Original Research Article

Using in-house designed multiplex PCR for differential detection of *Mycobacterium avium paratuberculosis*, *M. bovis* and *M. smegmatis* from faecal samples of dairy animals in parallel to Ziehl-Neelsen staining, faecal culture, IS*900* PCR and TaqMan real-time PCR

ABSTRACT:

Aim: Mycobacterial infections due to *Mycobacterium avium Paratuberculosis* (MAP) (Paratuberculosis), and *M. bovis* (Tuberculosis) in dairy animals are potential public health risk that needs to be diagnosed at the earliest for effective prevention. In-addition, *M. smegmatis* (non-tuberculous mycobacterium) is a saprophytic mycobacterium that may interfere with diagnosis of mycobacterial infection besides causing opportunistic infections. This study aims at rapid differential detection of these organisms from faecal samples of cattle and buffaloes using in-house designed multiplex PCR, besides other techniques for aiding in the accuracy of diagnosis.

Methodology: Faecal samples (n=268) were collected per-rectum from suspected animals and screened by Ziehl-Neelsen staining and graded as low, medium and high shedders; Then faecal culture in middlebrook 7H11 media for isolation of MAP from faecal samples of medium to high shedders (n=150). All the samples were subjected to in-house designed multiplex PCR targeting MAP, *M. bovis* and *M. smegmatis*; besides using IS*900* conventional PCR and TaqMan real-time PCR for detection of MAP.

Result: A total of 237 (88.43%) samples were found to be positive for Acid-fast bacilli, out of which 49 (20.68%), 106 (44.73%) and 82 (34.60%) samples were from low, medium and high shedders respectively. And only 4 (2.67%) samples were found to be culture positive for MAP. Whereas, multiplex PCR detected 16 (5.97%) faecal samples to be positive for MAP, and none were positive for *M. bovis* and *M. smegmatis*. However, IS*900* conventional PCR and IS*900* TaqMan real-time PCR detected 19 (7.09%) and 29 (10.82%) faecal samples to be positive for MAP respectively.

Conclusion: Thus, the *in-house* multiplex PCR can be used for screening of mycobacterial infections and also for the rapid detection of MAP like the IS*900* PCR. After all, a large proportion of the Acid-fast bacilli in the faecal samples maybe from other mycobacteria or non-mycobacterial Acid-fast bacilli that needs to be studied further.

Key words: Paratuberculosis, Tuberculosis, Non-tuberculous Mycobacteria, IS900, Multiplex PCR, Faecal culture

1. INTRODUCTION

Mycobacterial infections in dairy cattle and buffaloes, having a potential public health risk and huge economic significance, are the Paratuberculosis or Johne's Disease (caused by *Mycobacterium avium paratuberculosis,* MAP) and Tuberculosis (caused primarily by *M. bovis*, to a lesser extent by *M. tuberculosis* and also by *M. caprae*) [1, 2, 3] which are endemic in many regions of our country, with the prevalence rate of 29.0% (28.6% in buffalo and 29.8% in cattle) and 5.38% respectively, in Northern India [4, 5]. MAP has impact on food safety and are also believed to be associated with Crohn's disease, which is a chronic inflammatory bowel disease in humans [6, 7, 8, 9]. Bovine TB, though neglected since long, is an emerging zoonotic disease which can cause multiple drug resistant infections [10, 11, 12]. In a report to the WOAH, during January 2017 to June 2018, 82 (44%) of the 188 countries and territories reported the presence of bovine TB [3]. Both the JD and TB are WOAH listed diseases and causes chronic infections, resulting in economic losses to the dairy industry due to loss of production, morbidity, mortality and ban on international trade [13].

Besides, sporadic infections by other non-tuberculous mycobacteria of environmental origin are also reported. One such example is the *M. smegmatis*, which is a saprophytic, rapid growing, atypical, non-tuberculous mycobacteria (NTM) and has the potential of causing opportunistic infection in animals [2] as well as humans [14].

MAP causes chronic intermittent diarrhea and are shed in feces intermittently [15], other NTM may live as commensals or cause opportunistic infection and are also shed in feces [2]. *M. bovis* are also intermittently excreted in feces, especially in case of swallowing from respiratory secretion in case of active pulmonary TB or in case of open generalized TB in multiple organs [2].

