RESEARCH ARTICLE

Evaluation of IS900 PCR for Detection and IS1311 PCR via Restriction Enzyme Analysis for Strain Typing of *Mycobacterium avium* subspecies *paratuberculosis* in Ruminant Fecal Samples

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Abstract

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes chronic granulamatous infectious enteritis known as Johne's disease in ruminants and is characterized by progressive diarrhea, weight loss, and reduced productivity. MAP shedding in feces makes fecal samples valuable for diagnostic screening. Based on clinical symptoms, 220 fecal samples from cattle and buffaloes with a history of chronic diarrhea were collected. Each sample was stained with Ziehl- Neelsen staining technique to detect acid fast positive bacteria. The DNA was collected from highly acid fast bacterial samples and was subjected to isolation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Out of 71 acid fast bacterial samples; 22 were highly acid fast positive. Only 2 samples were found to be positive in isolation for MAP. DNA from these isolates were then extracted and subjected to IS900 PCR hence, confirming the presence of MAP in the samples. The positive samples were also subjected to IS1311 PCR and were analyzed by Restriction enzyme analysis (REA). The PCR-REA confirmed the presence of 'Cattle type' of strain of the two isolates. Sequence analysis further demonstrated a high degree of similarity between the two isolates and strong phylogenetic identity with previously reported global MAP sequences, with the highest similarity (99.3%) to a Chinese MAP isolate (MW546854_China).

Key words: IS900 PCR, IS1311 PCR, *Mycobacterium avium* subspecies *paratuberculosis*, PCR-REA, Phylogenetic analysis.

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Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the cause of a chronic, granulamatous, infectious enteritis disease known as paratuberculosis or Johne's disease, in different ruminant species worldwide. Johne's disease is responsible for significant economic losses globally. The disease is one of the main causes of the livestock industry's unprofitable returns due to decrease in milk production, lower fertility rates and increase in high rate of premature culling and mortality (Peek *et al*., 2018). The animals infected with sub-clinical and clinical MAP infection shed live bacteria in milk and feces, increasing the risk of transmission to young animals and other animals liable to the infection. The disease's impact in India has not been estimated, despite the fact that it causes high morbidity, lower output, and increased culling. However, reports of MAP in northern India's cattle, buffalo, sheep, and goats are frequent (Singh *et al*., 2008). The test and cull approach used by JD control programme calls for a very delicate and focused diagnostic methods. The gold standard for the diagnosis of MAP is fecal culture but due to its slow growth rate can take up to 12 to 16 weeks (Chiodini *et al*., 1984).

There are two major subgroups of *M. paratuberculosis* strains: Sheep type strains and cattle type strains based on restriction fragment length polymorphism analysis (RFLP) and cultural traits (Collins *et al*., 1990). The sheep type strain is known to affect both sheep and goat populations, whereas, the cattle type strain infects wide range of species

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including cattle, sheep goats and even humans. Although, IS900 RFLP analysis and genome restriction on pulsed field gel electrophoresis are frequently used typing methods performed on DNA extracted from cultures used to distinguish MAP into "sheep-type" (S) or type I, "cattle-type" (C) or type II, and "intermediate" or type III isolates, these techniques are expensive, laborious and require large quantities of good quality DNA, which is challenging in the case of extremely slow-growing type of strains (Pavlik *et al*., 1994). In parts of the world where mixed grazing is preferred; differentiation between sheep and cattle type strains should be done for the control and eradication of the Johne's disease. Mobile genetic elements are also called as Insertion sequences and are commonly used targets in polymerase chain assays for the detection of various mycobacterial diseases (McAdam *et al*., 1994). An insertion sequence present in MAP specific to it is IS900 which is a target for quick identification of MAP by PCR. The IS900 gene, a member of the IS116 family, is currently the sole insertion sequence identified as unique to *M. paratuberculosis* (Green *et al*., 1989). A technique known as polymerase chain reaction- restriction enzyme analysis (PCR-REA) was developed based on the polymorphism present in the insertion sequence IS1311 (Collins *et al*., 1989). The MAP genome contains 17 copies of the insertion sequence IS1311. The IS1311 PCR-REA is a simple and quick technique which can be used to differentiate between both the cattle and sheep strains of MAP based on C/T polymorphism at base position 223. As compared to other gene or the insertion sequences present in the genome of MAP, IS1311 insertion sequence was found to have seven to ten copies in the genome, hence making it as helpful target to develop highly sensitive PCR assays (Marsh *et al*., 1999). The objective of the current work was to standardize a direct fecal PCR for MAP identification and IS1311 PCR-REA for genotyping of the MAP isolates.

