

Diagnosis of Mycobacterial infections (Tuberculosis and Paratuberculosis) in tissue samples using molecular (in-house multiplex PCR, PCR and TaqMan real-time PCR), histopathology and immunohistochemical techniques

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Abstract. An in-house multiplex PCR designed for differential detection of *M. bovis*, *M. avium paratuberculosis* (MAP) and *M. smegmatis* in tissue samples, besides PCR and TaqMan real-time PCR targeting *esxA* (ESAT-6) and *esxB* (CFP-10) gene of *M. tuberculosis* complex (MTC) and IS900 gene targeting MAP were carried out in the present study. Tissue samples comprising of lung, mediastinal lymph node, intestine and mesenteric lymph node from cattle and buffaloes (n=16) suspected to have died of tuberculosis (TB) or paratuberculosis (JD) were collected at post-mortem. Out of 16 animals, only 4 (25%) cases were positive for *M. bovis* by in-house designed multiplex PCR, where as 9 (56.25%) cases were positive for MTC by both conventional and real-time PCR targeting *esxA* (ESAT-6) and *esxB* (CFP-10) genes. Out of 9 TB positive animals, 2 (22.22%) had co-infection with MAP as confirmed by IS900 PCR, real-time PCR and also by in-house multiplex PCR. No samples were found positive for *M. smegmatis* by the in-house multiplex PCR. Analytical sensitivity of in-house multiplex primers targeting *M. bovis*, MAP and *M. smegmatis* were 170 fg/µl, 300 fg/µl and 51 fg/µl of genomic DNA respectively. Analytical sensitivity of primers for both conventional and real time PCR targeting *esxA* were 8 pg/µl and 800 fg/µl, *esxB* were 800 fg/µl and 80 fg/µl and IS900 were 30 fg/µl and 3 fg/µl of *M. tuberculosis* and MAP genomic DNA respectively. The tissue samples were also subjected to histopathology and Immunohistochemical (IHC) staining using ESAT-6 and CFP-10 antibodies for TB and anti-JD anti-serum (raised in rabbit) for JD. All the animals positive for TB (9 of 9) and JD (2 of 2) in PCR and real-time PCR were also positive in histopathology and IHC. Thus, combination of molecular, histopathology and IHC techniques increased the accuracy of TB and JD diagnosis, all of which had same sensitivity and specificity except the in-house multiplex PCR which was comparatively less sensitive in *M. bovis* detection. However, the molecular methods were found to be more convenient and rapid, capable of making the diagnosis within 6 hours. The study indicated that Real-time PCR was ten times more sensitive than the conventional PCR.

INTRODUCTION

Mycobacterium falls under the category of 'Hazard group-III organisms' (OIE, 2004). The most important mycobacterial infections in cattle and buffalo includes Tuberculosis (TB) and Paratuberculosis (Johne's Disease or JD) which are chronic and wasting diseases. Both TB and JD have zoonotic potential and are

endemic in Indian dairy herds causing severe economic losses due to morbidity, decrease in production and mortality (Singh *et al.*, 2008; Sharma *et al.*, 2011).

TB is prevalent in wide range of livestock, including domestic and wild animals, besides humans. It is caused by the pathogenic mycobacterial species, *Mycobacterium tuberculosis* complex

(MTC) comprising of *M. bovis*, *M. tuberculosis*, *M. africanum*, *M. canneti*, *M. microti*, *M. pinnipedii* and *M. caprae* (Olsen *et al.*, 2010). TB in milch animal is mainly caused by *M. bovis*. *M. tuberculosis* (Ocepek *et al.*, 2005) and *M. caprae* (Prodinger *et al.*, 2005) has also been reported to cause TB in cattle. In Indian dairy cattle the overall prevalence rate of TB is 5.38% (Sharma *et al.*, 2011). Secondly, JD, caused by MAP, is characterized by progressive enteritis in domestic and wild ruminants. It has a prevalence rate of 29.0% (28.6% in buffalo and 29.8% in cattle) in Northern India (Singh *et al.*, 2008).

TB and JD are difficult to diagnose by conventional lab methods, i.e. culture, acid-fast staining and microscopy, which are low in sensitivity and does not identify the species of mycobacteria causing the disease (Parsons *et al.*, 2002). Further, granulomatous lesions may be confused with those caused by *Nocardia*, *Corynebacterium* and other granuloma causing organisms (Shitaye *et al.*, 2007; Grist, 2008). Therefore, molecular methods like PCR, multiplex PCR, real-time PCR and histopathology or IHC are required for the confirmatory diagnosis of the disease.