The slow growth rate of the bacteria of Mycobacterial Tuberculosis Complex and MAP is a disadvantage for using it in the diagnostic point of view, since it delays rapid diagnosis by conventional techniques i.e. culture and microscopy, though it is considered the gold standard [1, 16, 15, 17]. Therefore, molecular diagnostics like PCR targeting different specific genes are much more convenient and rapid diagnostic techniques used in the modern laboratories for the confirmatory diagnosis of mycobacterial infections [15, 17]. However, as no test is 100% sensitive and specific [15, 17], further research are needed to develop newer diagnostic techniques. In this study, an in-house designed multiplex-PCR which differentiates mycobacterial infections caused by MAP*, M. bovis* and *M. smegmatis* was used in faecal samples of cattle and buffaloes for differential diagnosis of JD, TB and NTM infection. Further, other multiple diagnostic approaches, viz., direct microscopic examination of Acid-fast bacilli, faecal culture, molecular detection of MAP by conventional IS*900* PCR and TaqMan real time PCR was used for aiding in the accuracy of diagnosis.

2. MATERIALS AND METHODS

2.1 Source of samples

Fecal samples (n = 268) from cattle (Sahiwal, Jersey cross and HF cross) and buffaloes (Murrah and Nili-Ravi) of 2 years and above with a history or incidence of cases of chronic intermittent diarrhea were collected per-rectum from dairy farms in and around Ludhiana, Punjab.

2.2 Microscopic examination of faecal samples

All the faecal samples were subjected to Ziehl-Neelsen (Acid-fast) staining and after microscopic examination, the positive samples were graded as low (1-4 bacilli/10 fields), medium (5-15 bacilli/10 fields), and high shedders (>15 bacilli/10 fields) as per the number of bacilli present per high power (HP, 1000x oil immersion) field on the smears.

2.3 Isolation of MAP from faecal samples

Attempts were made to isolate MAP by inoculation of faecal samples (n=150, which were highly positive on ZN staining) in Middlebrook *7H11* media supplemented with mycobactin J (1µg/ml) and also without mycobactin J, after following appropriate decontamination steps [18]. The cultures were incubated at 37°C and were observed periodically upto 6 months post inoculation and the growth observed was

confirmed by IS*900* PCR using specific primers by Vary *et al*., (1990) [19] and also by in-house multiplex PCR [20] and TaqMan Realtime IS*900* PCR [21].

2.4 Extraction of DNA

DNA extraction from the faecal samples (n=268) were carried out using QIAamp Fast DNA Stool Mini kit (Qiagen) as per the manufacturer's instructions. The eluted DNA was stored at -20°C for further use.

2.5 In-house Multiplex PCR

Multiplex PCR primers were designed with the help of *in-silico* PCR targeting three mycobacterial species (Table 1). DNA was amplified by multiplex PCR using in-house developed primers targeting three mycobacterial species viz. MAP, *M. bovis* and *M. smegmatis* as per Brahma *et al*., (2017) [20]. A standard positive and negative control was also run along the samples. Amplicons of 187 bp, 571 bp and 628 bp were considered positive for MAP, *M. bovis* and *M. smegmatis* respectively.

Table 1: Primer Sequences for the in-house designed Multiplex PCR

Target Organism and strain		Primer Sequence	Location of primer gene sequence	Size of PCR product
M. avium subsp. *paratuberculosis* MAP4 CP005928.1	Forward	5′–CGCGCGTACCTGACAAAAC - 3′	562055 –562037	187 bp
	Reverse	5′– TCACCCTGACACTGACAGACA –3′	561869 –561889	
M. bovis strain SP38 CP015773.1	Forward	5′- GATGGTGGAACACGACCACT - 3′	4138314 -4138333	571 bp
	Reverse	5′- TTGATCGACCGTTCCGGTTT -3′	4138865 –4138884	
M. smegmatis MC2 155 CP009494.1	Forward	5′- ACCATGTCTATCTCAGTGTGCT -3′	3877883 –3877904	628 bp
	Reverse	5′- ACGCTCGAGGTCCACTACAA -3′	3878510 –3878491	

2.6 IS*900* PCR

DNA was amplified by PCR using primers which were species specific and based on the insertion sequence IS*900* by Vary *et al*., (1990) [19], [Forward (IS*900*/150C): 5- CCG CTA ATT GAG AGA TGC

GAT TGG - 3] [Reverse (IS*900*/921): 5- AAT CAA CTC CAG CAGCAG CGC GGC CTC G -3] designed to amplify a 229 base-pair target sequence. PCR was performed as per Brahma *et al*., (2017) [20]. Amplicons of 229 bp were considered positive for IS*900* PCR.

