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Molecular Detection of *Mycobacterium avium paratuberculosis* by Large Sequence Polymorphisms (LSPs)

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ABSTRACT

Background: *Mycobacterium avium subspecies paratuberculosis* is a significant pathogen causing Johne's disease. Rational evaluation of the prevalence of this organism in animals has been restricted by the lack of accurate diagnostic techniques. DNA microarray-based and computational study of *Mycobacterium avium* subsp. *paratuberculosis* strain K10 discovered 17 distinct large sequence polymorphisms present. Of these 17 LSP^{P}s, 7 were with a specificity of > 98% and in some cases, sensitivity of up to 95% suggesting that the LSPs are ideal for diagnosing *M. avium subsp. paratuberculosis*.

Methods: A total of 218 fecal samples were collected from cattle (n=180) and buffaloes (n=38) for detection of *M. avium subsp. paratuberculosis* (MAP) using Large Sequence Polymorphisms (LSPs) and *IS900* PCR. The fecal samples were subjected to Ziehl-Neelsen staining, isolation and PCR. A total of 90 fecal samples were found ZN staining positive and were subjected to isolation of MAP, growths were seen in two fecal samples after 16 weeks of incubation.

Result: Out of 218 fecal samples tested, 7 samples were positive by both LSP PCR *viz*; LSP^{P}2 (MAP0284), LSP^{P}4 (MAP0865), LSP^{P}11(MAP2154) LSP^{P}12 (MAP2182c), LSP^{P}12 (MAP2188c), LSP^{P}15 (MAP3774), LSP^{P}16 (MAP3815) and *IS900* sequence with an amplicon size of 600, 597, 375, 430, 719, 621, 611 and 229 bp. DNA was also extracted from the isolates (n=2) and were confirmed by PCR targeting Large Sequence Polymorphism (LSPs) and *IS900*. Detection of MAP in all the 7 fecal samples both by IS900 and by 7 LSP^{P}s indicates the detection potential of selected 7 LSP^{P}s (LSP^{P}2, LSP^{P}4, LSP^{P}11, LSP^{P}12 (MAP2182c), LSP^{P}12 (MAP2188c), LSP^{P}15, LSP^{P}16) in fecal samples of cattle and buffaloes suspected for Johne's disease.

Key words: Large Sequence Polymorphisms (LSPs) and *IS900* PCR, *Mycobacterium avium* subsp. *paratuberculosis* (MAP).

INTRODUCTION

Johne's disease (JD), a chronic granulomatous enteritis that affects ruminants worldwide and has a severe negative impact on productivity and results in major economic loss, is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It is an intestinal disease affecting both domestic and wild ruminants (Singh *et al*., 2014). It is contagious, persistent and frequently fatal. Clinically affected animals exhibit wasting and emaciation symptoms. The most typical route for *M. paratuberculosis* transmission is through the fecal-oral pathway. The quantity of organisms in the exposure and the animal's age has an effect on the intensity and rate at which the disease develops. When substantial faecal shedding is observed in late stages of disease, other mechanisms of transmission, such as transplacental and direct excretion in milk and colostrum, should not be disregarded and should be of special concern (Roller *et al*., 2020). PCR techniques that target the IS900 insertion element are the most common approach for MAP detection in faecal samples. This method is frequently used as a diagnostic marker for *M. avium subsp. paratuberculosis.* With a frequency of 14 to 18 copies per genome seen in the genomes of *M. avium subsp. paratuberculosis*, multicopy insertion elements known as *IS900* elements offer increased sensitivity for their detection (Bull *et al*., 2000).

Numerous polymorphic genomic regions have been identified through genome sequencing research that may be specific to the diagnosis of *M. avium* subsp. *paratuberculosis* known as Large Sequence Polymorphisms (LSPs). These Large Sequence Polymorphisms (LSPs), which are regions that are specific to *M. avium* subsp. *paratuberculosis* strain K10 that have been found so far, range in size from 2.9 to 66 kb and can be employed in nucleic acid-based diagnostic assays. A 38-kb fragment is defined as a *M. avium* subsp. *paratuberculosis*-specific putative pathogenicity island and contains a 19-kb sequence dubbed LSP^P 12 as well as a 98-kb stretch of the genome that includes elements called LSP^P 14 and LSP^P 15 (Stratmann *et al*., 2004) and these can be used in diagnosis of MAP.

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MATERIALS AND METHODS

The experiment was conducted in year 2021-2022 in department of veterinary microbiology, GADVASU, Ludhiana. Fecal samples (n=218) were taken from cattle (n=180) and buffaloes (n=38) from organized dairy farms in the state of Punjab that had a history of or occurrence of cases of chronic intermittent diarrhea. Following the collection of faecal samples, all of the samples were stained using the Ziehl Neelsen (acid fast) staining method for microscopic analysis. The samples were categorized positive or negative after staining based on the number of bacilli per High power (HP) field on the smears: 1+ (1-5 bacilli per HP field), 2+ (6–10 bacilli per HP field), 3+ (11-15 bacilli per HP field) and 4+ (>15 bacilli per HP field).