A number of PCR-based protocols have been developed for the detection of Mycobacteria belonging to the MTC. PCR targeting culture filtrate protein-10 (CFP-10) and early secretory antigenic target protein-6 (ESAT-6) encoded by *esxA* and *esxB* genes respectively, located in the RD1 region (*Rv3874* and *Rv3875* genes) of the genome which play an important role in the virulence of *M. tuberculosis* and related pathogenic mycobacteria are potential diagnostic tools (Dikshit *et al.*, 2012). Identification of MAP is currently based on detection of the insertion sequence IS900 by the means of conventional and real-time PCR, which can distinguish MAP from the other *M. avium* subspecies. All MAP strains have been found to harbour multiple copies of IS900 sequence (Vary *et al.*, 1990; Huntley *et al.*, 2005).

Multiplex PCR is a fast tool that allows the simultaneous amplification of more than one sequence of target DNA in a single reaction, saving time and reagents (Tanaka *et al.*, 2003). The present study was

undertaken to design and develop a multiplex-PCR which differentiates *M. bovis*, MAP and *M. smegmatis* in tissue samples of cattle and buffaloes. Further, this multiplex PCR was combined with other multiple approaches, viz., comparative accuracy of conventional PCR, real-time PCR, histopathology and IHC for confirmatory diagnosis of TB and JD.

MATERIALS AND METHODS

Tissue Sample collection

Fresh tissue samples from suspected cases of bovine TB (lungs and mediastinal lymph nodes, n=16) and JD (intestine and mesenteric lymph nodes, n=6) were collected in two separate containers, one in simple sterile container for PCR studies and the other in 10% Neutral Buffered Formalin (NBF) for histopathology and IHC.

Molecular Diagnosis

For PCR, DNA extraction was done using NucleoSpin (Machery-Nagel) tissue DNA extraction kit and stored in -20°C until further use.

In-house developed Multiplex PCR

Multiplex PCR primers were designed with the help of *in-silico* PCR targeting three mycobacterial species: *M. bovis*, MAP and *M. smegmatis* (Table 1).

The sensitivity of the in-house multiplex primers were assessed by using ten-fold serial dilution of the standard DNA of *M. tuberculosis* (IMTECH, Chandigarh), MAP (GENEKAM, Germany) and *M. smegmatis* (Microbiologics). Specificity of the primers were cross-tested individually against genomic DNA of *M. tuberculosis*, MAP, *M. smegmatis* and non-tuberculous mycobacterial species (*M. fortuitum* and *M. kansasii*) and non-mycobacterial species (*B. abortus*, *P. multocida* and *E. coli*).

DNA of suspected tissue samples were amplified by in-house multiplex PCR primers in 25 µl reaction volume, containing 12.5 µl of GoTaq® Green Master mix, 0.5 µl (25 pmol) each of 3 forward primers, 0.5 µl (25 pmol) each of 3 reverse primers, 1.5 µl of nuclease

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

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

free water and 8 µl of DNA template. Along with the test sample DNA, a known positive control DNA and a negative control were also amplified. Thermal cycling were performed in research thermal cycler (Eppendorf, Germany) and the cycling conditions were: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 66°C for 1 min, extension at 72°C for 2 min 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). Amplicons of 571 bp, 187 bp and 628 bp were considered positive for *M. bovis*, MAP and *M. smegmatis* respectively.

***esxA* (ESAT-6) and *esxB* (CFP-10) PCR**

The sensitivity of the *esxA* (ESAT-6) and *esxB* (CFP-10) primers were assessed in ten-fold serial dilution of the known concentration (8 ng/µl) of the standard genomic DNA of *M. tuberculosis*. The specificity of *esxA* and *esxB* gene of *M. tuberculosis* were tested by using non-tuberculous mycobacterial species (*M. fortuitum*, *M. kansasii* and *M. smegmatis*) and non-mycobacterial species (*B. abortus*, *P. multocida* and *E. coli*).