2.7 IS*900* TaqMan Real-time PCR

TaqMan real-time PCR assay for this study was done as per the method of Kim *et al*., (2002), [21] for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The MAP specific sequence IS*900* was targeted as this sequence is having the highest copy number. Primer and probe sequences used in the assay are: Forward (F2)-5'- AATGA CGGTT ACGGA GGTGG T- 3', Reverse (R2)- 5'- GCAGT AATGG TCGGC CTTAC C- 3' and probe (P2) – 5'-FAM-TCCAC GCCCG CCCAG ACAGG-TAMRA-3'.

TaqMan Real-time assay was performed with the Applied Biosystems (ABI) step one plus Real-time PCR as per Brahma *et al*., (2017) [20]. All the samples were run in duplicate and appropriate positive and negative control were also included in duplicate in each run.

3. RESULTS

3.1 Microscopic examination of faecal samples

Out of total 268 faecal samples, 237 (88.43%) were detected positive by ZN staining (Table 2, Figure 1). The results were interpreted according to the number of Acid-fast bacilli per 10 HP fields.

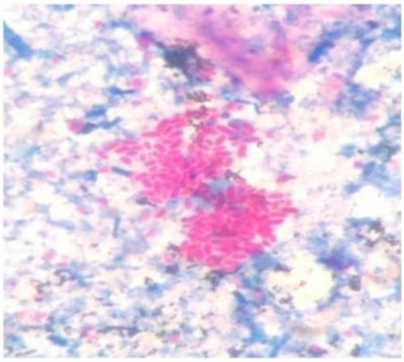

Fig. 1: Clumps of Acid-fast bacilli in faecal sample of high shedders (oil immersion, 1000x magnification).

3.2 Isolation of MAP from faecal samples

Out of the highly ZN positive faecal samples (n = 150) in microscopic examination that were processed for isolation of MAP in culture, 4 (2.67%) faecal samples were found positive for MAP (Table 2, Figure 2 and 3) after 4 months of incubation in the mycobactin supplemented media. The colonies appeared convex, soft, moist, glistening, off-white buffy colour. Colonies were typically between pinpoint and 0.5 to 1 mm in diameter. The animals positive for faecal culture were high degree (high shedders) positive by ZN staining. The samples were confirmed positive by IS*900* PCR (Figure 4), multiplex PCR and TaqMan real-time IS*900* PCR of the DNA extracted from the fecal culture colonies. No MAP colonies were observed in media without mycobactin J. In-fact, some of the inoculated tubes/media were dried up and some were contaminated with other bacterial and fungal growth despite the antibacterial antifungal treatment of the samples.

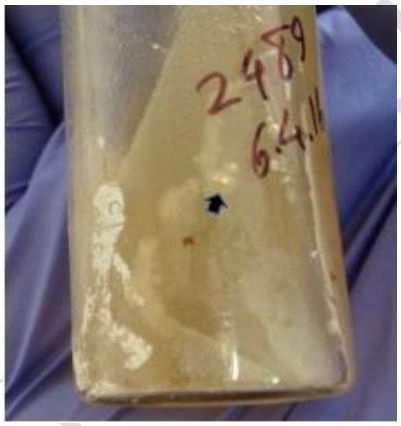

Fig. 2: Small raised off-white MAP colonies in mycobactin J supplemented Middlebrook *7H11* media

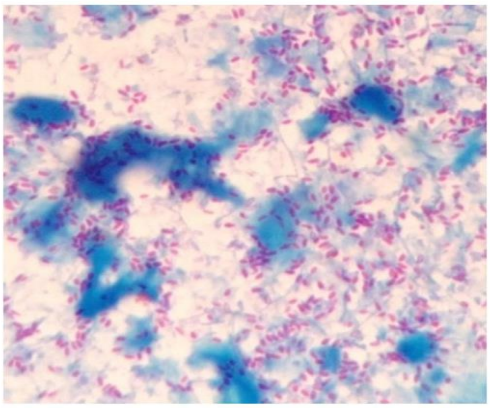

Fig. 3: Acid-fast staining of MAP colony in isolation (oil immersion 1000x magnification)

