma tests (IFN-γ) demonstrate good sensitivity and specificity, showing promise in early infection detection in cattle and sheep (Jungersen *et al*., 2012). Serological assays, especially ELISA, serves as a widely used tool for diagnosing JD, owing to its simplicity and cost-effectiveness (Karuppusamy *et al*., 2021). However, sub-clinically affected animals or animals in the incubatory phase of the disease may not possess serum antibody levels adequate to surpass the detection threshold in ELISA (Clark e*t al*., 2008). Moreover, the diagnostic sensitivity of commercial ELISA kits is poor, particularly in cases where fecal culture confirms the presence of JD in animals (Speer *et al*., 2006).

Polymerase chain reaction (PCR) is a rapid, sensitive, and reliable method requiring less time and sample volume. It reduces the risk of MAP infection, by identifying the infected animals at an early stage (Gardner *et al*., 2011). Most PCR protocols involve the identification of the IS900 insertion sequence of the 1451 bp segment that is repeated 15-20 times within the MAP genome (Singh *et al*., 2010). PCR significantly enhances the diagnosis of MAP, allowing for its direct detection from diverse clinical materials such as feces, blood, milk, as well as intestinal and mesenteric lymph nodes, with sensitivity ranging from 70% to 100% and a specificity of 100% (Logar *et al*., 2012). Molecular epidemiological approaches using IS1311 PCR-RFLP (restriction fragment length polymorphism) assay can be instrumental in unraveling useful insights into the routes of transmission of the agent. This method also aids in deciphering the genetic diversity of MAP present across various ruminant species, thereby facilitating the design of effective control strategies. The limited information available (Kaur *et al*., 2011; Dixit *et al*., 2023) regarding the prevalence of predominant MAP genotypes among small ruminants from the Punjab region of India has exclusively focused on fecal shedding of MAP. The potential transmission of MAP through milk as reported by Kaur *et al*. (2010) and its presumed zoonotic nature, underscores the need for the rapid and accurate detection of MAP. This is crucial not only to prevent transmission of MAP among animals but also to mitigate potential risks to human health. Therefore, keeping in view the performance and discriminatory power of molecular epidemiological assays for discerning the genetic diversity of MAP, the present study aimed to assess the efficacy of IS900 PCR protocol, for detecting the presence of the agent and identification of predominant MAP genotypes through the application of IS1311 PCR-RFLP, circulating among small ruminants in the Punjab (India), using milk as starting sample.

Materials and Methods

Samples collection and preparation

A total of 205 milk samples (103 from sheep and 102 from goats) were employed in this study. After thoroughly cleaning and drying the teats, about 20 ml of milk samples from both groups were carefully collected in sterile 30 ml sample tubes, ensuring the exclusion of initial milk by discarding the first few streaks onto the ground. These collected samples were transported to the laboratory while at 4°C. The samples were transferred into sterilized centrifuge tubes with a capacity of 15 ml, filling them up to a volume of 12 ml. The samples were centrifuged at 3000 rpm for 45 min, facilitating the separation of components based on their densities in three distinct layers (top fat layer, middle whey layer, and bottom sediment layer). After pipetting out the middle layer, the pellet (SNF) and cream were combined and introduced into a column containing 50 ml of 0.9% Hexadecyl Pyridinium Chloride (HPC) for decontamination. Following four-hour incubation in a shaking incubator (120 rpm) at room temperature, with subsequent overnight incubation, the cream and SNF were allowed to separate. After discarding the HPC, the cream and SNF were pooled into a 1.5 ml sterile collection tube. The pooled mixture underwent four washings of 5 min each with 1 ml Phosphate Buffer Saline (PBS) through centrifugation at 5000 rpm. The samples were then stored at -20°C until further use (Kaur *et al*., 2010).

DNA extraction from milk

The DNA isolation from milk samples followed the protocol outlined by van Soolingen *et al*. (1991). The pooled cream and pellet mixture were re-suspended in 450 µL TE buffer, followed by the addition of 50 µL lysozyme (10 mg/ml). The suspension underwent gentle vortexing and an overnight incubation at 37°C. Subsequently, 100 µL of 10% sodium dodecyl sulphate was added and gently mixed by inverting tubes 5-6 times, and then 50 µL of proteinase K (10 mg/ml) was added and mixed gently. The mixture was incubated at 55°C for two h. To this suspension, 200 µL of 5 M NaCl was added and mixed gently. Then, 160 µL cetyl trimethyl ammonium bromide (CTAB) (preheated to 65°C) was added, and the mixture was incubated at 65°C for 30 min. After incubation, an equal volume of chloroform-isoamyl alcohol (24:1) was added and vortexed thoroughly. The suspension was centrifuged at 8000 rpm for 15 min. Following centrifugation, the aqueous layer was transferred to a sterile collection tube. The chloroform-isoamyl alcohol step was repeated, and the aqueous layer was again transferred to a sterile collection tube. To the aqueous layer, 0.7 volumes (~560 µl) of isopropyl alcohol were added and stored overnight at -20°C to allow DNA precipitation. After precipitation, centrifugation was performed at 12000 rpm for 15 min. The supernatant was discarded, and the sediment was washed with 1 ml of 70% ethanol by centrifugation at 12000 rpm for 15 min. The supernatant was discarded, and the pellet was allowed to air dry. The DNA pellet was re-suspended in 30 µL TE buffer and stored at -20°C until further use.

