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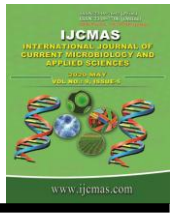

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Diagnosis of Paratuberculosis by Fecal Culture Followed by Nested-Polymerase Chain Reaction

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A B S T R A C T

Culture of clinical samples is the confirmatory test for the diagnosis of paratuberculosis but, it is time consuming due to its long incubation period, further it requires a skilled personnel to monitor the growth. Use of molecular techniques such as PCR is advantageous over the long incubation period in culturing of samples; more than eight months of incubation is needed to declare a sample to be negative. Two cattle farms (Cattle rehabilitation center and organized dairy farm) with different organizational setup were screened by culture of fecal samples. Ziehl-Neelsen (ZN) staining and nested-PCR were implied to confirm the growths on these samples after two months of inoculation on Herrold's Egg Yolk Medium (HYM). Overall, 33.67% samples confirmed the growth of *Map* bacilli and 25.00% samples confirmed the growth of acid-fast bacilli. In cattle rehabilitation center 50.00% samples confirmed the growth of *Map* bacilli and 35.63% shown to have acid-fast bacilli, whereas in the organized dairy farm it was 15.00% and 13.57%, respectively. Confirmation of growth of bacilli in samples belongs to age groups 0 - < 3 yrs and 3 - < 6 yrs were indicated, approximately same level of infection (χ^2; $p>0.05$), but significantly higher level of infection were confirmed in age group of ≥ 6 yrs (χ^2; $p<0.05$). Appreciable colonies were identified after the incubation of 7 to 9 months on cultures positive in both ZN stain and nested-PCR, but cultures negative in nested-PCR were failed to produce colonies even after incubation of one year. Study confirmed that variation in level of paratuberculosis in these two cattle farm with possible organizational set up influences. Present study concludes that, use of nested-PCR in conjunction with fecal culture reduce long incubation period to confirm *Map* bacilli and high percentage of paratuberculosis positive animals in old age group in both farms.

Introduction

Mycobacterium avium subspecies *paratuberculosis* (*Map*) causes chronic, granulomatous enteritis of ruminants, known as paratuberculosis or Johne's disease. Host acquires infection in early life via feco-oral route through the ingestion of contaminated colostrums, milk, water, or feed (Sweeney, 1996) and also possibly through intrauterine route (Seitz *et al.*, 1989). Disease can persist for several years in a subclinical phase without showing any clinical signs. However, once the disease enters the clinical phase, the wall of intestine get thickened which leads to diarrhea, weight loss, decreased milk

production and eventual death (Hasonova and Pavlik, 2006). Disease can be diagnosed by detection of viable bacilli (culture), genome (PCR), host immune response (Harris and Barletta, 2001), etc. Antibody detection techniques such as enzyme-linked immunosorbent assay (ELISA) are commonly used diagnostics (Collins *et al.*, 2005). However, host humoral immune response to bacilli is delayed and techniques which rely on humoral response are to be used in advanced stage of infection. Meantime, infected animals may contaminate the environment or pass infection to other susceptible animals. Culture of *Map* is considered as gold standard and confirmatory diagnosis of paratuberculosis, but is time consuming and requires 8 to 16 weeks of incubation. The application of molecular techniques in conjunction with culture may hasten the diagnostic procedure. PCR is most common and widely used molecular technique and has been adopted in different format. PCR using insertion sequence IS*900* has been routinely used to detect the *Map* genome but, similarities with other mycobacterium, particularly *Maa* (97% DNA homology) decreases its specificity. Furthermore, sequences related to IS*900* have also been identified in Wood pigeon mycobacterium (IS*902*), *M. intracellulare* (IS*1626*) and *Maa* (IS*901* and IS*1626*). Therefore a positive IS*900* should be confirmed by subsequent nested-PCR or by a PCR assay targeting another gene or sequence in *Map* genome.

Present study was designed to investigate the level of infection in two different cattle population with culture followed by ZN staining and nested-PCR for confirmation of disease. IS*900* and *f57* sequences have been used as complementary to each other in nested format to ascertain the *Map* bacilli. Results were also being compared with test Nested-PCR and ZN staining which was performed before culture of samples on HYM.