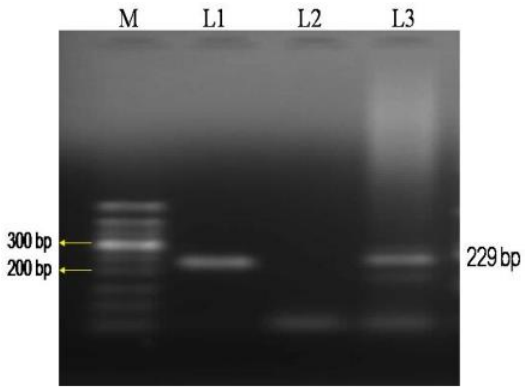

Fig. 4: Confirmation of the MAP isolate by IS*900* PCR (M= 50 bp ladder, L1= Control MAP DNA, L2= Negative control, L3= MAP positive isolate DNA)

3.3 In-house Multiplex PCR

All the faecal samples (n = 268) were tested by the in-house multiplex PCR for detection and differentiation of infections by the three target organisms. Out of total 268 faecal samples, 16 (5.97%) were found to be positive for MAP infection (medium and high shedders) and none were positive for *M. bovis* and *M. smegmatis.* The MAP positive faecal samples gave the specific amplicon size at 187 bp (Figure 5).

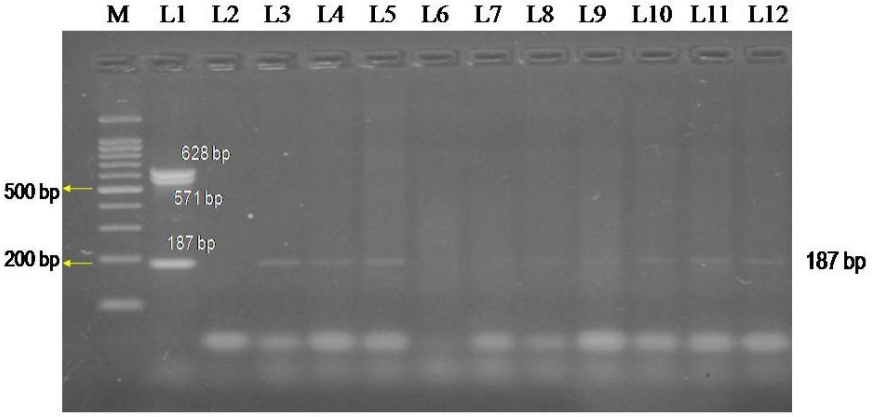

Fig. 5: Faecal samples positive for MAP by in-house Multiplex PCR (M= 100 bp plus ladder, L1= Multiplex positive control, L2= Negative control, L3-L12= Faecal samples positive for MAP by in-house multiplex PCR)

3.4 Molecular detection of MAP by IS*900* PCR

In the present study, a total of 19 (7.09%) faecal samples were detected positive (Table 2, Figure 6) out of all the 268 faecal samples processed. Out of the 19 MAP positive faecal samples, 3 samples had a single band at 229 bp, the rest 16 samples had an additional band at 150 – 200 bp besides the specific band at 229 bp. All the faecal samples detected positive for MAP by IS*900* PCR were from medium and high shedders showing the characteristic clumps of small and strongly Acid-fast rods. However, in comparison with the results of in-house multiplex PCR, only 12 (4.5%) out of 19 IS*900* PCR positive samples were also Multiplex PCR positive. Rest 4 (1.5%) and 7 (2.6%) number of samples were exclusively positive by multiplex PCR and IS*900* PCR respectively.