Isolation of MAP from fecal samples

After the initial screening of fecal samples with Ziehl Neelsen (acid fast) staining, the highly positive acid-fast bacilli (AFB) positive fecal samples were treated with 0.95% Hexadecyl pyridinium chloride (HPC) for decontamination and then were inoculated on Middlebrook 7H10 media slants supplemented with mycobactin J (1 µg/ml) (OIE Terrestrial Manual, 2014). After inoculation, the slants were incubated at 37°C and were periodically observed for a period of 6 months (Quinn and Carter, 1994). The observed growth if any, was stained with acid fast stain and DNA was extracted from the samples as well as isolates using a commercially available DNA extraction kit (Qiagen).

Polymerase chain reaction

Large sequence polymorphism (LSP)-targeting primer sets, including LSP^P 2 (MAP0284), LSP^P 4 (MAP0865), LSP^P 11 (MAP2154), LSP^P 12 (MAP2182c), LSP^P 12 (MAP2188c), LSP^P 15 (MAP3774), LSP^P 16 (MAP3815) (Table 1) and *IS900* sequence (Table 2), were used to amplify the extracted DNA from faecal samples (n=218) (Semret *et al*., 2005 and Vary *et al*., 1990). Each PCR experiment employed a 25 µl volume that contained 12.5 µl of GoTaq® Green Master Mix (2X), 1 µl of forward primers (25 pmol), 1 µl of reverse primers (25 pmol), 8 µl of DNA template and 2.5 µl of nuclease-free water. Along with all of the test samples, a positive control *Mycobacterium avium* subsp. *paratuberculosis* (K10) (IMTECH, Chandigarh) DNA was also amplified.

LSP amplification started with a denaturation stage at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds and extension at 72°C for 2 minutes (Semret *et al*., 2005). The cyclic conditions for IS900 amplification were as follows: an initial denaturation at 95°C for 5 minutes, 40 cycles of denaturation at 95°C for 45 seconds, primer annealing at 62°C for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 10 minutes were performed after (Vary *et al*., 1990). On a 1.5% agarose gel, the amplified PCR products were separated and the Gel Documentation System was used to display them (Alpha Innotech). PCR amplicons of 600 bp, 597 bp, 375 bp, 430 bp, 719 bp, 621 bp and 611 bp were considered positive for LSP^P 2 (MAP0284), LSP^P 4 (MAP0865), LSP^P 11 (MAP2154), LSP^P 12 (MAP2182c), LSP^P 12 (MAP2188c), LSP^P 15 (MAP3774), LSP^P 16 (MAP3815) and PCR amplicons of 229 bp were considered positive for *IS900*.

RESULTS AND DISCUSSION

In this experiment a total of 218 fecal samples from cattle and buffaloes with a history or incidence of cases of chronic intermittent diarrhea were screened out of which 90 animals were detected positive by acid-fast staining and findings were interpreted based on the number of acid-fast bacilli per HP field. These 90 ZN staining positive fecal samples were subjected to isolation of MAP, growths were seen in two fecal samples after 16 weeks of incubation and the colonies were diffused greyish and off white. The isolates from these tubes were further subjected to acid fast staining which showed acid-fast bacilli under 100x oil emulsion.

Molecular detection of MAP by large sequence polymorphism (LSPs) and IS900 PCR

DNA extracted from fecal samples as well as isolates using QIAmp DNA Purification kit was amplified using different sets of primers targeting large sequence polymorphism (LSP^Ps) *viz*; LSP^P2 (MAP0284), LSP^P4 (MAP0865), LSP^P11 (MAP2154)

Table 1: Primer sequence used in LSPs for MAP detection.

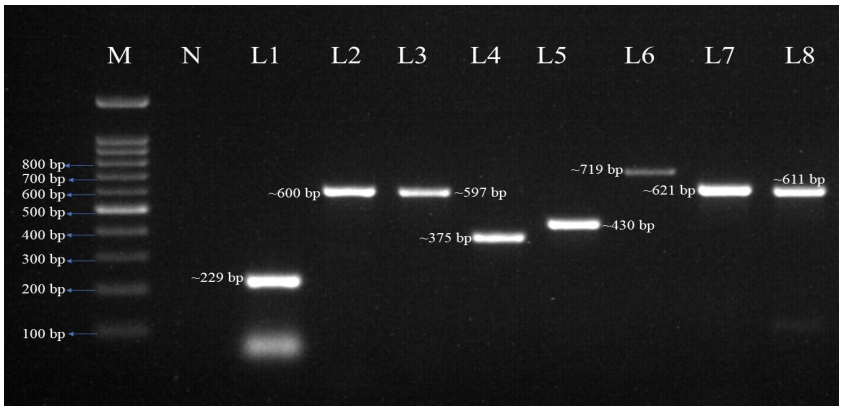
Sequence	PCR target	Forward primer	Reverse primer	Product size (bp)
LSP^P2	MAP0284	CCAGTTACCTTCGACGAGGA	CTCTGTTGGATTCCCGTTTG	600
LSP^P4	MAP0865	TCCTCTCCTTCGTCACCAAC	ATCTCAGACAGTGGCAGGT	597
LSP^P11	MAP2154	GTCGGACCAGGGTTGAGAT	GGATAAGGCCGCATACAAAC	375
LSP^P12	MAP2182c	TCTGAACCCGGCTACACAC	CATGCCGGTGTGAGTACAA	430
	MAP2188c	GCCCGTAGAAGTGGACGAT	GATGACGGTCGCCAATCC	719
LSP^P15	MAP3774	AGCAGTGGACGAGGCAAC	GAGGGCGTAGAACTCTGTG	621
LSP^P16	MAP3815	GTAATGGCGCATTCTCTGGT	CCCTTGATCTCGACACTGGT	611