Materials and Methods

Animal population and sample collection

Fecal samples were collected directly from rectum of animals and kept in plastic bag; a cold chain was maintained till the samples were processed. Two cattle farms were chosen; one was unproductive cattle rehabilitation center *i.e.* Gaushala (Barsana, Mathura, Uttar Pradesh) which maintains unproductive, orphan, discarded animals. A total of 160 sick cattle with the history of chronic diarrhea and ill health, were selected for sample collection. Another was instructional cattle dairy farm, Pantnagar (Uttarakhand), maintains the animals for milk production. Here samples were collected from 140 cattle without given preference to sick animals. Details of fecal samples collected with respect to age are mentioned in table 1.

Processing and Culture of fecal samples

Fecal samples were decontaminated in 0.75% (W/V) Hexadecylpyridium chloride (HPC) to remove contaminant microorganism (OIE *Terrestrial Manual*, 2008). Decontaminated sediment (100 µl) was inoculated on Herrold's Egg Yolk Medium (HYM) containing mycobactin-J (2 µg/ml) in duplicates. HYM was prepared according to the composition mentioned in OIE *Terrestrial manual* (2008) and was modified with addition of chloramphenicol (50 µg/ml), penicillin (100 U/ml) and amphotericin-B (50 µg/ml). The slants were incubated at 37°C at least for 4 months with regular observation. Any visible growth was confirmed by ZN staining, followed by nested-PCR. Slants which did not give positive results with PCR even after four month of incubation declared *Map* negative, but observed up to 1 year.

Confirmation of *Map* by nested-PCR

Slants with suspected growth of ≥ 2 months old were taken for genomic DNA isolation as well as ZN staining. Methods described by Kauppinen *et al.,* (1994) were followed with suitable modifications for DNA isolation. Confirmation of *Map* bacilli was done based on presence of IS*900* and *f57* sequence. Primer pairs, PCR reaction compositions, thermocyclic and electrophoretic conditions were followed as prescribed by Vansnick *et al.,* (2004). Briefly, all samples were screened for IS*900* insertion sequence which corresponds to 572bp amplicon. Positive amplicons were again amplified with different internal primer pair which corresponds to 452bp amplicons (Nested-PCR). Samples which were positive in both amplification of IS*900* were again screened for presence of *f57* sequence in same way as IS*900*. Amplicons of 432bp and 424bp respectively in first and second amplification for *f57* sequence were expected in positive cases. Samples which had given all four amplicons of IS*900* and *f57* were designated as positive otherwise declared negative for *Map*.

Results and Discussion

Cultures of fecal samples were weekly monitored for any growth and contaminations. As contamination was the main complication (Nielsen *et al.,* 2004) in the initial few weeks of incubation but it was resolved by subculture to new slant. Contamination requires more attention since tubes need to be incubated for longer duration. Typical colonial morphology of *Map* is difficult to identify particularly in primary isolation, however small (1 mm diameter), colorless, translucent, hemispherical, smooth and glistening colony would be expected after 8 weeks of growth (Sevilla *et al.,* 2007). Most of tubes didn't show any appreciable growth at two month of incubation. Slow growth of *Map* in primary culture and adaptation on artificial media retard its multiplication. More than 25 hrs (Thorel *et al.,* 1990) is required to complete a multiplication cycle, even though 1.2 days to 10 days generation time have also been estimated (Elguezabal *et al.,* 2011). Some of tubes had minute transparent colonies after the incubation of 7 to 9 months (Figure 1A and 1B). Standard laboratory working on *Map* suggests; colonies are appreciable any time after 5 weeks to 6 months of incubation (Gwozdz, 2010) in primary isolation and recommends negative samples should not be discarded without incubation of 6 to 8 months (de Juan *et al.,* 2006). Further, monitoring of growth should be regular after 4 weeks of incubation (Whipple *et al.,* 1991). ZN stain suggested growth of concern bacilli was undergoing even they were not appreciated on naked eye (Figure 2).

All tubes were screened by nested-PCR after two month of inoculation. Primer pairs correspond to IS*900* and *f57* sequences separately were used in nested manner (Vansnick *et al.,* 2004). Preliminary screening by ZN staining shown 25.00% samples together from both farms had growth of acid-fast bacilli (Table 1). However, confirmation based on presence of IS*900* and *f57* sequences (Figure 3) in nested-PCR revealed 33.67% samples had *Map* bacilli. In individual farms, acid-fast positive culture was 35.63% and 13.57%, respectively, in cattle rehabilitation center and dairy farm. Confirmed cases of *Map* in both farms were 50.00% and 15.00%, respectively (Table 1). Although, level of paratuberculosis in the places from where these samples were collected, was 20 to 30% (Mishra *et al.,* 2009; Singh *et al.,* 2010; Singh *et al.,* 2013). The way of sampling, particularly from cattle rehabilitation center was supposed to be the causal factor of high level of paratuberculosis diagnosed. Farm crowded with aged, discarded, apparently