Materials And Methods

The study was conducted after the approval of IAEC/CPCSEA, GADVASU, Ludhiana, India (GADVASU/CPCSEA/20/03/2019).

Collection, Decontamination of Fecal Samples and DNA Extraction

A total of 220 fecal samples of cattle and buffaloes suffering from chronic, intermittent diarrhea were collected from organized dairy farms of Ludhiana, Punjab. The fecal samples were stained with Ziehl-Neelsen staining and were microscopically examined and classified either as positive or negative (5->10 bacilli per HP field) on the smears. The highly positive fecal samples were subjected to decontamination as per OIE Terrestrial Manual 2014's methodology in which 2 gram of feces was collected in 20 mL of distilled water and was mixed thoroughly by keeping it on a shaker for about 30 min. About 5 mL of supernatant was collected and transferred to 20 mL of 0.95% HPC in half strength BHI broth. It was left undisturbed at room temperature for 18 h. The sediment was transferred (0.1 mL) to Middlebrook 7H10 Media and was kept for incubation and was observed periodically for a period of 6 months. DNA was extracted from the fecal samples by kit as well as conventional method from the isolates and was used as a template for carrying PCR reactions.

IS900 PCR

The DNA template extracted from the isolates was subjected to IS900 PCR for confirmation of MAP as this insertion sequence is specific to MAP. IS900 PCR was performed by using primers specified by Vary *et al*. (1990). For preparing a reaction mixture of 25 µL, 12.5 µL of GoTaq® Green Master Mix, 1 µL (25 pmol/µL) of forward and reverse primer each, 2.5 µL of DNA and 8 µL of nuclease-free water were added. Thermal cycling was carried out with the initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 45 s, primer annealing at 62 °C for 45 s, extension at 72 °C for 45 sec, and final extension at 72 °C for 10 min. The products were analyzed by agarose gel electrophoresis and the presence of PCR product of 229 bp was evaluated.

IS1311 PCR-REA

In order to confirm MAP identification after IS900 PCR, an IS1311 PCR was performed using M56 and M119 primers (Garrido *et al.,* 2000). For the amplification of the DNA an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 45 s, primer annealing at 62°C for 45 s, extension at 72°C for 45 s, and final extension at 72°C for 10 min were used. By using the IS1311 PCR-REA method, each MAP DNA sample derived from mycobacterial isolates or fecal samples was genotyped. Amplified product showing a band size of 608 bp was considered as positive after visualizing it in 1.5 % agarose gel stained with ethidium bromide. The IS1311 polymerase chain reaction-restriction endonuclease analysis was used to detect any genetic differences or relatedness among the mycobacterial isolates. Briefly, a 30 µL volume reaction including 10 µL of positive *IS1311* PCR product, 3 µL of 10X buffer (Thermoscientific), and 2 units of each endonuclease *HinfI* and *MseI* (Thermoscientific) was carried out. The reaction mixture was incubated at 37° C for 2 h. After performing agarose gel electrophoresis on a 2.5% high resolution agarose gel stained with ethidium bromide, band patterns were seen and a genotype profile interpretation was carried out (Sevilla *et al.,* 2005).

Results and Discussion

ZN Staining and Isolation of Samples

After screening of 220 fecal samples, 71 samples were found to be acid fast positive, out of which 22 samples were highly acid fast positive. These fecal samples were subjected to isolation of *Mycobacterium avium* subsp. *paratuberculosis*. MAP was isolated from two highly acid fast positive fecal samples in which growth was seen after 16 weeks of incubation. The colonies appeared as dispersed grayish and

off white (Fig. 1). The isolates were subjected to acid fast staining, and it revealed acid-fast bacilli in a 100x oil emulsion.

Fig. 1: Growth of *Mycobacterium avium paratuberculosis* on Middle brook 7H10 media.

IS900 PCR

The DNA extracted from the two isolates from the highly positive acid fast samples was subjected to conventional IS900 PCR using published primers where it amplified a product of 229 bp (Whittington *et al.,* 1998), hence confirming the presence of MAP in the isolates (Fig. 2). For this, MAP from the standard culture was used as a positive control and primers were also standardized using this standard culture.