The primer sequences for ESAT-6 were: Forward- 5'-GTACCAGGGTGTCCAGCAA AA-3' and Reverse- 5'-CTGCAGCGCGTT GTTCAG-3' (Rogerson *et al.*, 2006); and for CFP-10 were: Forward- 5'-ATGGCA GAGATGAAGACCGATGCCGCT-3' and

Reverse- 5'-TCAGAAGCCCATTTGCGA GGACAGCGCC-3' (Dikshit *et al.*, 2012) giving a product size of 61 bp and 302 bp respectively. Tissue sample DNA were amplified by *esxA* (ESAT-6) and *esxB* (CFP-10) PCR, for detection of MTC. PCR for both the primers were done in reaction volume of 25 µl containing 12.5 µl of GoTaq® Green Master mix, 1 µl of forward primer (10 pmol), 1 µl of reverse primer (10 pmol), 4.5 µl of nuclease free water and 6 µl of DNA template. Along with the test sample DNA, a known positive control DNA and a negative control were also amplified. Thermal cycling conditions for ESAT-6 were as follows: initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 65°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min. Cycling conditions for CFP-10 were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 63°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. Amplicons of 61 bp and 302 bp were considered positive for ESAT-6 and CFP-10 PCR respectively.

***esxA* (ESAT-6) and in-house designed *esxB* (CFP-10) TaqMan real-time PCR**

Sensitivity and specificity tests for both the primer-probes were done similar to conventional *esxA* (ESAT-6) and *esxB* (CFP-10) PCR. ESAT-6 TaqMan Real-Time PCR

was done for detection of MTC as per the method of Rogerson *et al.*, (2006). Primer and probe sequences for *esxA* (ESAT-6) are: Forward- 5'-GTACCAGGGTGTCCAGCA AAA-3' and Reverse- 5'-CTGCAGCGCG TTGTTTCAG-3' and Probe- 5'-GGGACGCC ACGGCTACCG-3', labeled with fluorescent reporter dye VIC on the 5' end and the quencher dye NFQ-MGB on the 3' end. The primer and probe for *esxB* (CFP-10) TaqMan real-time PCR were self designed in the department for detection of MTC. The in-house *esxB* (CFP-10) primer sequences are: Forward- 5'-GGCGACCTGAAAACCC AGAT-3' and Reverse- 5'-TGCTTATTGGC TGCTTCTTGGA-3' and Probe- 5'-CCCT GCAACGAACCTG-3', labeled with the fluorescent reporter dye FAM on the 5' end and the quencher dye NFQ-MGB on the 3' end. The *esxA* (ESAT-6) and in-house *esxB* (CFP-10) real-time primers were obtained from Invitrogen and Applied Biosystems respectively.

Amplification and detection of DNA in TaqMan real-time PCR was performed with the Applied Biosystems (ABI) Step one plus Real-Time PCR. The reaction mixture for each reaction were: TaqMan Master Mix (2X) 10 µl, Primer-Probe Mix (20 X) 1 µl, Nuclease free water 7 µl and DNA template 2 µl. Each reaction including positive and negative control were run in duplicate. The cycling conditions were as per Step one plus Real-Time PCR software: Initial denaturation at 95°C for 10 min for 1 cycle, denaturation at 95°C for 15 sec for 40 cycles, annealing and extension at 60°C for 1 min for 40 cycles, final extension at 60°C for 30 sec for 1 cycle. CT based quantification of the *esxA* (ESAT-6) and in-house *esxB* (CFP-10) genes were also recorded and analysed as per Step one plus Real-Time PCR software.

IS900 PCR

Sensitivity of IS900 PCR was assessed in ten fold serial dilutions of MAP genomic DNA (3 ng/µl). Specificity of IS900 PCR was assessed by using non-tuberculous mycobacterial species (*M. fortuitum*, *M. kansasii* and *M. smegmatis*) and non-mycobacterial species (*B. abortus*, *P. multocida* and *E. coli*).

JD suspected tissue sample DNA were amplified by MAP species specific PCR based on the insertion sequence IS900. The sequences are - Forward (IS900/150C): 5'-CCG CTA ATT GAG AGA TGC GAT TGG-3' and Reverse (IS900/921): 5'-AAT CAA CTC CAG CAGCAG CGC GGC CTC G-3' designed to amplify a 229 bp target sequence (Vary *et al.*, 1990; Huntley *et al.*, 2005). Reaction volume of 25 µl containing 12.5 µl of GoTaq® Green Master mix, 1 µl of forward primer (10 pmol), 1 µl of reverse primer (10 pmol), 2.5 µl of nuclease free water and 8 µl of DNA template were used for PCR. Along with the test sample DNA, a known positive control DNA and a negative control were also amplified. Thermal cycling conditions were as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 45 sec, annealing at 62°C for 45 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 min.