healthy and diseased animals make the ground for high level of paratuberculosis in cattle rehabilitation center. Crowding of animals in small premises had also built the ground for easy and rapid transmission of infection among susceptible hosts. Continuous mixing of old and new animals in farm might hasten the rate of infection. In addition, nested-PCR is sensitive and specific technique (Herthnek and Bolske, 2006; Wells *et al.,* 2006: Douarre *et al.,* 2010) and hence able to confirm the growth of bacilli on culture which was incubated for ≥ 2 months. Sub-normal level of bacilli may be enriched during incubation of two months and might be diagnosed as positive both in zn staining and nested PCR. In contrary, organized dairy farm had comparatively low level of infection; it may be attributed to method of sample collection (not biased toward the sick animals) and organizational setup of the farm.

Growths confirmed by ZN staining/Nested-PCR in age groups 0 - <3 yrs and 3 - <6 yrs (Table 1) had approximately same level of infection (χ^2; $p>0.05$). This might be due to small sample size. However, level of paratuberculosis in advanced age group (≥6 yrs) was high (χ^2; $p<0.05$). Similar conclusion has also been given by Nielsen *et al.,* (2013). This could be explained as infection build up in animals was cumulative due to long incubation and was diagnosed only after animals excrete bacilli in feces (Crossley *et al.,* 2005) or immune system response to bacilli and both occur late in case of paratuberculosis.

Table.1 Number of positive cases from two cattle farm with respect to age in different diagnostics on fecal samples for paratuberculosis. *Cattle rehabilitation center (Goshala, Barsana, Mathura, Utter Predesh); **Instructional cattle dairy farm (Pantnagar, Uttarkhand); [a]Samples grown on HYM and stained after a fixed incubation period; [b]PCR was performed on culture grown on HYM; P- No. of positive samples; T-Samples tested. Figure in parentheses indicate percentage

Age	Unorganized farm*		Organized dairy farm**		Grand total	
	P (%)	T	P (%)	T	P (%)	T
ZN Staining[a]						
0 - <3 yrs	10 (41.67)	24	2 (12.50)	16	12 (30.00)	40
3 - <6 yrs	8 (36.36)	22	9 (15.25)	59	17 (20.99)	81
≥6 yrs	39 (34.21)	114	8 (12.31)	65	47 (26.26)	179
Sub-total	57 (35.63)	160	19 (13.57)	140	75 (25.00)	300
Nested PCR[b]						
0 - <3 yrs	15 (62.50)	24	2 (12.50)	16	17 (42.50)	40
3 - <6 yrs	12 (54.55)	22	8 (13.56)	59	20 (24.69)	81
≥6 yrs	53 (46.49)	114	11 (16.92)	65	64 (35.75)	179
Sub-total	80 (50.00)	160	21 (15.00)	140	101 (33.67)	300

Figure.1 Growth of Map on HYM after A: 7 months and B: 9 months of incubation.

