RESEARCH ARTICLE

Molecular Detection and Typing of *Mycobacterium avium* Subspecies *paratuberculosis* from Fecal Samples of Small Ruminants

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Abstract

The chronic nature of Jhone's disease and prolonged incubation period of the agent often complicate the diagnosis of this disease. The present study was undertaken with the objective to compare fecal microscopy using Ziehl-Nielsen (ZN) staining method and Polymerase Chain Reaction assay for detection of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in small ruminants. A total of 205 fecal samples of sheep and goat were first microscopically examined using ZN staining method and later tested for presence of MAP DNA through IS900 PCR. Win Episcope 2.0 software was used to determine agreement between the test assays. Of the total 205 fecal samples, 78 (38.0%) were positive by ZN staining method and 48 (23.4%) by IS900 PCR. Moderate level of agreement (0.517) was observed between the test assays. Restriction enzyme analysis of DNA amplicons from IS1311 PCR revealed that all positive samples belonged to Bison type MAP strain. Single predominant strain (Bison type) among all ruminants indicates its ability to infect multiple host species, possibility of interspecies transmission and endemic co-circulation.

Key words: Bison type strain, IS1311 PCR REA, IS900 PCR, *Mycobacterium avium* subspecies *paratuberculosis,* Small ruminants.

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Introduction

Jhone's disease (JD), caused by *Mycobacterium avium* subspecies paratuberculosis (MAP), is a chronic granulomatous infectious enteritis of ruminants. The disease is mainly transmitted through feco-oral route; however transmammary and *in utero* transmission of the agent has also been reported (Lambeth *et al.,* 2004). Despite low incidence of clinical disease and mortality rate, it can severely impact the overall productive efficiency of the affected animals resulting in huge economic losses (Rasmussen *et al.,* 2021).

Disease is diagnosed on the basis of clinical signs, detection of acid fast bacilli in the feces and host immune response. However acid fast staining method has low sensitivity and specificity (Laga *et al.,* 2014). Isolation of the organism is difficult due to slow growth and the contamination of culture by competing bacterial and fungal agents (Nielsen *et al.,* 2004). The *in-vivo* intra-dermal test lacks specificity (Kennedy *et al.,* 2014) and sero-assays (AGID, CFT) suffer from low sensitivities in sub-clinically infected animals.

Polymerase Chain Reaction (PCR) is, sensitive, reliable, easy to perform, requires less amount of sample and has high throughput. Most of PCR protocols involve detection of IS900*;* an insertion sequence, which has 1451 bp segment and is repeated 15-20 times in MAP genome. The uniqueness of sequences is that the 5› portion of IS900 provides a source of probes and primers for the specific and sensitive detection of *M. paratuberculosis* (Singh *et al.,* 2010). Molecular epidemiological approaches using IS1311 PCR-REA assay can help in elucidation of information about transmission of the agent, genetic diversity of MAP circulating among different ruminant species and in designing control strategies. The present study was carried out to check the suitability of molecular assay (IS900 PCR protocol) for detection of this agent and identification of MAP strains prevalent in Punjab region of India by IS1311PCR-Restriction Endonuclease Analysis (REA).

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Materials and Methods

Collection and Processing of Samples

A total of 205 fecal samples (102 from goats and 103 from sheep) were collected directly from rectum under hygienic conditions in a plastic bag. The samples were stored at –20

Table 1: Comparison of IS900 fecal PCR and Ziehl-Nielsen staining

Tests	ZN staining Negative	ZN staining Positive				TOTAL
		1+ (1-4)*	2+ (5-10)*	3+ (10-15)*	4+ (>15)*	
IS900 Positive	7	12	16	10	3	48
IS900 Negative	121	26	8	2	0	157
TOTAL	**128**	**38**	**24**	**12**	**3**	**205**

* Number of bacilli per field

°C till use. Two gram feces were finely ground into paste in sterile pestle mortar using about 10 mL distilled water. The mixture was transferred into a 15 mL tube and subjected to centrifugation at 839 x g for 45 min at room temperature. Supernatant was discarded and smear was prepared from interface layer for Ziehl–Nielsen (ZN) staining.