Table 2: Primer sequence used in *IS900* for MAP detection.

Primer	Sequence	Product size
Forward (IS900/150C)	5′-CCG CTA ATT GAG AGA TGC GAT TGG-3′	229 bp
Reverse (IS900/921)	5′-AAT CAA CTC CAG CAG CAG CGC GGC CTC G-3′	

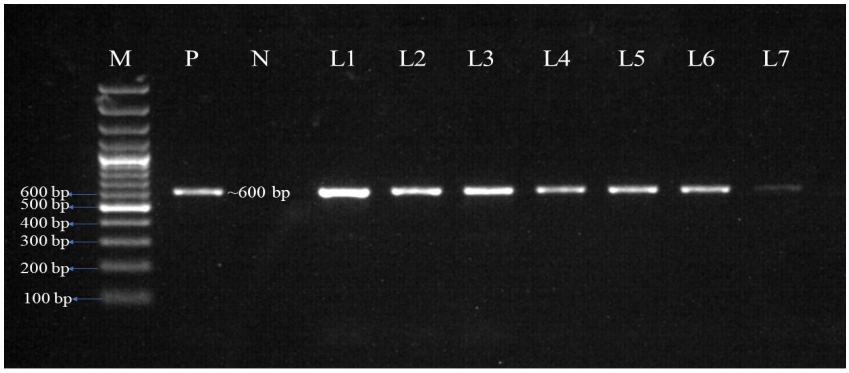
LSP^{P}12 (MAP2182c), LSP^{P}12 (MAP2188c), LSP^{P}15 (MAP3774), LSP^{P}16 (MAP3815) and *IS900* sequence (Semret *et al*., 2005 and Vary *et al*., 1990) with an amplicon size of 600, 597, 375, 430, 719, 621, 611 and 229 bp. DNA extracted from the standard culture of MAP was used as positive control in all the PCR reactions and the primers were initially standardized using standard culture DNA (Fig 1). Out of 218 fecal samples tested, 7 samples were positive by both LSP PCR (Fig 2, 3, 4, 5, 6, 7 and 8) and *IS900* PCR (Fig 10). DNA was also extracted from the isolates (n=2) were confirmed by PCR targeting Large Sequence Polymorphism (LSPs) (Fig 9) and *IS900* (Fig 10).

Various methods, such as fecal smears, fecal culture and PCR, can be used to diagnose paratuberculosis in feces samples. Goal of present study was to use culture and molecular approaches to detect *Mycobacterium avium* subsp. *paratuberculosis* (MAP). In the chronic stage of infection, MAP organisms are shed in feces, hence fecal samples can be examined to diagnose paratuberculosis. For the initial screening of MAP, Ziehl–Neelsen staining can be used (Rathnaiah *et al*., 2017). The number of acid-fast bacteria present in the sample determines the success of microscopic screening of feces (Ris *et al*., 1988). Although acid-fast staining is the simplest, fastest and most cost-



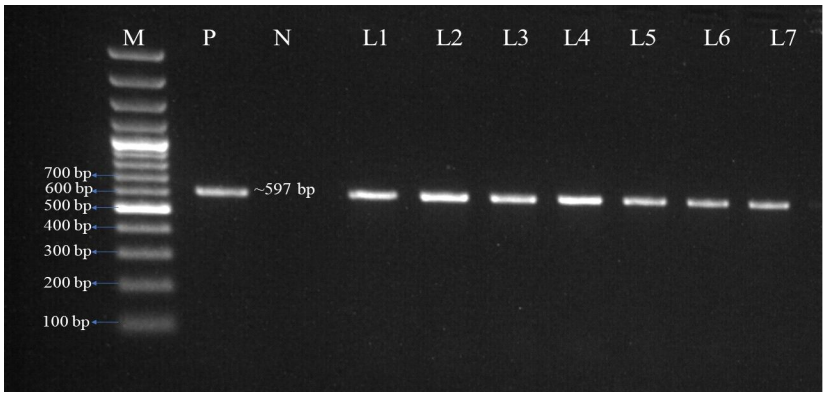
M: Marker (100 bp DNA ladder), N: Negative control.
L1=Positive standard IS900 PCR (229bp).
L2= Positive standard for LSP^{P}2 (MAP0284) (600 bp).
L3= Positive standard for LSP^{P}4 (MAP0865) (597 bp).
L4= Positive standard for LSP^{P}11 (MAP2154) (375 bp).
L5= Positive standard for LSP^{P}12 (MAP2182c) (430 bp).
L6= Positive standard for LSP^{P}12 (MAP2188c) (719 bp).
L7= Positive standard for LSP^{P}15 (MAP3774) (621 bp).
L8= Positive standard for LSP^{P}16 (MAP3815) (611 bp).