IS900 TaqMan real-time PCR

Sensitivity and specificity of IS900 TaqMan real-time PCR using MAP specific IS900 primer probe was done similar to conventional IS900 PCR. All the reactions including positive and negative control were run in duplicate.

The TaqMan real-time PCR for detection of MAP was done as per the method of Kim *et al.* (2002). Primer and probe sequences are: Forward (F2)-5'-AATGA CGGTT ACGGA GGTGG T-3', Reverse (R2)-5'-GCAGT AATGG TCGGC CTTAC C-3' and probe (P2)- 5'-TCCAC GCCCG CCCAG ACAGG-3'. The probe was labeled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) on the 5' end and the quencher dye *N*-, *N*-, *N*-, *N*-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end. Primers and probes specific for MAP IS900 sequence DNA were obtained from Applied Biosystems.

Amplification and detection of DNA by IS900 TaqMan Real-time PCR was done as per Step one plus Real-Time PCR software i.e. similar to the *esxA* and *esxB* TaqMan real-time PCR conditions.

Histopathology and Immunohistochemical detection of anti-TB and anti-JD antibodies

For histopathology and immunohistochemical detection of anti-TB (ESAT-6 monoclonal and polyclonal, CFP-10 polyclonal) and anti-JD (polyclonal) antibody; all tissue samples separately collected and fixed in 10% NBF, were processed as per conventional methods (Phom *et al.*, 2016), paraffin blocks were prepared, 4–6 µm sections were cut and stained with Hematoxylin and Eosin (H&E). For immunohistochemical studies, 4–5 µm thick paraffin tissue sections were spread on superfrost positively charged microscopic slides (Fisher Scientific, USA). Antigen retrieval was done in EZ antigen retrieval solutions using EZ-Retriever System (BioGenex Laboratories Inc., California). After endogenous peroxidase and non-specific protein blocking, the sections were incubated with standardised dilution of anti-TB and anti-JD antibodies (Table 2) in a humidified chamber at 4°C overnight. Secondary antibody conjugated with HRP (Vector Laboratories, USA) was added and

incubated for 30 min at room temperature. Antigen-antibody complex was visualized using ImmPACT DAB Peroxidase Substrate Kit (Vector Laboratories, USA) followed by counterstaining with hematoxylin. Omission of primary antibody was used as a negative control. Presence or absence of Mycobacterial antigens were evaluated by observing the stained cells showing positive reactivity (macrophages, giant cells, epithelioid cells) using light microscopy under oil immersion (Purohit *et al.*, 2007; Phom *et al.*, 2016).

RESULTS

In-house developed Multiplex PCR

The sensitivity of *M. bovis*, MAP and *M. smegmatis* primers were as little as 170 fg/µl, 300 fg/µl and 51 fg/µl of genomic DNA respectively (Table 3). None of the organisms other than the specific standard DNA had amplification at the standardised annealing temperature i.e. 65.5–68°C which clearly indicates the specificity of in-house multiplex PCR.

Table 2. Panel of antibodies used for Immunohistochemical characterization of TB and JD

S. No.	Antibody	Company	Clonity	Dilution used
1.	ESAT-6 Monocloal	Thermo Scientific	Monoclonal	1:100
2.	ESAT-6 Polyclonal	Thermo Scientific	Polyclonal	1:100
3.	CFP-10	Thermo Scientific	Polyclonal	1:100
4.	Anti-JD raised in rabbit	Raised by Dr. VK Gupta, Palampur	Polyclonal	1:2500

Table 3. Sensitivity of primers against standard DNA

Primers	Target organism	Standard DNA concentration (ng/µl)	Sensitivity in conventional PCR (fg/µl)	Sensitivity in real-time PCR (fg/µl)
In-house designed Multiplex PCR	<i>M. bovis</i>	1.7	170	–
	MAP	3	300	–
	<i>M. smegmatis</i>	5.1	51	–
ESAT-6	<i>M. tuberculosis</i>	8	8.0 pg/µl	800
CFP-10	<i>M. tuberculosis</i>	8	800	80
IS900	MAP	3	30	3

The in-house multiplex PCR could differentiate between TB and JD infections in the tissue samples. Out of 16 TB and 6 JD suspected cases, 4 were positive for TB and 2 were positive for JD (Figure 1). However, none of the tissue samples were found to be positive for *M. smegmatis*.

