Rapid differential detection of selected organisms from faecal samples of cattle and buffaloes by using in-house designed IS900 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 the 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. Only 4 (2.67%) samples were culture positive for MAP. Whereas, multiplex PCR detected 16 (5.97%) faecal samples as 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 as positive for MAP respectively.

Conclusion: Thus, the *in-house* multiplex PCR can be used for screening 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 may be from other mycobacteria or non-mycobacterial Acid-fast bacilli that need to be studied further.

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

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1. INTRODUCTION

Mycobacterial infections in dairy cattle and buffaloes, having a potential public health risk and huge economic significance, are 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 an impact on food safety and is 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 for long, is an emerging zoonotic disease which can cause multiple drug-resistant infections [10, 11, 12]. In a report to the WOAH, from January 2017 to June 2018, 82 (44%) of the 188 countries and territories reported the presence of bovine TB [3]. Both JD and TB are WOAH-listed diseases and cause 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 *M. smegmatis*, which is a saprophytic, rapid-growing, atypical, non-tuberculous mycobacteria (NTM) and has the potential to cause opportunistic infection in animals [2] as well as humans [14].

MAP causes chronic intermittent diarrhoea and is shed in faeces intermittently [15], other NTM may live as commensals or cause opportunistic infection and are also shed in faeces [2]. *M. bovis* is also intermittently excreted in faeces, 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 from 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 modern laboratories for the confirmatory diagnosis of mycobacterial infections [15, 17]. However, as no test is 100% sensitive and specific [15, 17], further research is needed to develop newer diagnostic techniques. In this study, an in-house designed

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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. 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

Faecal 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 diarrhoea 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 up to 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) was carried out using the 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

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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 CAG CAG 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 has the highest copy number. Primer and probe sequences used in

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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 controls were also included in duplicate in each run.

3. RESULTS

3.1 Microscopic examination of faecal samples

Out of 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.

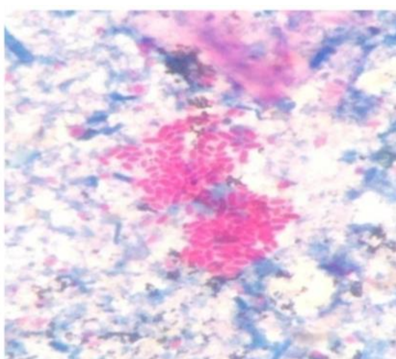

Fig. 1: Clumps of Acid-fast bacilli in faecal sample of high shedders (oil immersion, 1000x magnification).

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 faecal culture colonies. No MAP colonies were observed in media without mycobactin J. Some of the inoculated tubes/media were dried

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up and some were contaminated with other bacterial and fungal growth despite the antibacterial antifungal treatment of the samples.

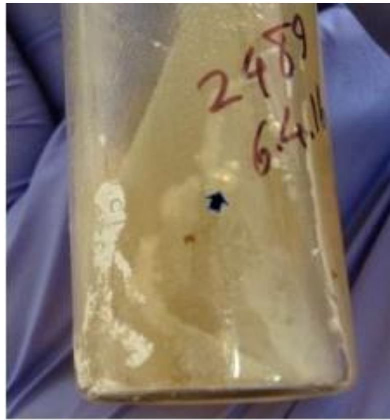

Fig. 2: Small raised off-white MAP colonies in mycobactin J supplemented Middlebrook *7H11* media

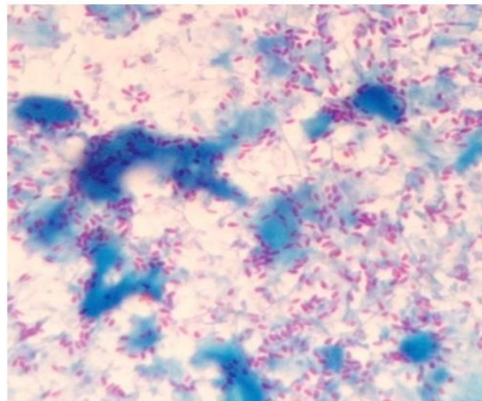

Fig. 3: Acid-fast staining of MAP colony in isolation (oil immersion 1000x magnification)

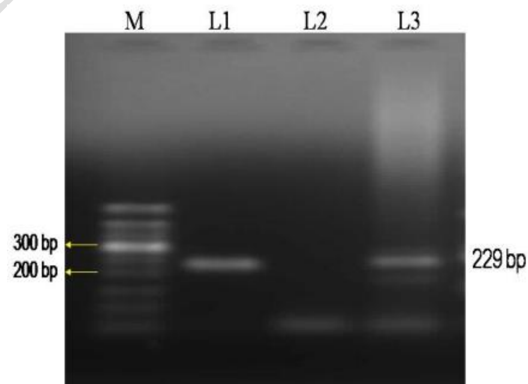

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Fig. 4: Confirmation of the MAP isolate by IS*900* PCR (M= 50 bp ladder, L1= Control MAP DNA, L2= Negative control, L3= MAP positive isolate DNA)

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 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).