DNA Extraction

Method described by Garrido *et al.* (2000) was used for DNA extraction with minor modifications. After centrifugation, the interface layer was transferred to 50 mL column of 0.9% HPC (w/v in distilled water) for decontamination at room temperature for 18 h. After decontamination supernatant was discarded and remaining sediment (~100 µL) was transferred to sterile 1.5 mL micro-centrifuge tube and was given four washings with 1 mL PBS by centrifugation at 2336 x g each for 5 min. After washing, 50 µL of distilled water was added to the sediment and mixed. The mixture was subjected to four cycles of heating (95 °C for 20 min) and thawing (rapid chilling on ice for 20 min) followed by centrifugation at 5724 x g for 5 min. The resulting supernatant (10 µL) was used as template in the PCR.

DNA Amplification and Restriction Enzyme Analysis

For DNA amplification nucleotide primers P90B and P91B described by Millar *et al.* (1995) were used. IS900 PCR was carried out as per the method adopted by Marsh *et al.* (1999). IS1311 PCR was carried out on the DNA extracted from fecal samples using M56 and M119 primers as described by Whittington *et al.* (2001). The reaction mixture composition and thermocycling conditions were similar to IS900 PCR, except that annealing temperature was set at 62 °C. The IS1311 PCR products were subjected to restriction digestion using *Hinf I* and *Mse I* (New England Biolabs, Ipswich Massachusetts) restriction endonuclease enzymes IS1311 PCR-REA was performed as per the method adopted by Sevilla *et al.* (2005). The reaction mixture comprised of 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. Reaction mixture was incubated at 37 °C for 1.5 h in water bath. Following incubation, products of restriction enzyme analysis were allowed to migrate electrophoretically in 4% agarose gel. Genotypes were identified by fragment migration patterns as described by Whittington *et al.* (2001).

Results and Discussion

Out of total 205 fecal samples 78 (38.0%) were positive by ZN staining, of which 36 (34.9 %) and 42 (41.2%) samples belonged to sheep and goat, respectively. ZN staining method is quick preliminary method of diagnosing acid fast bacilli in fecal and tissue samples, but the low specificity and high rate of false positive results in ZN staining limits its use. Fecal IS900 PCR (Fig. 1) detected 48 samples as positive, of which 20 samples were of sheep and 28 from goats. All +4 grade ZN positive samples were positive in IS900 fecal PCR assay and PCR positivity decreased with decrease in number of acid-fast bacilli in the samples (Table 1). Of 128 ZN negative samples, 7 (5.4 %) were positive by IS900 PCR. A moderate level of agreement (0.517) was observed between the two assays. Samples positive for IS900 PCR were found positive in IS1311 PCR also (Fig. 2). IS1311 PCR products were subjected to restriction digestion.

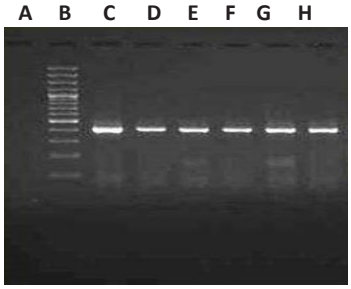

Fig. 1: Gel electrophoresis of IS900 PCR product from fecal samples of sheep and goats.
Lane A -Negative-Control, lane B- 100 bp PLUS DNA ladder™ and lane C Positive control. Lane D-H - PCR product of 413 bp

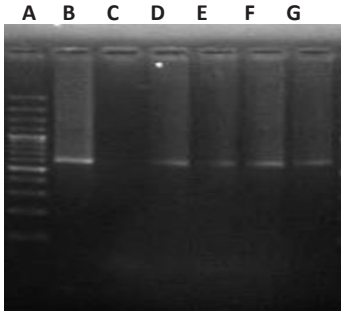

Fig. 2: Gel electrophoresis of IS1311 PCR product from fecal samples of sheep and goats.
Lane A - 100 bp PLUS DNA ladder™, Lane B– Positive control, lane C- negative Control and Lane D-G - PCR product of 608 bp.

Since cultural isolation of MAP from infected small ruminant is difficult and time consuming, PCR has proven useful in detection of this agent from these animals. Its high throughput and robustness allows a rapid detection in about 30 h compared to culture. However, assay performance of PCR is decreased because of lysis-resistant cell wall of MAP and presence of high amount of PCR inhibitors in the feces (Varela *et al*., 1994). Intermittent shedding of MAP in low numbers in sub-clinically infected animals also reduces the likely hood of obtaining a sample containing the bacilli (Koets *et al.,* 2015). Performance of IS900 based PCR detection system is also affected as this sequence is not present in all strains of MAP due to the variation of genomic DNA; moreover mycobacteria other than MAP harbor IS900 like insertion sequences (Englund *et al.,* 2002), affecting the test outcome. IS900 like regions of environmental mycobacteria, *M. cookii* and *M. scrofulum* share 94 and 79 % homologies, respectively, with IS900 region of MAP, suggesting that IS900 PCR can result in false positive results. Therefore result interpretation on basis of IS900 PCR alone should be done with caution. Semret *et al.* (2006) recommended that a positive IS900 PCR should be confirmed by subsequent sequencing or PCR assay targeting another gene in MAP. In the present study IS1311 PCR analysis of samples positive for IS900 was carried out for identification and genotyping of MAP.