Table 1: Results of IS900 milk PCR for *Mycobacterium avium* subspecies *paratuberculosis*

Tests	Animals	Positive	Negative	Total	% positivity	Average positivity (%)
Milk PCR (IS900)	Sheep (103)	16	87	103	15.50	18.05
	Goat (102)	21	81	102	20.60	

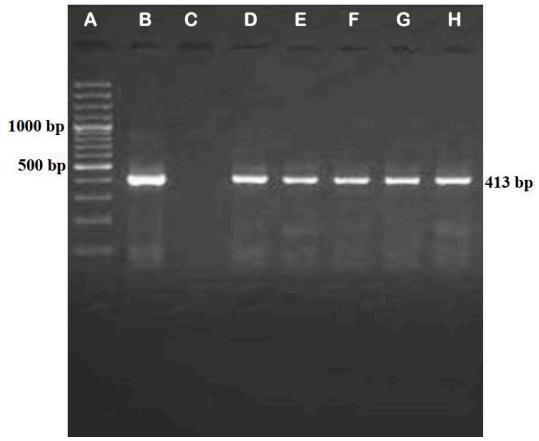

Fig. 1: Gel electrophoresis of IS900 PCR product from milk samples of sheep and goats. Lane A: 100 bp PLUS DNA ladder, Lane B: Positive control, Lane C: Negative Control, and Lanes D-H: Amplified PCR product of 413 bp

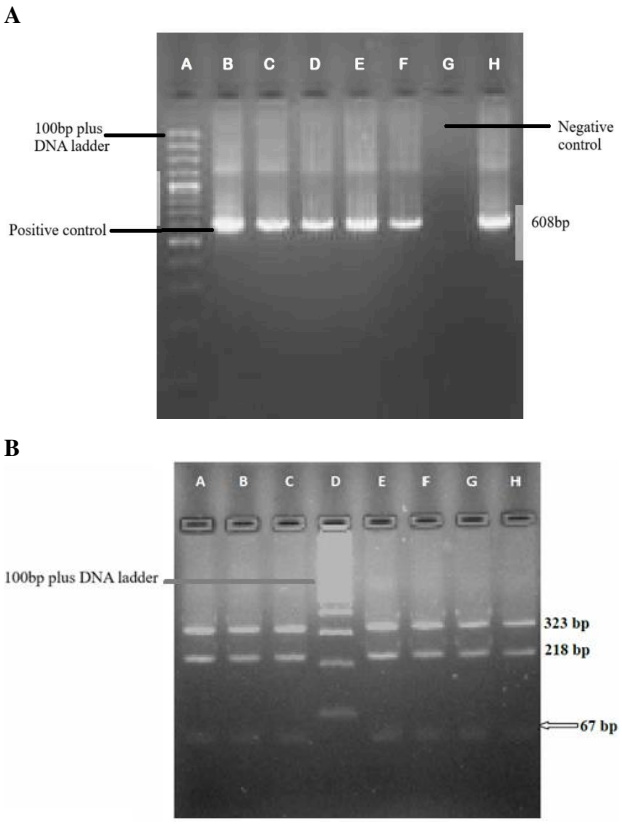

Fig. 2: (**A**) Gel electrophoresis of IS1311 PCR product from milk samples of sheep and goats. Lane A: 100 bp PLUS DNA ladder TM, Lane B: PCR product from standard DNA, Lane G: Negative control, and Lanes C-F and H: PCR product of 608 bp, and (**B**) Gel electrophoresis: *Hinf I* and *Mse I* restriction endonuclease analysis of IS1311 PCR products showing Bison type MAP from milk samples of sheep and goats. Lanes A-C and E-H: MAP *Bison* type with predicted band size of 67, 218, and 323 bp, and Lane D: 100 bp PLUS DNA ladder