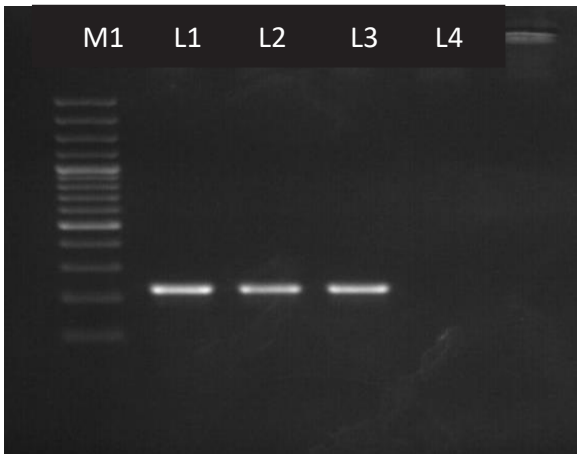

Fig. 2: Agarose gel electrophoresis showing an amplicon of 229 bp of *IS900* of MAP from isolates. M1: marker (100 bp DNA ladder), L1: positive control, L2, L3: Positive sample for IS900 of MAP, L4: Negative control.

IS1311 PCR

IS1311 PCR was performed after identification by IS900 PCR. The two isolates from highly acid fast positive samples were subjected to IS1311 PCR using published primers where it showed an amplified product of 608 bp (Garrido *et al*., 2000). By using the IS1311 PCR-REA method, each MAP DNA sample derived from Mycobacterial isolates of the fecal samples was genotyped (Fig. 3).

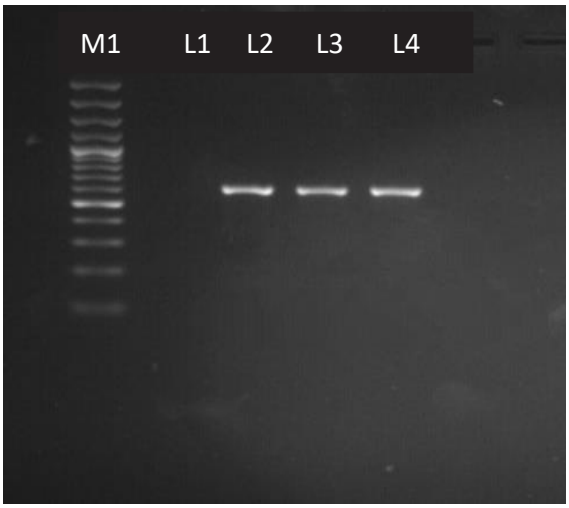

Fig. 3: Agarose gel electrophoresis showing an amplicon of 608 bp of *IS1311* of MAP from isolates. M1: marker (100 bp DNA ladder), L1: Positive control, L2, L3: Positive sample for IS1311 of MAP, L4: Negative control.

Restriction Enzyme Digestion and Analysis

The IS1311 PCR-REA was used to detect any genetic differences or relatedness among the Mycobacterial isolates. By using the IS1311 PCR-REA method, each MAP DNA sample derived from Mycobacterial isolates or fecal samples was genotyped. Restriction endonucleases analysis of IS1311 PCR product by *Hinf /MseI* enzymes showed digestion pattern containing four bands 67 bp, 218 bp, 285 bp, and 323 bp (Sevilla *et al.,* 2005), hence confirming the presence of Cattle type of strain of the isolates (Fig. 4).

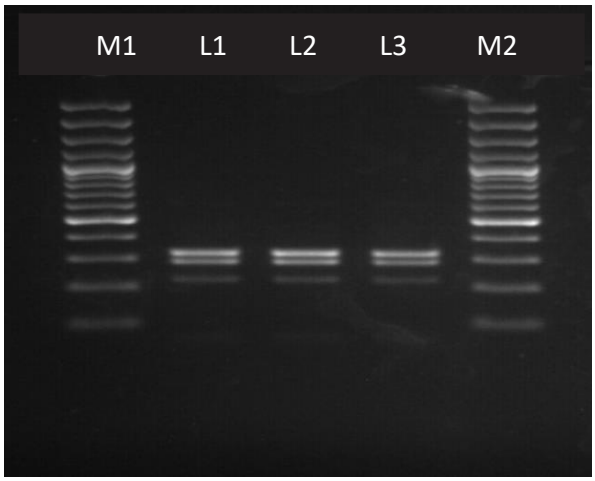

Fig. 4: Agarose gel electrophoresis showing Restriction enzyme analysis pattern of standard cultures and isolates. M1: marker 100 bp DNA ladder, L1: positive control -323 bp /285 bp /218 bp /67 bp, L2: Isolate 1(Hinf) -323 bp /285 bp /218 bp/ 67 bp, L3: Isolate 2 (Hinf)- 323 bp/285 bp/ 218 bp/ 67 bp, M2: 100 bp DNA ladder.