Out of total 48 positive fecal samples, nine samples could not be typed because of insufficient amplification in PCR. The bands were very faint and became almost invisible after restriction digestion. This could be due to insufficient amount of mycobacterial DNA present in the samples. Rest of the samples taken for IS1311 REA gave a restriction pattern (67, 218 and 323 bp) characteristic of Bison type (Fig. 3). Restriction pattern (67, 218, 285 and 323 bp) belonging to Cattle type was not observed in any of the samples. Molecular epidemiological tools have proven to be of immense use in identification and tracking of pathogens. The approaches make combined use of molecular techniques and evolutionary biology to identify differences among species, sub-species, strains and sub-types at gene level. These robust techniques have helped in identification and characterizing the evolution of a pathogen and at the same time evaluation of surveillance programmes aimed at reducing the prevalence of a disease to a justifiable level. PCR-REA has been widely explored for differentiation of *Mycobacterium avium* subspecies from Bison, cattle and sheep strains of MAP (Whittington *et al.,* 2001). The basis for this test involves point mutations within the open reading frame of a highly conserved gene, which results in the loss of a restriction enzyme recognition site. When a portion of the gene is amplified using PCR and subsequently digested with the appropriate restriction enzymes, fragments of different lengths are obtained as a result of the specific polymorphisms. This strategy has been also used with point mutations in the IS1311 element (Harris and Barletta, 2001).

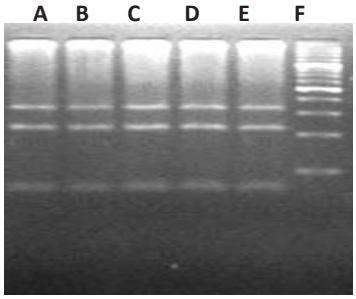

Fig. 3: Gel electrophoresis: *Hinf I* and *Mse I* restriction endonuclease analysis of IS1311 PCR products showing Bison type from fecal samples of sheep and goats.
Lane A-E- *M. paratuberculosis* Bison type with predicted band size of 67, 218 and 323 bp. Lane F – 100 bp PLUS DNA ladder™

The results of the previous molecular epidemiological studies from India, report very high prevalence of 'Bison type' MAP from domestic animals (Sonawane *et al.,* 2016). Sheep type has not so far been reported from India. Observations of Liapi *et al.* (2015) indicate host adaptation of different MAP strains. C strains have been found in cattle and other species (goat, alpaca, humans), while S strains are predominated in sheep. In present study no host specificity could be observed for the RFLP types identified. The goat herd had been grazing on pasture fertilized with manure from cows likely to have JD, which indicates the possibility of interspecies transmission of MAP isolates. The presence of single predominant RFLP type (Bison) among both sheep and goats indicates the interspecies transmissibility of Bison type MAP. Moreover natural infection of sheep and goat with Bison type MAP has also been documented by Sevilla *et al.* (2005) and Singh *et al.* (2007). MAP Bison type has also been recovered from cattle and buffalo (Yadav *et al.,* 2008) suffering from terminal Johne's disease.

Conclusions

The high positivity of MAP in feces of sheep and goats is a matter of concern as the disease decreases the productivity of the animals. In the absence of proper diagnosis, animals continue to shed MAP in the environment and pose a threat to other animals. Intensive grazing of sheep and goat on pastures and manure contamination of forages pose a great risk to other animals. There is no specific treatment for Johne's disease, but it is important to identify animals which continue to shed the organism, in order to prevent the spread of infection to other animals. Further, its likely zoonotic nature indicates that humans are equally at risk of contracting Crohn's disease. Presence of Bison type strain supports the interspecies transmissibility and endemic co-circulation of this strain. The information gained will be very useful if more such studies are conducted at regional and national level, so that it could be used to formulate a pan state coordinated monitoring and surveillance programme to reduce significantly, the economic losses livestock owners have to face.

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