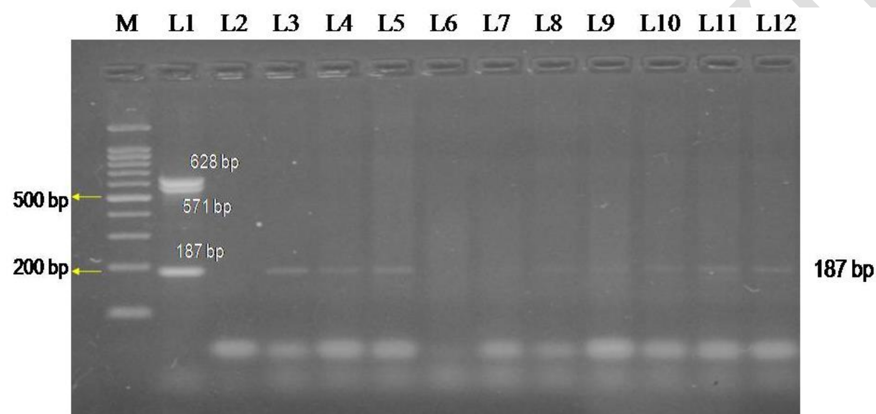

Fig. 5: Faecal samples positive for MAP by in-house Multiplex PCR (M= 100 bp plus ladder, L1= Multiplex positive control, L2= Negative control, L3-L12= Faecal samples positive for MAP by in-house multiplex PCR)

3.4 Molecular detection of MAP by IS*900* PCR

In the present study, 19 (7.09%) faecal samples were detected positive (Table 2, Figure 6) out of all the 268 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, comparing the results of in-house multiplex PCR, only 12 (4.5%) out of 19 IS*900* PCR positive samples were also Multiplex PCR positive. However, 4 (1.5%) and 7 (2.6%) samples were exclusively positive by multiplex PCR and IS*900* PCR respectively.

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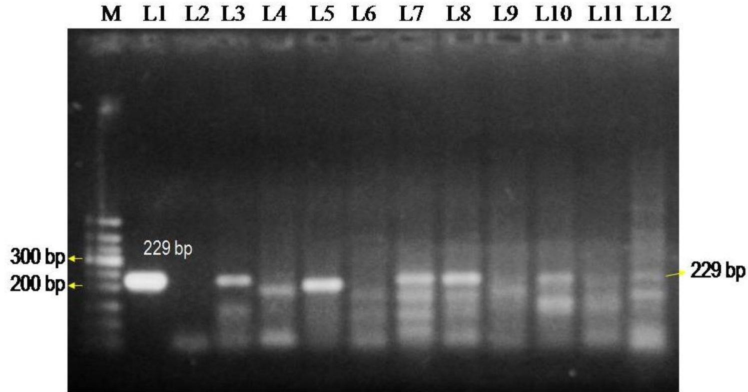

Fig. 6: Samples positive for MAP by IS*900* PCR (M= 50 bp ladder, L1= Positive control MAP DNA, L2= Negative control, L3-L12= Faecal samples positive for MAP)

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

Out of the 268 faecal samples processed, 29 (10.82%) faecal samples whose C_T values were below 36 (Table 2, Figure 7) were considered positive, and the remaining 239 faecal samples whose C_T values were above 36 were considered negative based upon the 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.

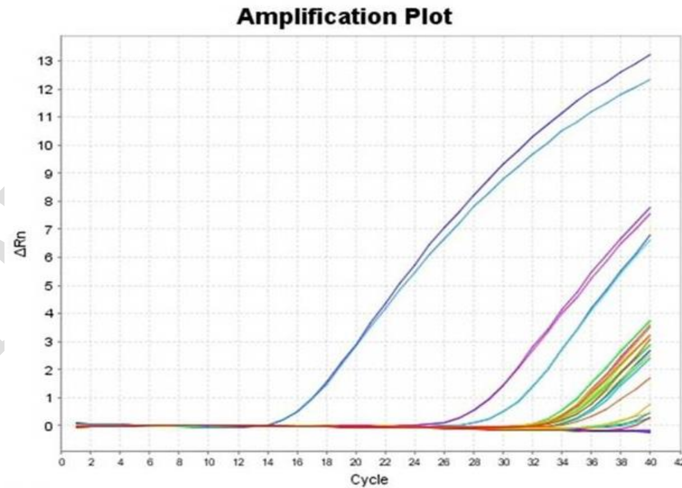

Figure 7: Amplification Plot of MAP DNA in faecal samples using real-time PCR TaqMan Assay