Fig 1: Agarose gel electrophoresis showing an amplicon of ~ 229 bp, 600 bp, 597 bp, 375 bp, 430 bp, 719 bp, 621 bp and 611 bp from standard culture of MAP (K10).



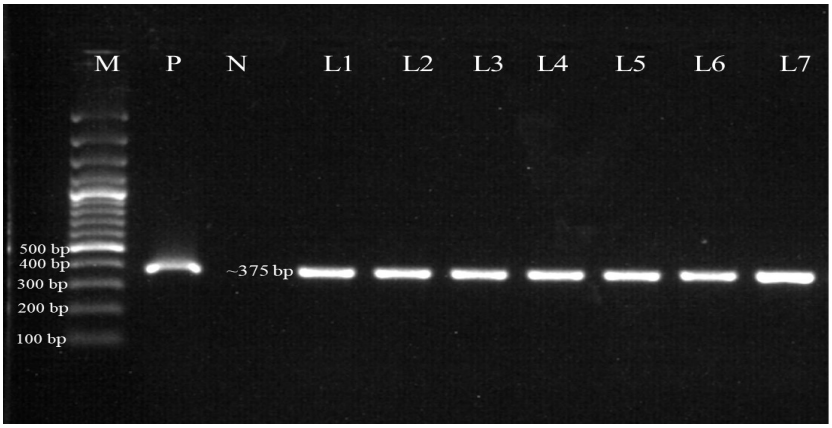
M: Marker (100 bp DNA ladder), N: negative control.
P= Positive standard of LSP^{P}2 (MAP0284), N= Negative control.
L1, L2, L3, L4, L5, L7: Positive sample for MAP from fecal samples.

Fig 2: Agarose gel electrophoresis showing an amplicon of ~ 600 bp of MAP from fecal samples targeting LSP^{P}2 (MAP0284).



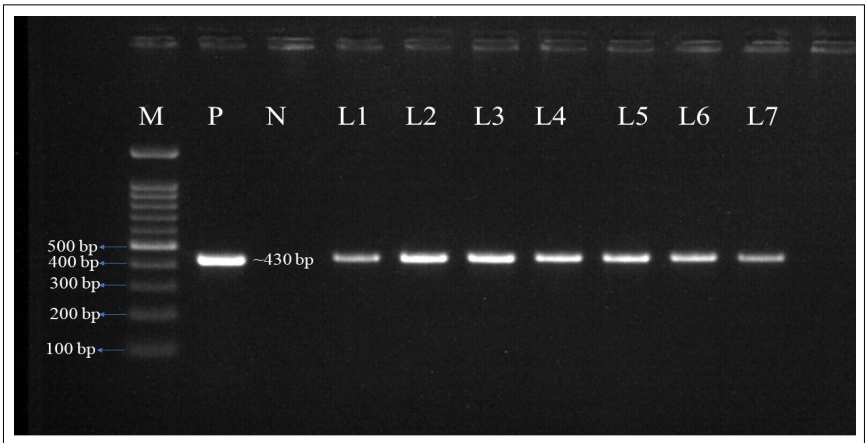
M: Marker (100 bp DNA ladder), N: Negative control.
P= Positive standard of LSP^{P}4 (MAP0865), N= Negative control.
L1, L2, L3, L4, L5, L7: Positive sample for MAP from fecal samples.

Fig 3: Agarose gel electrophoresis showing an amplicon of ~ 597 bp of MAP from fecal samples targeting LSP^{P}4 (MAP0865).



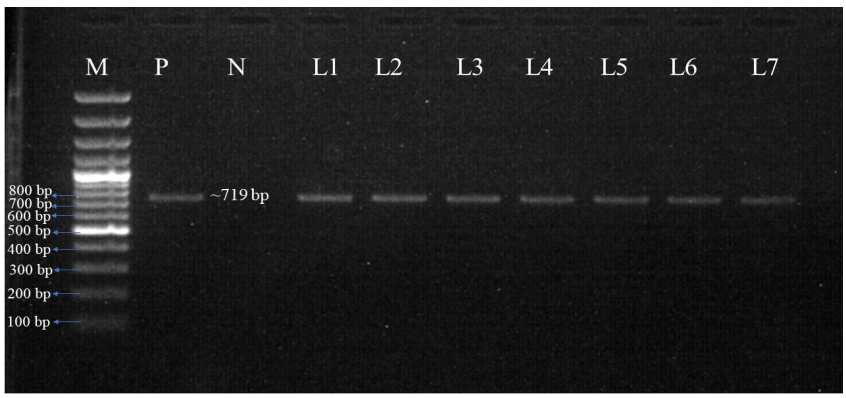
M: Marker (100 bp DNA ladder), N: Negative control.
P= Positive standard of LSP^{P}11 (MAP2154), N= Negative control.
L1, L2, L3, L4, L5, L7: Positive sample for MAP from fecal samples.