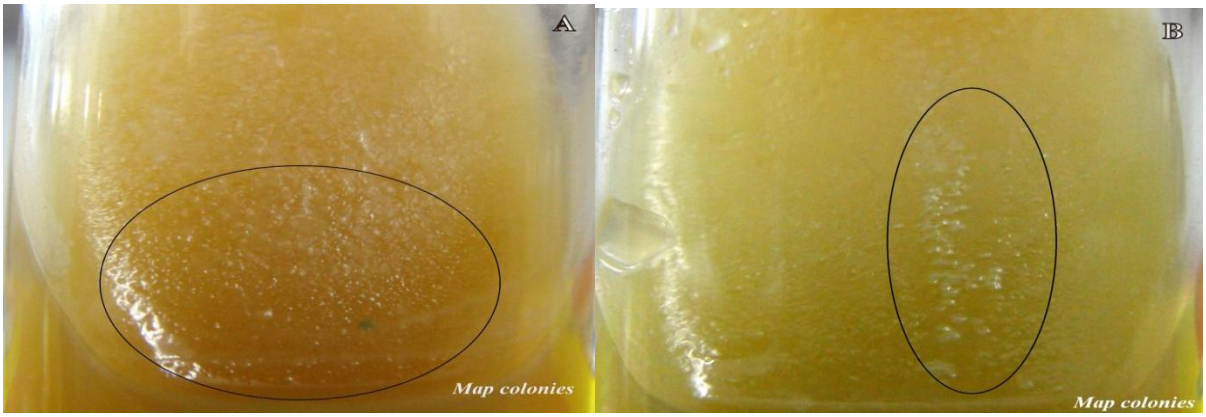


Figure.2 ZN stained bacilli which was incubated ≥ 2 months on HYM

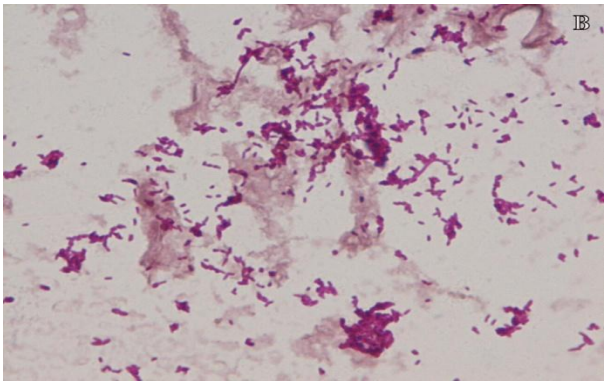
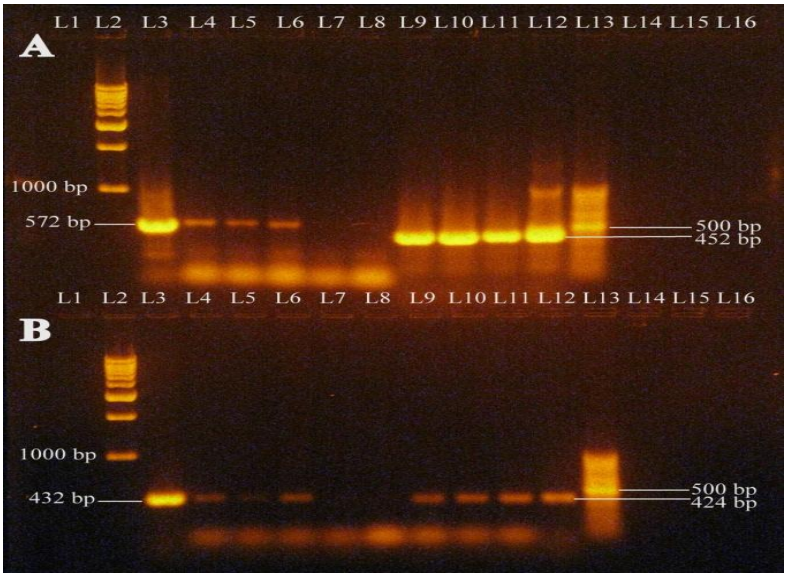


Figure.3 Amplicons of A: IS*900* and B: *f*57 sequences of *Map* from samples which was incubated ≥ 2 months on HYM



Nested-PCR with same primer pairs and ZN staining has also been performed on fecal samples (Mohan *et al.,* 2013), but percentage of positive samples diagnosed was low. Several reasons could be postulated; one of them was the organic matters of feces, which could be interfered in DNA isolation, some of them were identified as polymerase inhibitor (Widjojoatmodjo *et al.,* 1992). Host might excrete sub-optimal level, erratic shedding of bacilli; particularly in earlier stage of disease. Decontamination procedure also reduced the viable bacilli in clinical samples (Reddacliff *et al.,* 2003) significantly. Taking these in consideration, culture was performed on HYM, from decontaminated fecal samples. A considerable number of negative samples in each age group of animals from both farms were diagnosed positive in respective tests (Nested-PCR and ZN staining). Culture of clinical samples is always confirmatory to disease. In case of paratuberculosis, fecal culture is routinely used and found superior to other tests, even though it helps in calculating the diagnostic efficiency of different techniques (Martinson *et al.,* 2008; Sharma *et al.,* 2008; Douarre *et al.,* 2010; Sonawane and Tripathi, 2013).

Present study concludes that nested-PCR (IS*900* and *f57*) used in conjunction with fecal culture can be used to reduce the need of long incubation for culture of samples on HYM. Different organizational setup of farms might be responsible for unusual level of paratuberculosis diagnosed particularly in cattle rehabilitation center but high percentage of paratuberculosis positive animals in old age group in both farms.

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