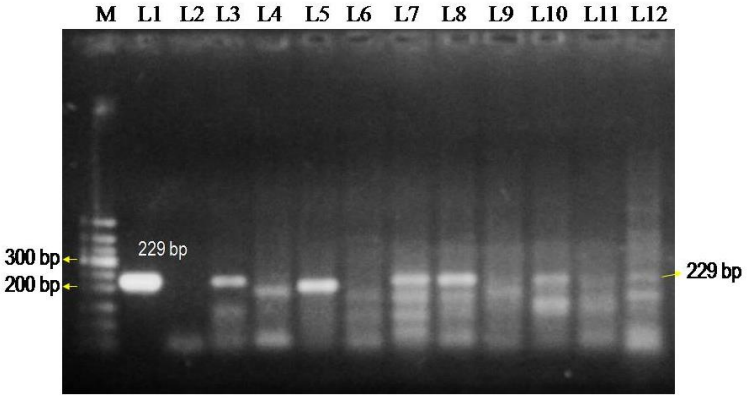

Fig. 6: Samples positive for MAP by IS*900* PCR (M= 50 bp ladder, L1= Positive control MAP DNA, L2= Negative control, L3-L12= Faecal samples positive for MAP)

3.5 Detection of MAP by IS*900* TaqMan Real-time PCR

Out of the 268 faecal samples processed, C_T values of the 29 (10.82%) faecal samples ranging from 14 - 36 (Table 2, Figure 7) were considered positive while rest of 239 faecal samples whose C_T values were above 36 were considered negative based upon our results of standardization of real time PCR protocol. IS*900* MAP specific TaqMan real time PCR was 10 times more sensitive than the conventional PCR and detected MAP even from the low shedders.

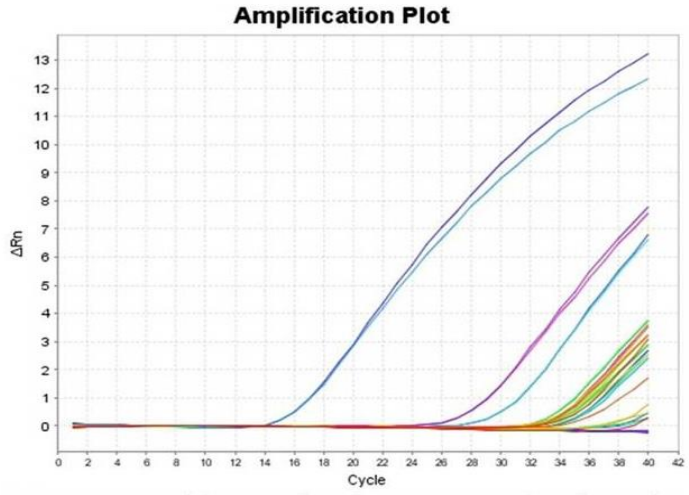

Figure 7: Amplification Plot of MAP DNA in faecal samples using real-time PCR TaqMan Assay

Table 2: Result of different diagnostic approaches used on faecal samples

Category of Faecal Shedders (No. of Acid-fast bacilli per 10 HP fields)	Ziehl-Neelsen staining for Acid-fast Bacilli (n=268)	Faecal culture of MAP (n=150)	In-house multiplex PCR (n=268)	IS*900* Conventional PCR (n=268)	IS*900* TaqMan Real-time PCR (n=268)	
	No. of Positive	No. of Positive	No. of Positive	No. of Positive	No. of Positive	C_T Value Range
Low (1-4 bacilli/10 fields)	49 (20.68 %)	0	0	0	10 (34.48%)	31 to 36
Medium (5-15 bacilli/10 fields)	106 (44.73 %)	0	4 (25%)	7 (36.84%)	7 (24.14%)	26 to 30
High (>15 bacilli/10 fields)	82 (34.60 %)	4 (2.67%)	12 (75%)	12 (63.16%)	12 (41.38%)	14 to 25
Total Positive	237 (88.43%)	4 (2.67%)	16 (5.97%)	19 (7.09%)	29 (10.82%)	
Total Negative	31 (11.57%)	146 (97.33%)	252 (94.03%)	249 (92.91%)	239 (89.18%)	

4. DISCUSSION

Mycobacteria causes a wide variety of diseases in human and animals, especially *M. bovis* and *M. tuberculosis* being the major pathogenic species to bovines and humans, respectively [1, 3]. Moreover, MAP and NTM within the *Mycobacterium* genus have become increasingly important in recent years due to human infections, particularly in the immunocompetent individuals [8, 14, 9]. Species that mainly infect cattle and buffaloes include *M. bovis* and MAP, though opportunistic infections by NTM also occur in animals [2].