Fig 4: Agarose gel electrophoresis showing an amplicon of ~ 375 bp of MAP from fecal samples targeting LSP^{P}11 (MAP2154).



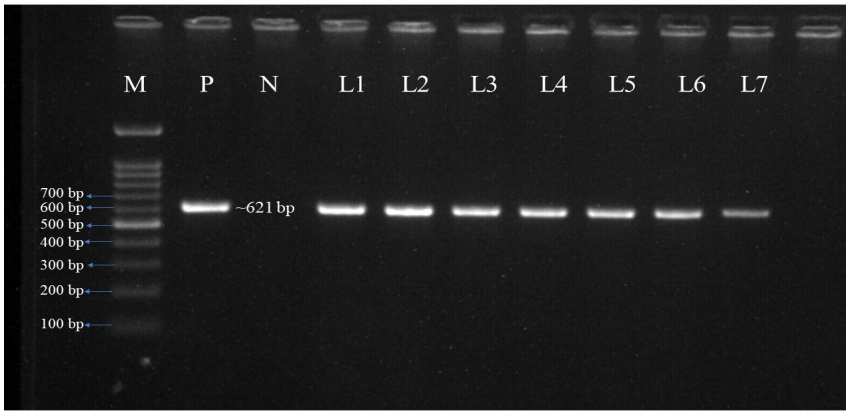
M: Marker (100 bp DNA ladder), N: Negative control.
P= Positive standard of LSP^{P}12 (MAP2182c), N= Negative control.
L1, L2, L3, L4, L5, L7: Positive sample for MAP from fecal samples.

Fig 5: Agarose gel electrophoresis showing an amplicon of ~ 30 bp of MAP from fecal samples targeting LSP^{P}12 (MAP2182c).



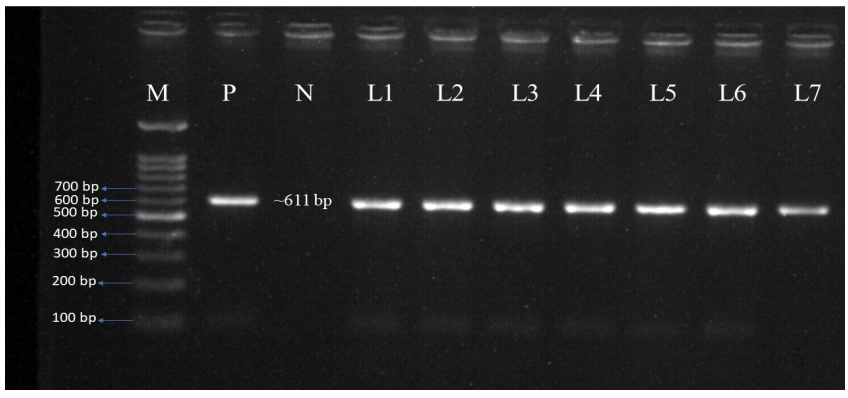
M: Marker (100 bp DNA ladder), N: Negative control
P= Positive standard of LSP^{P}12 (MAP2188c), N= Negative control.
L1, L2, L3, L4, L5, L7: Positive sample for MAP from fecal samples.

Fig 6: Agarose gel electrophoresis showing an amplicon of ~ 719 bp of MAP from fecal samples targeting LSP^{P}12 (MAP2188c).



M: Marker (100 bp DNA ladder), N: Negative control
P= Positive standard of LSP^{P}15 (MAP3774), N= Negative control.
L1, L2, L3, L4, L5, L7: Positive sample for MAP from fecal samples.

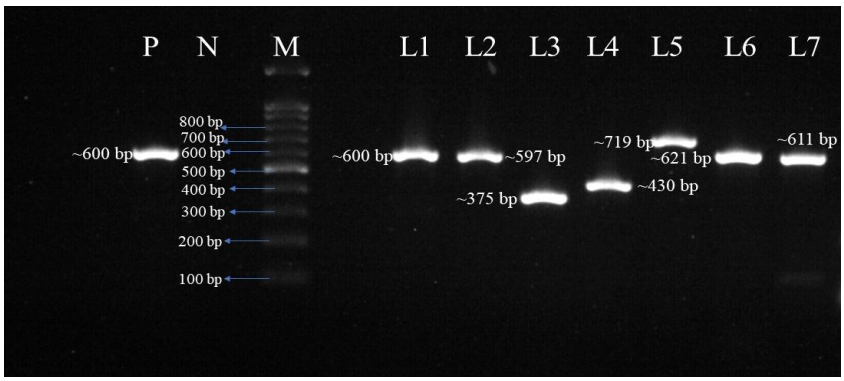
Fig 7: Agarose gel electrophoresis showing an amplicon of ~ 621 bp of MAP from fecal samples targeting LSP^{P}15 (MAP3774).