DNA amplification and restriction enzyme analysis

Nucleotide primers P90B and P91B described by Millar *et al*. (1995) were used for DNA amplification. The method described by Marsh *et al*. (1999) was used to carry out IS900 PCR. IS1311 PCR protocol described by Whittington *et al*. (1998) was adopted for DNA amplification sing M56 and M119 primers. The IS1311 PCR products were subjected to restriction digestion using *Hinf I* and *Mse I* (New England Biolabs, Ipswich Massachusetts) restriction endonuclease enzymes. Restriction enzyme analysis (REA) of IS1311 PCR products was performed as per the method adopted by Sevilla *et al*. (2005). The reaction mixture comprised 0.25 µL of each enzyme (10 U/µL), 5 µL of HPLC water, 5 µL of 10X buffer (provided with enzyme) and 40 µL PCR product. The reaction mixture was incubated at 37°C for 1.5 h in water bath, following which the products of restriction enzyme analysis were subjected to electrophoresis in 2% agarose gel and identified as per the method described by Whittington and Sergeant (2001).

Results

Milk PCR identified 37 positive cases (16 sheep and 21 goats) out of a total of 205 samples (Table 1 and Fig. 1). Samples positive for IS900 were subjected to IS1311 PCR analysis to enhance the accuracy of MAP identification. All samples positive for IS900 PCR were also positive in the IS1311 PCR (Fig. 2A). The IS1311 RFLP analysis of all samples exhibited a restriction pattern (67, 218, and 323 bp) corresponding to the *M. avium* subsp *paratuberculosis* Bison-type exclusively (Fig. 2B). Notably, none of the samples displayed the restriction pattern 285 and 323 bp, *M. avium* subsp *paratuberculosis* corresponding to sheep type or 67, 218, 285, and 323 bp corresponding to cattle type *M. avium* subsp *paratuberculosis* or *M. avium* subsp. *avium* 134, 189, and 285. In the present study, no discernible host specificity was identified for the observed RFLP types.

Discussion

Johne's disease, a chronic wasting diarrheal illness, affecting ruminants, poses diagnostic challenges due to the prolonged incubation period of the agent. IS900 PCR is an effective tool in detecting MAP in raw milk (Pillai and Jayarao, 2002), pasteurized milk (Anzabi and Hanifian, 2012), cheese and milk powder (Donaghy *et al*., 2011) and meat and drinking water (Gill *et al*., 2011). IS900 PCR is effective in detecting MAP in asymptomatic animals, highlighting its potential for early

disease diagnosis (Raveendran *et al*., 2011). Numerous studies have reported the presence of MAP in the milk of both clinically affected and asymptomatic sheep and goats. Singh and Vihan (2004), Nebbia *et al*. (2006), and Dimareli-Malli (2010) observed MAP in the milk of animals with a history of paratuberculosis. Nebbia *et al*. (2006) also suggested that raw milk might act as a potential transmission route. The detection of MAP has also been reported in the milk of both vaccinated and unvaccinated dairy goats, indicating that the bacterium can be present in milk irrespective of vaccination status (Djonne *et al*., 2003). Consequently, the use of raw milk emerges as a non-invasive method for both screening and confirming MAP infection (Sukumar *et al*., 2014).

DNA extraction was performed on pooled cream and pellet samples. According to Millar *et al*. (1996), MAP bacilli are primarily partitioned into the cream and pellet fractions after centrifugation, with only a few in the whey and their distribution in pellet and cream portions is reported to be equal (Giese and Ahrens, 2000). The whey fraction has been found to contain significantly higher concentrations of PCR inhibitors than the cream and pellet (Schrader *et al*., 2012). The use of both pellets and cream has been shown to enhance the recovery of MAP from milk (Giese and Ahrens, 2000). The fecal shedding of MAP bacilli in milk is influenced by the infection status of the animal as well as the stage of lactation (Stabel *et al*., 2014). In this investigation, milk samples obtained from animals exhibiting substantial fecal shedding, the details of which are outlined elsewhere (Dixit *et al*., 2023), tested positive for MAP. MAP DNA was detected in sheep and goat milk, although irregularly excreted. Furthermore, the highest number of positive samples was observed in milk collections taken shortly after parturition as compared with subsequent samplings. This observation corroborates with the results reported by Stabel *et al*. (2014), who found that viable MAP was primarily isolated from milk and colostrum during the early lactation period (days in milk, DIM 0-60) in sub-clinically and clinically infected cows, while minimal positive samples were detected in both mid (DIM 60-240) and late (DIM 240-305) lactation stages. Despite the seronegative status of asymptomatic animals, MAP has been reported in milk, which may potentially be due to the low sensitivity of serological tests in identifying infections during the asymptomatic or incubatory phase of the disease (Englund *et al*., 2001). However, Nebbia *et al*. (2006) observed the intermittent presence of MAP DNA in the milk of asymptomatic sheep and goats, with a higher prevalence in seropositive animals. This suggests that MAP transmission via milk poses a risk even for healthy animals, and the infection of lambs and kids through this route is likely under normal breeding conditions (Ayele *et al*., 2018).