Phylogenetic Analysis

To compare and determine the phylogenetic relationships of the MAP isolates (Cattle 1 Current study, Cattle 2 Current study) were sequenced and a phylogenetic tree

was constructed using the neighbor joining method and bootstrap analysis (Fig. 5). On sequence analysis, it was revealed that the isolates of MAP showed a higher degree of sequence identity among themselves. The two isolates from our current study showed maximum identity with the previously reported strains. It was evident from the analysis that the MAP isolates of the current study and those of the MAP isolates reported from India and other parts of the world had a high degree of sequence similarity (97.0-99.3%). However, among the isolates from the other parts of the world, it showed maximum identity with the Chinese isolate (MW546854_China), showing a percent identity of 99.3%.

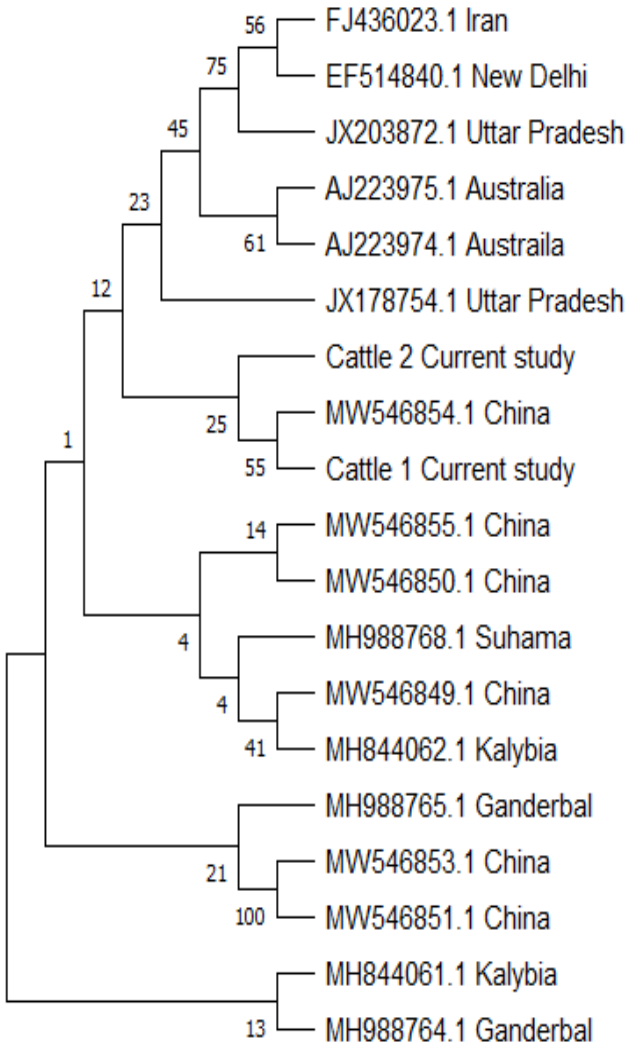

Fig. 5: Phylogenetic tree constructed using Maximum likelihood method and bootstrap analysis by using Mega11 software.

The findings of this study validated that IS900 and IS1311 PCR procedures can both be used to confirm identification and typing of MAP. As compared to other gene or insertion sequences present in the genome of MAP, the IS1311 insertion sequence was found to have 7-10 copies in the genome, hence, making it a helpful target to develop highly sensitive PCR assays. Analysis of the isolates by PCR-REA can identify genetic similarity or variations attributed to the host and geographical factors. It can prove useful in epidemiological tracing of the new cases of paratuberculosis as slow growth rate of the pathogen in artificial media hinders its epidemiological studies. The DNA from both the isolates of the fecal samples of the MAP can be genotyped. Our present study genotyped the two isolates as the 'Cattle type' strains. Hence, this technique can be used as rapid and an easy alternative to genotype the strains as compared to the other cumbersome techniques like PFGE and RFLP which require larger quantities of good quality DNA, which is difficult in case of slow growing *Mycobacterium avium* subspecies *paratuberculosis*.

Conclusion

IS1311 PCR followed by restriction enzyme analysis of DNA extracted from fecal samples revealed that both isolates of *Mycobacterium avium* subspecies *paratuberculosis* belong to the cattle ('C') strain type, and sequence and phylogenetic analyses showed that these isolates share high sequence identity with each other and with previously reported strains, particularly a Chinese MAP isolate with 99.3 % identity, highlighting their close genetic relatedness. These findings contribute to the understanding of MAP genetic profiles in ruminants and emphasize the importance of combining conventional and molecular approaches for accurate detection and characterization of MAP in epidemiological studies.

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