M: Marker (100 bp DNA ladder), N: Negative control.
P= Positive standard of LSP^{P}16 (MAP3815), N= Negative control.
L1, L2, L3, L4, L5, L7: Positive sample for MAP from fecal samples.

Fig 8: Agarose gel electrophoresis showing an amplicon of ~ 611 bp of MAP from fecal samples targeting LSP^{P}16 (MAP3815).

effective way of diagnosis, its specificity and sensitivity are limited because it is difficult to distinguish between different members of the family Mycobacteriaceae and other acid-fast bacteria (Manning and Collins, 2001). Although acid-fast staining is of limited and benefits in the early stages of infection, it can be helpful in diagnosing the disease as it progresses. According to Whitlock *et al*. (2000), the majority of Johne's disease-affected animals were moderate shedders. The "gold standard" for JD diagnosis is the isolation of MAP by culturing (OIE, 1996). The fact that MAP requires mycobactin J to grow in Middle brook media can be used to distinguish it from other acid-fast bacteria. Because, MAP grows slowly (12-16 weeks for colony formation on solid media), culture-based diagnosis takes a long period but it is considered more sensitive than most other detection procedures (Ellingson *et al*., 2000). But the decontamination technique has been found to be damaging to MAP growth. The decontamination procedures followed during the sample processing for isolation also decreases the number of viable mycobacteria (Whittington and Sergeant, 2001). Decontamination of samples with low concentrations of MAP may result in a negative culture result



M: Marker (100 bp DNA ladder), P: Positive control, N: Negative control.

L1= Positive for LSP^{P}2 (MAP0284) (600 bp).

L2= Positive for LSP^{P}4 (MAP0865) (597 bp).

L3= Positive for LSP^{P}11 (MAP2154) (375 bp).

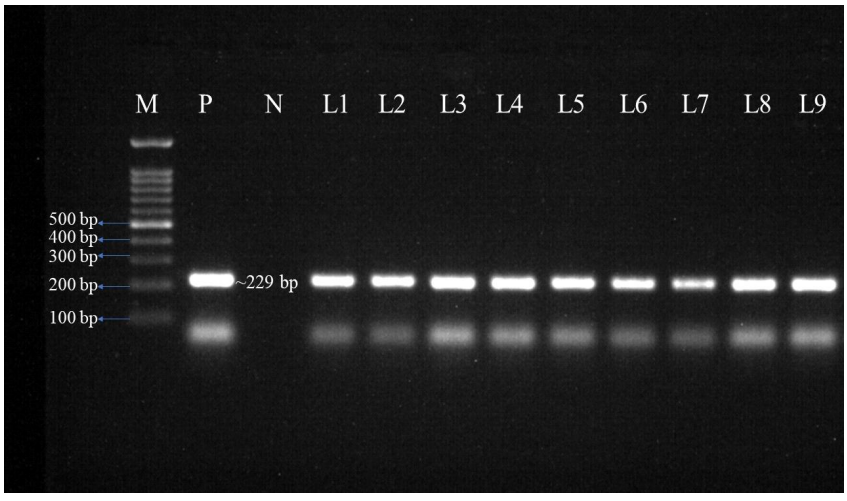
L4= Positive for LSP^{P}12 (MAP2182c) (430 bp).

L5= Positive for LSP^{P}12 (MAP2188c) (719 bp).

L6= Positive for LSP^{P}15 (MAP3774) (621 bp).

L7= Positive for LSP^{P}16 (MAP3815) (611 bp).

Fig 9: Agarose gel electrophoresis showing an amplicon of MAP isolate from fecal sample.



M: Marker (100 bp DNA ladder), N: Negative control.

P= Positive standard of IS900 PCR (229bp), N= Negative control.

L1, L2, L3, L4, L5, L6, L7: Positive sample for MAP from fecal samples.

L8 and L9: Positive isolates for MAP from fecal samples.

Fig 10: Agarose gel electrophoresis showing an amplicon of ~ 229 bp of MAP from fecal samples and isolates.

(Clark *et al*., 2008). Overgrowth of contaminating bacteria and fungus pose a problem, while culturing the samples. All of these variables make isolation of MAP colonies using culture techniques problematic, resulting in lower viable MAP recovery and, as a result, fewer culture positive animals. According to reports, fecal culture can only detect upto 15 to 25% of sub-clinically infected animals (Stabel, 1998). As a result, a highly sensitive and quick PCR-based test was employed to identify MAP in clinical samples. PCR IS900 insertion element is specific to MAP (Chaubey *et al*., 2016). With fecal samples, PCR tests are approximately 70% sensitive and 85% specific (Clark *et al*., 2008).