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

Category of	Ziehl-	Faecal	In-house	IS*900*	IS*900* TaqMan Real-

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Faecal Shedders (No. of Acid-fast bacilli per 10 HP fields)	Neelsen staining for Acid-fast Bacilli (n=268)	culture of MAP (n=150)	multiplex PCR (n=268)	Conventional PCR (n=268)	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 humans 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 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 the diagnosis of mycobacterial infection included culture and microscopy. The microscopic examination of faecal samples by ZN staining revealed mostly from the category of medium (44.73%) and high (34.60%) faecal shedders. This finding is partially

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similar to the other studies, where they found that the majority of MAP-infected animals belonged to the category of low or moderate faecal shedders [22, 23]. In fact, Acid-fast staining is a conventional diagnostic method routinely used for showing the presence of MAP in faecal 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 faecal samples is considered the gold standard for the diagnosis of MAP [15]. However, culturing of *M. bovis*, MAP and *M. tuberculosis* requires an 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 to inactivate contaminating microbes that may over-grow samples during the long incubation periods reduces the viable population of mycobacteria [27, 28]. In our study, despite a maximum number of ZN-positive animals from medium to high shedders, only 4 (2.67%) samples from high shedders (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 faecal samples might have lowered the recovery of viable MAP cells and thus the lesser number of culture-positive animals as described by Fawzy *et. al*., (2015) [26], Whittington and Sergeant, (2001) [27], Medeiros *et. al*., (2012) [28]. Similar to our study, Espejo & Wells (2009) [29], could identify heavy shedders through faecal culture. The culture media may also have an important role in the growth of the MAP, as the growth of MAP on Herrold's egg yolk agar was found to be more efficient than the Middlebrook 7H9 agar, Middlebrook 7H11 agar and modified Lowenstein-Jensen media [30]. However, despite these limitations, culture-based methods are still considered to be the gold standard for diagnostic purposes [31], even

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though they may not be as sensitive and reliable as other diagnostic tests for the detection of MAP infection [32].

Although microbiological culture is still considered the gold standard method for diagnosis, molecular diagnostic approaches are much easier and more rapid in terms of diagnosis and are increasingly used in modern laboratories [14]. Molecular diagnostic methods like PCR have proven to be a powerful tool for the specific detection of mycobacterial signature DNA sequences from all types of samples [33]. PCR is stated as a more sensitive method than culture for the 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 [34]. In a study, the sensitivity of faecal culture and PCR assay in the detection of MAP was 52.5% and 90% respectively [35]. The faecal PCR can be used as an alternative method on pooled faecal samples for the detection of heavy shedders [29]. 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 [36]. Multiplex PCR can be of great value for the differential detection of 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 [37, 38]. 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 [39]. Sinha *et. al*., (2016) [40], 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 the 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 [41] and faecal samples in this study. The shedding of *M. bovis* in faeces is stated to be intermittent [15] or there may be the presence of some PCR inhibitors, and probably there was no *M. smegmatis* infection in the dairy herd. Moreover, the in-house multiplex PCR detected MAP in 4 samples (medium shedders) that were negative

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by both conventional and real-time IS*900* PCR. After all, most of the acid-fast organisms detected in ZN staining of the faecal samples 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 faeces, which is similar to the detection limit for culture [42]. IS*900* PCR is a rapid and routine method for MAP detection from different sources including faeces, milk, intestinal tissues and mesenteric lymph nodes [9, 43]. The sensitivity of conventional PCR in the detection of MAP was 10^3 organisms and there are reports of IS*900* PCR detecting as low as 1 pg/µl of MAP DNA [44]. Plain *et al.*, (2014) [45], using a multistage protocol involving the recovery of MAP cells from a faecal 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. We could detect an even lower quantity of MAP DNA i.e. up to 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. Similarly, Tripathi *et al.*, (2009) [46], using multiplex PCR targeting two different genes IS*900* and F57 of MAP found the previous to detect more positive samples than the latter. The detection of a 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 [47].

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, faeces, tissues and environmental samples) and thus offers hope for the 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 using real-time PCR [15, 48, 49]. 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 [50]. IS*900* real-time PCR is generally more sensitive than culture because of the

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chemical treatment used in processing 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 faecal culture. Such a difference in sensitivity was also reported in another study by Sanftleben, (1990) [51]. Real-time PCR was capable of detecting <3 genomic DNA copies with a 99% probability or using cells directly in the reaction, 12 cells can be detected with a 99% probability [52]. In our study, the IS9*00* TaqMan real-time PCR detected 29 (10.82%) MAP positive faecal samples and was the most sensitive method for the 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]. After all, though these new advanced techniques are rapid and increases diagnostic efficiency, it is very difficult to diagnose the disease before the faecal shedding of MAP [53]. Therefore, diagnosis of the disease at an early stage is essential for effective control and prevention.

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 real-time PCR was more sensitive than that of conventional and multiplex PCR. A large-scale study is required to determine whether this in-house multiplex PCR assay is adequate for paratuberculosis and tuberculosis control programs. After all, a large proportion of the Acid-fast bacilli in the faecal samples may be from other mycobacteria or non-mycobacterial Acid-fast bacilli that need to be studied further.

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