Mycobacteria, other than MAP, possess IS900-like elements, sharing nucleotide sequences with up to 94% similarity to MAP IS900 (Englund *et al*., 2002). This similarity raises concerns about the specificity of IS900 PCR, potentially leading to false-positive results. Thus, caution is advised when interpreting results solely based on IS900 PCR. Semret *et al*. (2006) recommend confirming a positive IS900 PCR outcome through subsequent sequencing or PCR assays targeting alternative genes in MAP. Keeping this in view samples positive for IS900 were subjected to IS1311 PCR analysis to enhance the accuracy of MAP identification and genotyping. The IS1311 RFLP analysis of all samples exhibited a restriction pattern corresponding to the Bison type exclusively and none of the samples displayed the cattle type restriction pattern (67, 218, 285, and 323 bp). The findings corroborate with Singh *et al*. (2009) and Singh *et al*. (2015) who reported MAP DNA from Indian-origin samples exclusively corresponding to the Bison type. IS1311 PCR-RFLP has been employed in various studies for the identification of MAP in animals. Traveria *et al*. (2014) used this method to compare MAP strains in sheep and cattle in Argentina, while Machackova-Kopecna *et al*. (2005) applied IS1311 PCR RFLP analysis to investigate paratuberculosis and avian tuberculosis infections in red deer. The IS1311 PCR-RFLP is a robust method and easy-to-perform assay for differentiation of Bison-type Indian MAP isolates from Non-Indian MAP isolates, as highlighted by Singh *et al*. (2015). A high prevalence of 'Bison type' MAP from domestic animals in India has also been reported by (Kumar *et al*., 2007; Sharma *et al*., 2008; Kaur *et al*., 2011). Sheep type has not so far been reported from India.

Strain identification serves as a valuable tool in molecular epidemiological investigations, understanding the origin of infection, disease transmission dynamics and development of rational and more effective control measures tailored to specific scenarios. PCR-RFLP has been extensively used for the discrimination of MAP from *Mycobacterium avium* subspecies *avium* and for distinguishing bison, cattle, and sheep type MAP (Englund, 2003; Douarre *et al*., 2012; Traveria *et al*., 2014). This method relies on identifying point mutations within the open reading frame of a highly conserved gene, leading to the loss of a recognition site for a restriction enzyme (Dixit *et al*., 2023). Amplification of the gene through PCR and digestion with specific restriction enzymes produces fragments of different lengths due to the distinct polymorphisms. Studies by Whittington and Sergeant (2001), Singh *et al*. (2009), and Singh *et al*. (2015) have observed host adaptation among various strains of MAP.C strains have been identified in diverse species, encompassing cattle, goats, alpacas, and humans, whereas S strains are predominant in sheep (Szteyn *et al*., 2020). In the present study, no discernible host specificity was identified for the observed RFLP types. The animals had been browsing on pastures, fertilized with dung from cows likely to have JD, indicating the likelihood of interspecies transmission of MAP strains, which is consistent with the observations made by Dixit *et al*. (2023). The exclusive identification Bison type RFLP in both sheep and goats again underscores its potential interspecies transmission. This situation may be attributed to the endemic co-

circulation of Bison type MAP among ruminants, facilitated by uncontrolled animal movement in endemic areas and specific husbandry practices. Moreover, the introduction of replacement animals into a herd with an unknown status of JD may play a pivotal role in the transmission of MAP. Natural infection of sheep and goats with Bison-type MAP has been documented by Sevilla *et al*. (2005) and Singh *et al*. (2007). Additionally, Bison-type MAP has been recovered from cattle and buffalo suffering from terminal Johne's disease (Yadav *et al*., 2008). Outside India, MAP Bison type has been recovered only from bison to date (Whittington and Sergeant, 2001).

The application of PCR-based methods for the detection and strain differentiation of MAP DNA in milk holds great promise for improved disease control, enhancing food safety, and safeguarding human and animal health alike. The detection of MAP DNA in the milk of sheep and goats is a cause for concern due to its adverse impact on animal productivity and human health. Without accurate diagnosis, infected animals persist to shed MAP in the environment, posing a significant threat to other livestock. The extensive grazing habits of sheep and goats, coupled with manure contamination of forages increase the risk of infection to other animals. The presence of the Bison-type strain highlights its interspecies transmissibility and endemic co-circulation. These findings emphasize the need for additional studies at regional and national levels, offering valuable insights for the development of comprehensive, monitoring and surveillance programs. Such initiatives aim to significantly mitigate the economic losses faced by livestock owners.

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Conflict of interest

The authors have no conflict of interest to declare.

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