In a comparative genome study conducted by Semret *et al*. (2005) to detect the genomic differences between *M. avium* subsp. *paratuberculosis* and its close relative *M. avium* subsp. *avium,* two types of large sequence polymorphisms (LSPs): those present in the *M. avium* subsp. *avium* but missing in the *M. avium* subsp. *paratuberculosis* (LS) and those only present in the *M. avium* subsp. *paratuberculosis* (LS) were found. Distribution of 3 LS and 17 LS across a panel of 383 *M. avium* complex isolates was examined for their potential use for accurate diagnostic test development. Of the 17 LS, only 7 (LSP^P2 (MAP0284), LSP^P4 (MAP0865), LSP^P11(MAP2154) LSP^P12 (MAP2182c), LSP^P12 (MAP2188c), LSP^P15 (MAP3774), LSP^P16 (MAP3815) were seen highly specific (>98%) and in some cases highly sensitive (up to 95%) for *M. avium* subsp. *paratuberculosis* indicate the LSPs best suited for *M. avium* subsp. *paratuberculosis* diagnostics. Additionally, Paustian *et al*. (2005) discovered genetic differences between *M. avium* subsp. *paratuberculosis* and other *M. avium* complex organism and described seven regions unique to only *M. avium* subsp. *paratuberculosis* (LSP^P2 (MAP0284), LSP^P4 (MAP0865), LSP^P11(MAP2154) LSP^P12 (MAP2182c), LSP^P12 (MAP2188c), LSP^P15 (MAP3774), LSP^P16 (MAP3815). Sohal *et al*. (2009) conducted a study in which different markers such as IS900, LSPs, were used to characterize MAP 'Bison type' S5 and this study showed the polymorphic profile for LSP^P 4 was also seen in MAP 'Bison type' S5. LSP^P 2, LSP^P 4, LSP^P 12 and LSP^P 15 amplified showing their presence in MAP S5.

Early detection of paratuberculosis can be aided by molecular detection technologies. The development of PCR technologies has increased the specificity and sensitivity of laboratory diagnosis (Vary *et al*., 1990). The insertion *IS900* is thought to be exclusive to *Mycobacterium avium* subsp. *paratuberculosis* and can be used for detection of MAP in fecal samples. Vary *et al*. (1990) also reported the presence of mycobacterial insertion sequence *IS900* in multiple copies and the DNA probes that hybridize to this sequence were found to be highly specific for MAP and synthesized primers for *IS900* DNA sequences. This resulted in highly specific direct detection of MAP DNA in feces from cattle with Johne's disease. The results of bacterial culture and *IS900*-specific PCR were also compared by Sivakumar *et al*. (2005) to detect *M. avium* subsp. *paratuberculosis* (MAP) from the intestinal and mesenteric lymph node tissues of water buffaloes with paratuberculosis (JD) lesions and found PCR to be more sensitive than bacterial culture in detecting subclinical paratuberculosis. Efficacy of fecal culture and IS900 Polymerase chain reaction (PCR) assay was compared by Soumya *et al*. (2009) in 40 fecal samples of dairy cattle and found the PCR assay to be more sensitive 90% (36/40) than fecal culture 52.5% (21/40).

CONCLUSION

Detection of MAP in all the 7 fecal samples both by IS900 and by 7 LS indicates the detection potential of selected 7 LS (LSP^P2, LSP^P4, LSP^P11, LSP^P 12 (MAP2182c), LSP^P 12 (MAP2188c), LSP^P15, LSP^P16) in fecal samples of cattle and buffaloes suspected for JD. They can also have a potential use for the development of an accurate diagnostic test.

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Conflict of interest: None.

REFERENCES

Bull, T.J., Taylor, H.J., Pavlik, I., Zaatari, F. and Tizard, M. (2000). Characterization of IS900 loci in *Mycobacterium avium* subsp. *paratuberculosis* and development of multiplex PCR typing. Microbiology. 146: 2185-97.

Chaubey, K.K., Gupta, R.D., Gupta, S., Singh, S.V., Bhatia, A.K., Jayaraman, S., Kumar, N. *et al*. (2016). Trends and advances in the diagnosis and control of paratuberculosis in domestic livestock. Veterinary Quarterly. 36: 203-27.

Clark, J.D.L., Koziczkowski, J.J., Radcliff, R.P., Carlson, R.A. and Ellingson, J.L.E. (2008). Detection of *Mycobacterium avium* subspecies *paratuberculosis*: Comparing fecal culture versus serum enzyme-linked immunosorbent assay and direct fecal polymerase chain reaction. Journal of Dairy Science. 91(7): 2620-7. DOI: 10.5281/zenodo. 3979943.

Ellingson, J.L., Stabel, J.R., Bishai, W.R., Frothingham, R. and Miller, J.M. (2000). Evaluation of the accuracy and reproducibility of a practical PCR panel assay for rapid detection and differentiation of *Mycobacterium avium* subspecies. Molecular and Cellular Probes. 14: 153-61.