In this study, conventional diagnostic methods used for diagnosis of mycobacterial infection included culture and microscopy. The microscopic examination of fecal samples by ZN staining revealed mostly from the category of medium (44.73%) and high (34.60%) fecal shedders. This finding is partially similar to the other studies, where they found that majority of MAP infected animals belonged to the category of low or moderate fecal shedders [22, 23]. In fact, Acid-fast staining is a conventional diagnostic method routinely used for showing the presence of MAP in feces and tissue samples of JD [24]. A tentative diagnosis of JD can be made if small clumps of strongly Acid-fast bacilli, of three or more organisms of about 0.5–1.5 µm in diameter are found. However, it is a known fact that ZN staining does not distinguish between MAP and other mycobacteria or non-mycobacterial Acid-fast bacteria [15].

The cultural isolation of MAP from fecal samples is considered the gold standard for diagnosis of MAP [15]. But, culture of *M. bovis*, MAP and *M. tuberculosis* require incubation period ranging from weeks to months [25]. Moreover, culturing these organisms requires special media and supplements and is therefore costly and time consuming. In addition, these slow growing bacteria usually exhibit a low plating efficiency, where only a proportion of viable cells in a culture will grow into colonies; making the culture method difficult and unreliable to detect low numbers of cells present in a sample [26]. Moreover, the need for treatment of clinical samples with harsh chemicals in order to inactivate contaminating microbes that may over-grow samples during the long incubation periods, in-fact reduces the viable population of mycobacteria [27, 28]. In our study, in spite of maximum number of ZN positive animals from medium to high shedders, only 4 (2.67%) samples (out of 150) had characteristic MAP colony growth which were

also successfully confirmed as MAP by multiplex PCR, IS*900* conventional as well as TaqMan real-time PCR. Problems like contamination of culture by overgrowth of competing bacterial and fungal agents and drying of the inoculated media due to long incubation period were also commonly seen. In addition, the decontamination step of the fecal samples might have lowered the recovery of viable MAP cells and thus lesser number of culture positive animals as described by Fawzy *et. al*., (2015) [26], Whittington and Sergeant, (2001) [27], Medeiros *et. al*., (2012) [28]. However, despite these limitations, culture-based methods are still considered to be the gold standard for diagnostic purposes [29], even though it may not be as sensitive and reliable as other diagnostic tests for the detection of MAP infection [30].

Although microbiological culture is still considered the gold standard method for diagnosis, molecular diagnostic approaches are much easier and rapid in terms of diagnosis and are increasingly used in modern laboratories [14]. Molecular diagnostic methods like PCR has proven to be a powerful tool for the specific detection of mycobacterial signature DNA sequences from all types of samples [31]. PCR is stated as a more sensitive method than culture for detection of Mycobacteria as low levels of MAP shed in the feces may not be detected by culture and PCR allows us to detect viable as well as non-viable microorganisms [32]. Moreover, multiplex PCR has the advantage of simultaneous amplification of more than one sequence of target DNA in a single reaction, saving time and reagents [33]. Multiplex PCR can be of great value for the differential detection of the chronic mixed infections with mycobacterial species. The accuracy of multiplex PCR for differential detection of *M. bovis* and *M. tuberculosis* was found to be 100% in terms of specificity and could detect as little as 20 pg of genomic DNA [34, 35]. A multiplex PCR designed to discriminate Mycobacterium avium complex organism targeting 16s rRNA, DT1, IS*900*, IS*901* and IS*1311* had an analytical sensitivity of 10 fg of MAP, 5-10 fg of *M. avium avium* and 2-5 fg of DNA from other mycobacterial species [36]. Sinha *et. al*., (2016) [37], using nested multiplex PCR targeting *IS6110*, *MTP40* and *32kD alpha antigen* encoding genes specific for Mycobacterium tuberculosis complex and NTM found that, the sensitivity of their assay was 97.1% for pulmonary and 91.4% for extra-pulmonary TB cases with specificity of 100% and 93.3% respectively, showing its reliability. In this study, the in-house designed multiplex PCR detected MAP in 16 (5.97%) faecal samples. Although, the in-house multiplex PCR successfully detected both MAP and *M. bovis* in tissue samples [20], so-far no *M. smegmatis* has been detected in clinical samples comprising of tissue [20],