***esxA* (ESAT-6) and *esxB* (CFP-10) PCR**

The detection limit of *esxA* (ESAT-6) and *esxB* (CFP-10) primers were 8 pg/μl and 800 fg/μl respectively (Table 3). None of the organisms other than *M. tuberculosis* had

amplification for *esxA* (ESAT-6) and *esxB* (CFP-10) gene. Out of the 16 animals suspected to have died of TB, 9 (56.25%) were found confirmed TB positive by both the ESAT-6 (Figure not included) and CFP-10 PCR (Figure 2).

***esxA* (ESAT-6) and *esxB* (CFP-10) TaqMan real-time PCR**

The detection limit of the *esxA* (ESAT-6) and *esxB* (CFP-10) TaqMan real-time PCR were upto 800 fg/μl and 80 fg/μl respectively (Table 3). None of the organisms other than

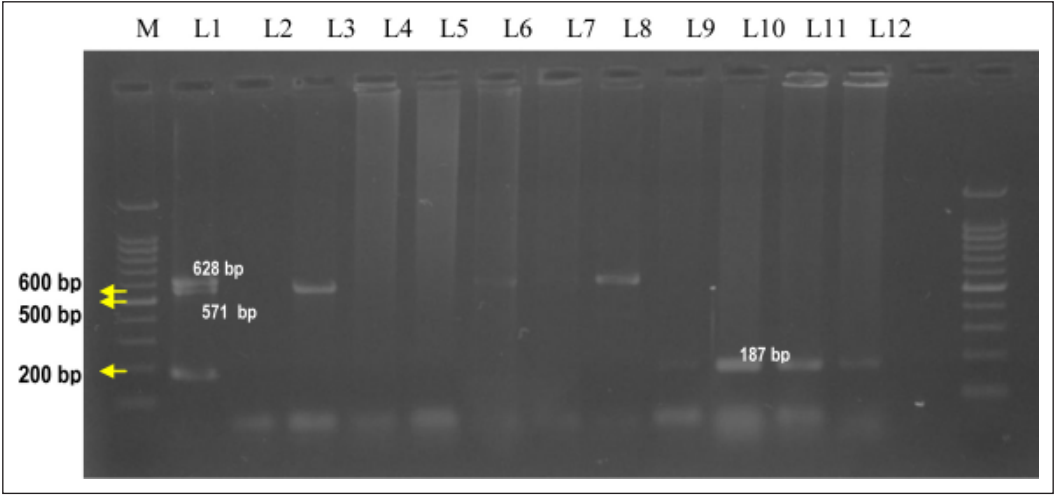


Figure 1. Amplification of DNA from the samples using in-house multiplex primers. [Lane M – 100 bp ladder, L1 – Positive control (*M. tuberculosis*), L2 – Negative control, L3-12 – Tissue samples].

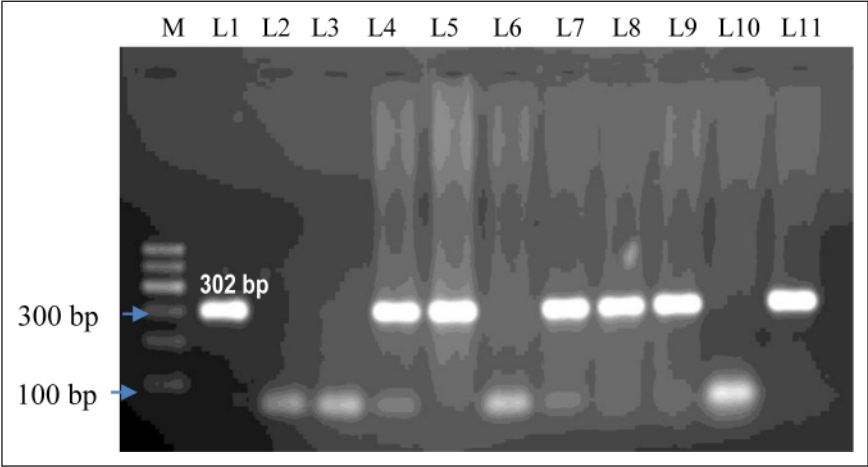


Figure 2. Amplification of DNA from the samples using *esxB* (CFP-10) primers. [Lane M – 100 bp ladder, L1 – Positive control (*M. tuberculosis*), L2 – Negative control, L3-L11 – Tissue samples].

