

Faecal Culture and IS900 PCR Assay for the Detection of *Mycobacterium avium* subsp. *paratuberculosis* in Bovine Faecal Samples

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Mycobacterium avium subsp. *paratuberculosis* is a pathogen that causes Johne's disease in animals and is implicated in Crohn's disease in humans. Culture of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) from faeces has been considered the gold standard for the diagnosis of paratuberculosis for many years. However, direct faecal polymerase chain reaction (PCR) is becoming more widely used, demonstrating similar sensitivity and specificity to culture. In the present study, faecal culture and IS900 Polymerase chain reaction (PCR) assay of faecal samples was done on 200 clinically suspected cases of Johne's disease in dairy cattle. One isolates appeared only on the mycobactin J supplemented media at 8–16 weeks post-inoculation. A total of 7 faecal samples out of 200 samples were detected positive by IS900 PCR assay for *Mycobacterium avium* subsp. *paratuberculosis* (MAP) yielding an expected product of size 229 bp. The sensitivity of the IS900 PCR was assessed by making ten fold serial dilutions of the known concentration (5 ng/μl) of the standard genomic DNA of MAP. The detection limit of the IS900 PCR was upto 5 pg/μl.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*, faecal culture, IS900 PCR.

Paratuberculosis (Johne's disease) is a chronic enteric progressive disease, characterized clinically by chronic or intermittent diarrhea, emaciation, and death. The disease has worldwide distribution and economic impact on ruminant livestock production (Stabel, 1998). The disease is considered one of the most important diseases of ruminant population (Tripathi *et al* 2002 and Sivakumar *et al* 2005). Johne's disease cause huge economic losses and has high impact on dairy and beef cattle industry due to premature culling of animals, reduced weight gain, reduced feed

efficiency, reduced carcass value, reduced milk production, increased susceptibility to mastitis and reproductive disorders leading to increased calving intervals, reduced fertility and additional veterinary costs (Hasonova and Pavlik 2006). *Mycobacterium avium* subsp. *paratuberculosis* is biochemically relatively inactive and identification is currently based on mycobactin dependency and detection of the insertion sequence IS900 by the means of the polymerase chain reaction (Collins *et al* 1989).

Faecal culture may remain the most definitive test to date for identification of both sub-clinically and clinically infected cattle but the prolonged incubation period (12- 16 weeks) required for accurate diagnosis was a distinct disadvantage (Stabel and Whitlock 2001). In addition, the need of a specialized medium for

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propagation of the organism and frequent contamination is often a problem when culturing MAP from faecal specimens. Despite some of these disadvantages, faecal culture is still considered as the “gold standard” diagnostic test for the detection of Map (Sweeney *et al* 1992). It is the only test that does not produce false positive results (100% specificity) (OIE 2004). Although the ‘gold standard’ for detection and confirmation of viable or more specifically culturable. MAP remains isolation of the organism in culture, PCR has made rapid detection of this fastidious, slow-growing organism possible in milk, faeces and clinical samples (Grant *et al* 2000, Pillai and Jayarao 2002 and Fang *et al* 2002).

The most widely used target gene for detection of MAP IS900 was first described in 1989 (Green *et al* 1989) and presently considered specific for MAP. The MAP genome has 15 to 20 copies of the insertion element, and the sequenced strain K-10 has 17 copies (Li *et al* 2005). This high copy number target gives an increased sensitivity compared to systems targeting single copy genes, which makes it popular in molecular diagnostic methods for paratuberculosis. IS900 has a unique nucleotide sequence which can be specifically detected by hybridization or PCR techniques (Collins *et al* 1993 and Moss *et al* 1992). Sequences of IS900 proved to be highly sensitive and specific markers of MAP among other slowly growing, acid-fast bacteria, because IS900 has been detected in all reference and vaccine strains as well as in field isolates of MAP from several hosts but never in other bacterial species (Ahrens *et al* 1995 and Whipple *et al* 1990).

Since Johne’s disease is so insidious within an animal population, early diagnosis and proper management are considered to be the most useful tools for controlling the disease within domestic livestock herds. Keeping the above points in mind the present study was planned with the objective to detect *Mycobacterium avium*

subsp. paratuberculosis in bovine fecal samples using faecal culture and IS900 PCR assay.

MATERIALS AND METHODS

Collection of samples

The present study was carried out to diagnose paratuberculosis using faecal culture and PCR assay on the DNA extracted from faecal samples of cattle and buffaloes suspected for infection with *Mycobacterium avium* subsp. *paratuberculosis* on the basis of history of chronic intermittent diarrhoea, emaciation and cachectic condition. Faecal (n=200) samples from cattle and buffaloes with a history of chronic intermittent diarrhoea were collected from dairy farms in Ludhiana, Punjab.

Laboratory Techniques

Isolation of MAP from faecal samples

Attempts were made to isolate *Mycobacterium avium* subsp. *paratuberculosis* (MAP) by inoculation of the AFB positive faecal samples on Middlebrook 7H10 media supplemented with mycobactin J (1¼g/ml) (OIE Terrestrial Manual 2008) after following appropriate decontamination procedure by Merkai and Richards (1972). The cultures were incubated at 42° C and were observed periodically for a period of 6 months post inoculation and the growth observed was confirmed by IS900PCR using phenol-chloroform method of DNA extraction (van Embden *et al* 1993).

DNA Extraction

The extraction of genomic DNA from faecal samples was done using commercially available bacterial genomic DNA Isolation Kit (HiPurA DNA Purification kit) as per the manufacturer’s instructions.