Manning, E.J. and Collins, M.T. (2001). *Mycobacterium avium* subsp. *paratuberculosis*: Pathogen, pathogenesis and diagnosis. Revue Scientifique et Technique. 20: 133-50.

Office International Des Epizooties, (1996). Paratuberculosis (Johne's disease). Section 3.1, Chapter 3.1.6, OIE, Paris, France.

OIE Terrestrial Manual. (2014). Chap 2.1.15 - Paratuberculosis (Johne's Disease).

Paustian, M.L., Kapur, V. and Bannantine, J.P. (2005). Comparative genomic hybridizations reveal genetic regions within the *Mycobacterium avium* complex that are divergent from *Mycobacterium avium* subsp. *paratuberculosis* isolates. Journal of Bacteriology. 187(7): 2406-415.

Quinn, P.J., Carter, M.E., Merkey, B. and Carer, G.R. (1994). Clinical Veterinary Microbiology. Mosby, Wolfe Publishing.

Rathnaiah, G., Zinniel, D.K., Bannantine, J.P., Stabel, J.R., Gröhn, Y.T., Collins, M.T. and Barletta, R.G. (2017). Pathogenesis, Molecular Genetics and Genomics of *Mycobacterium avium* subsp. *paratuberculosis*, the etiologic Agent of Johne's Disease. Frontiers in Veterinary Science. 4: 187. https://doi.org/10.3389/fvets.2017.00187.

Ris, D.R., Hamel, K.L. and Ayling, J.M. (1988). The detection of Mycobacterium paratuberculosis in bovine feces by isolation and the comparison of isolation with the examination of stained smears by light microscopy. New Zealand Veterinary Journal. 36: 112-14.

Roller, M., Hansen, S., Knauf-Witzens, T., Oelemann, W.M.R., Czerny, C.P., Wahed, A.A.E. and Goethe, R. (2020). *Mycobacterium avium* subspecies *paratuberculosis* infection in zoo animals: A review of susceptibility and disease process. Frontiers in Veterinary Science. 7: 24. doi: 10.3389/fvets.2020. 572724.

Semret, M., Alexander, D.C., Turenne, C.Y., de Haas, P., Overduin, P., van Soolingen, D., Cousins, D. and Behr, M.A. (2005). Genomic polymorphisms for *Mycobacterium avium* subsp. *paratuberculosis* diagnostics. Journal of Clinical Microbiology. 43(8): 3704-12.

Singh, S.V., Gupta, S., Chaubey, K.K., Rawat, K.D., Kumar, N., Sohal, J.S., Singh, S., Tiwari, R., Chakraborty, S. and Dhama, K. (2014). Johne's disease (JD) in a high yielding Holstein Friesian cattle dairy farm in India. Journal of Biological Sciences. 14(3): 195-3.

Sivakumar, P., Tripathi, B.N. and Singh, (2005). Detection of *Mycobacterium avium* subsp. *paratuberculosis* in intestinal and lymph node tissues of water buffaloes (Bubalus bubalis) by PCR and bacterial culture. Veterinary Microbiology. 108: 263-70.

Sohal, J.S., Sheoran, N., Narayanasamy, K., Brahmachari, V., Singh, S. and Subodh, S. (2009). Genomic analysis of local isolate of *Mycobacterium avium* subspecies *paratuberculosis*. Veterinary Microbiology. 134: 375-382.

Soumya, M.P., Pillai, R.M., Antony, P.X., Mukhopadhyay, H.K. and Rao, V.N. (2009). Comparison of faecal culture and IS900 PCR assay for the detection of Mycobacterium avium subsp. paratuberculosis in bovine faecal samples. Veterinary Research Communications. 33(7): 781- 91.

Stabel, J.R. (1998). Johne's disease: A hidden threat. Journal of Dairy Sciences. 81(1): 283-88. doi: 10.3168/jds.S0022-0302(98)75577-8.

Stratmann, J., Strommenger, B., Goethe, R., Dohmann, K., Gerlach, G.F., Stevenson, K., Li, L.L., Zang, Q., Kapur, T. and Bull, T.J. (2004). A 38-kilobase pathogenicity island specific for *Mycobacterium avium* subsp. *paratuberculosis* encodes cell surface proteins expressed in the host. Infection and Immunity. 72: 1265-74.

Vary, P.H. anderson, P.R., Green, E., Taylor, J.H. and McFadden, J.J. (1990). Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. Journal of Clinical Microbiology. 28: 933-37.

Whitlock, R.H., Wells, S.J., Sweeney, R.W. and Van Tiem, J. (2000). ELISA and fecal culture for paratuberculosis (Johne's disease): Sensitivity and specificity of each method. Veterinary Microbiology. 77: 387-98.

Whittington, R.J. and Sergeant, E.S. (2001). Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp. *paratuberculosis* in animal populations. Australian Veterinary Journal. 79(4): 267-78.