blood [38] and faecal samples in this study. In fact, shedding of *M. bovis* in feces is stated to be intermittent [15] or there may be presence of some PCR inhibitors; and probably there was absence of any infection with *M. smegmatis* in the dairy herd. Moreover, the in-house multiplex PCR detected MAP in 4 samples (medium shedders) that were negative by both conventional and real-time IS*900* PCR. In fact, most of the acid-fast organisms in the feces may be from NTM other than *M. smegmatis* or other non-mycobacterial acid-fast bacilli, which need to be differentiated by further extensive studies.

The detection limit of direct PCR on faecal samples for paratuberculosis infection is stated to be around 100 organisms per g of feces, which is similar to the detection limit for culture [39]. IS*900* PCR is a rapid and routine method for MAP detection from different sources including feces, milk, intestinal tissues and mesenteric lymph nodes [9, 40]. The sensitivity of detection of MAP by conventional PCR was 10^3 organisms and there are reports of IS*900* PCR detecting as low as 1 pg/µl of MAP DNA [41]. Plain *et al*., (2014) [42], using a multistage protocol involving the recovery of MAP cells from a fecal suspension, cell rupture by bead beating, extraction of DNA using magnetic beads, and IS*900* quantitative PCR found that the limit of detection of the assay was 0.0005 pg, and the limit of quantification was 0.005 pg MAP genomic DNA. In-fact, we could detect even lower quantity of MAP DNA i.e. upto 30 fg/µl using the IS*900* PCR [20], and detected MAP in 19 (7.09%) faecal samples in this study. Further, the IS*900* PCR detected MAP positive in 7 samples (medium shedders) which were multiplex PCR negative. The detection of slightly higher number of positive samples by IS*900* PCR may be due to the higher copy number of the IS*900* gene in a single MAP genome [7]. However, PCR amplification can also create false-negative results caused by a low number of MAP targets and/or PCR inhibitors [43].

In recent years, real-time PCR, being a rapid and highly sensitive diagnostic technique, have been extensively developed to detect MAP from different specimens (blood, milk, feces, tissues and environmental samples) and thus offers hope for detection of fastidious and slow growing microorganisms, such as MAP. However, this molecular tool is greatly influenced by the quality of nucleic acid samples. Therefore, a DNA extraction method that provides a high-quality DNA sample and a maximum bacterial DNA recovery is a critical step to use real-time PCR [15, 44]. The IS*900* TaqMan real-time PCR can identify as low as 1 organism in pure culture [21] and even as low as 5 pg of MAP specific DNA per assay [45]. IS*900* real-time PCR is generally more sensitive than culture because of the

chemical treatment used in processing of the sample that adversely affects the viability of a proportion of MAP present in the sample and leading to their non-recovery. In addition, the PCR target is present in multiple copies per cell. It is also likely that animals producing a positive real-time PCR result but a negative culture result were sub-clinically infected, shedding the bacteria in numbers below the threshold of detection by fecal culture. Such a difference in sensitivity was also reported in another study by Sanftleben, (1990) [46]. In fact, real-time PCR was capable of detecting <3 genomic DNA copies with 99% probability or alternatively, using cells directly in the reaction, 12 cells can be detected with 99% probability [47]. In our study, the IS*900* TaqMan real-time PCR detected 29 (10.82%) MAP positive faecal samples and was the most sensitive method for detection of MAP DNA, with the detection limit as low as 3 fg/µl i.e. 6 (3 fg/µl x 2 µl of DNA) fg/reaction in a 25 µl reaction volume [20].

5. CONCLUSION:

The *in-house* multiplex PCR can be used for screening of mycobacterial infections and also for the rapid detection of MAP like the IS*900* PCR. The parallel use of other multiple diagnostic approaches aided in the specific detection of MAP and revealed that the real time PCR was more sensitive than that of conventional and multiplex PCR. In fact, a large-scale study is required to determine whether this in-house multiplex PCR assay is adequate for paratuberculosis and tuberculosis control program. After all, a large proportion of the Acid-fast bacilli in the faecal samples maybe from other mycobacteria or non-mycobacterial Acid-fast bacilli that needs to be studied further.

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