IS900 PCR Protocol

DNA was amplified by PCR using Primers based on IS900 sequence (Table I). PCR was performed as per Vary *et al* (1990) with some

Table 1. Sequences of primers used in IS900 PCR for detection of MAP (Vary *et al* 1990)

Primers	Sequence	Size of PCR product
Forward (IS900/150C)	5'- CCG CTA ATT GAG AGA TGC GAT TGG - 3'	229bp
Reverse (IS900/921)	5'- AAT CAA CTC CAG CAG CAG CGC GGC CTC G -3'	

modifications. For the amplification of the IS900 sequence PCR reaction mixture was made in a final volume of 25 μ l containing ready to use GoTaq® Green Master Mix, (Promega), forward primer (10

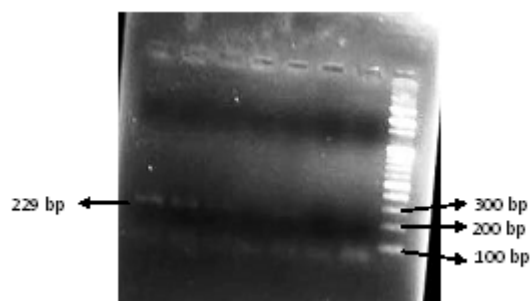


Fig. 1. Sensitivity of PCR using IS900 sequence of MAP



Fig. 2. Small raised slightly white colony of MAP

pmol), reverse primer (10 pmol), nuclease free water and DNA template. Cycling conditions were initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 45 sec, annealing of primers at 56°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min. PCR products were run by agarose gel electrophoresis and visualized in Gel Documentation System (Alpha Innotech)

Sensitivity of IS900 PCR

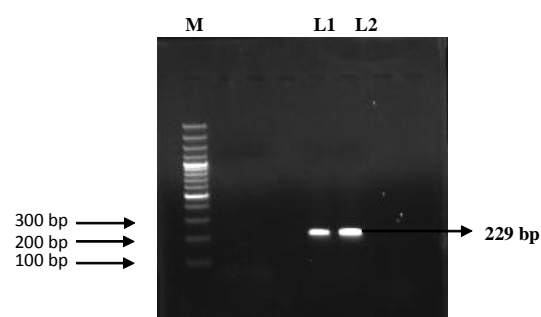
The sensitivity of the IS900 PCR was assessed by making a ten fold serial dilutions of the known concentration (5 ng/ μ l) of the standard genomic DNA of MAP. The detection limit of the IS900 was upto 5 pg/ μ l (Figure 1).

RESULT

In the present study, 200 faecal samples were processed for the isolation of MAP. After 5

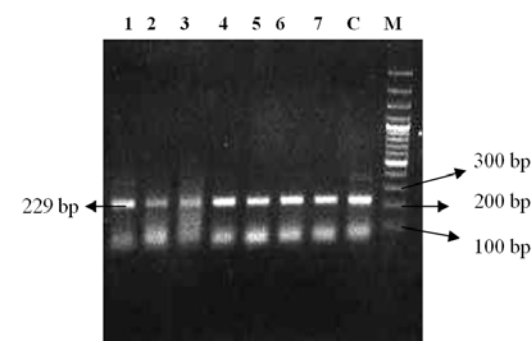


Fig. 3. Acid-fast positive MAP bacilli



M- 100 bp marker
L1- 229 bp band of isolate
L2- Control

Fig. 4. Confirmation of the MAP isolate by IS900 PCR



M-100 bp plus marker
C- control
L1-L7- 229 bp band

Fig. 5. Samples positive for MAP by IS900 PCR

months of incubation, only one faecal sample was found positive for faecal culture (Figure 2 and 3). The animal positive for faecal culture was high degree (4+) positive by ZN staining. The sample was confirmed positive by conventional PCR of the DNA extracted from the colonies (Figure 4). IS900 PCR was also employed directly on the DNA extracted from faecal samples. A total of seven faecal samples (4.29 percent) were detected positive (Figure 5) out of all the 200 faecal samples processed yielding an expected PCR product size of 229 bp.

DISCUSSION

The cultural isolation of MAP from faecal samples is considered as the gold standard (OIE, 1996) for diagnosis of MAP. However, culture, though considered more sensitive than most other detection techniques for MAP, is a slow and exacting procedure because of the long generation time of MAP organisms which may extend well beyond 12-16 weeks. Also, the decontamination procedures followed during the processing of faecal samples for culture inoculation also substantially decrease the number of viable MAP bacteria (Whittington and Sergeant 2001). In addition, contamination of culture by overgrowth of competing bacteria and fungal agents, chemical decontaminants used in isolation of MAP, intermittent shedding of acid-fast bacilli by subclinically affected animal and drying of the inoculated media due to long incubation periods is also a problem when culturing MAP from faecal specimens. All these factors make isolation of MAP colonies by culture techniques difficult with lower viable MAP recovery and lesser number of culture positive animals (Grant *et al* 2005 and Ruzante *et al* 2006).

All the faecal samples detected positive for MAP by IS900 PCR were from '3+' or '4+' ZN positive animals. The detection limit of direct PCR on faecal samples for paratuberculosis infection is stated to be around 100 organisms per g of faeces. This is similar to the detection limit for culture (Merkal *et al* 1987). Different types of samples pose different barriers to extraction and PCR. Unfortunately, faecal samples also belong to the samples that are quite difficult to process. It is the method of extraction of mycobacterial DNA from

clinical samples rather than the PCR process itself which is the chief bottleneck in detection of MAP by molecular techniques (Englund 2002). PCR is stated as a more sensitive method than culture for detection of Mycobacteria as low levels of MAP shed in the faeces may not be detected by culture and PCR allows us to detect viable as well as non-viable microorganisms (Stabel *et al* 2002).

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