M. tuberculosis had amplification which clearly indicates the specificity of *esxA* and *esxB* gene of MTC.

Similar to conventional PCR, 9 (56.25%) animals, whose CT values were between 22.2–36.9 by ESAT-6 (Figure 3) and CFP-10 (Figure 4) real-time PCR were considered positive for TB. Remaining samples whose CT values were equal to or greater than 37.5 were considered negative.

IS900 PCR

Sensitivity of conventional IS900 PCR was upto 30 fg/μl of genomic DNA (Table 3). None of the organisms other than MAP had amplification, indicating the specificity of IS900 PCR.

Out of total 16 animals, which included 6 cases suspected for JD, only 2 were positive for MAP by IS900 PCR from the tissue samples from both intestine and mesenteric

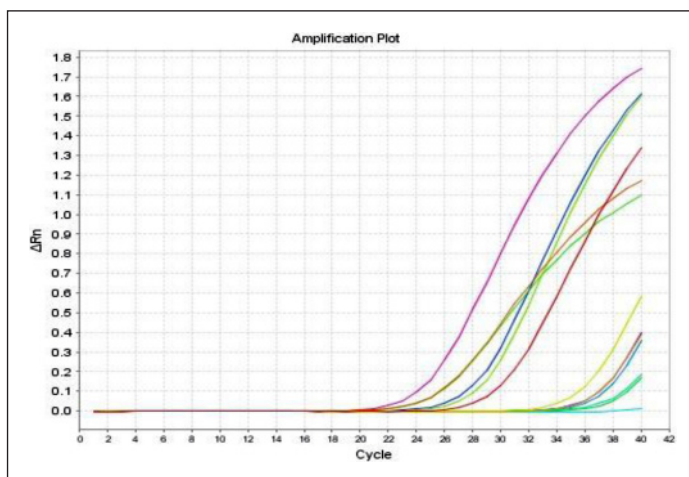


Figure 3. Amplification Plot of MTC DNA in the samples targeting *esxA* (ESAT-6) gene using TaqMan real-time PCR.

All the samples are differentiated by using different coloured lines. [Rn is the fluorescence of the reporter dye VIC or FAM divided by the fluorescence of a passive reference dye ROX (i.e., reporter signal normalized to the fluorescence signal). ΔRn is Rn minus the baseline; Rn and ΔRn are plotted against PCR cycle number].

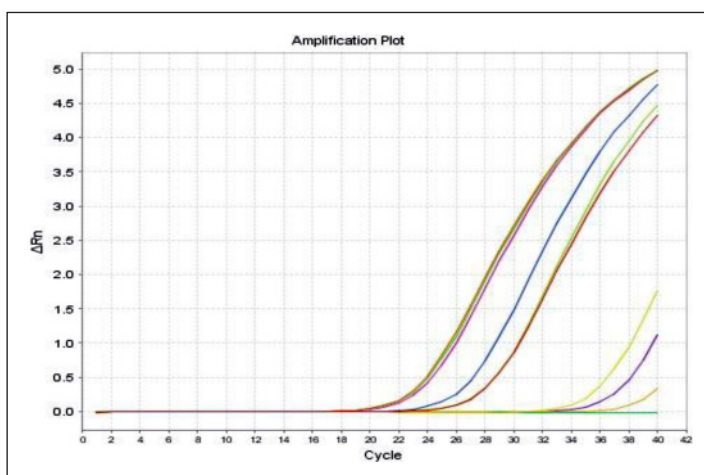


Figure 4. Amplification Plot of MTC DNA in the samples using in-house designed *esxB* (CFP-10) real-time primers by TaqMan real-time PCR.

All the samples are differentiated by using different coloured lines.

lymph nodes (Figure 5). Both of the JD positive animals had co-infection with TB.

IS900 TaqMan real-time PCR

Sensitivity of MAP specific IS900 TaqMan real-time PCR was upto 3 fg/ μ l of genomic MAP DNA (Table 3). None of the organisms other than MAP had amplification, indicating the specificity of IS900 TaqMan real-time PCR.

Same as conventional IS900 PCR, only 2 out of 6 JD suspected animals were detected

positive for JD by real-time PCR whose CT values were 18–23 (Figure 6).

Histopathology of TB and JD tissue samples

In the present study, both lungs and mediastinal lymph nodes had well-organized and poorly-organized granulomas, where majority of the granulomas were well-demarcated, having multifocal areas of central necrosis with calcification and circumscribed by fibrous encapsulation. The

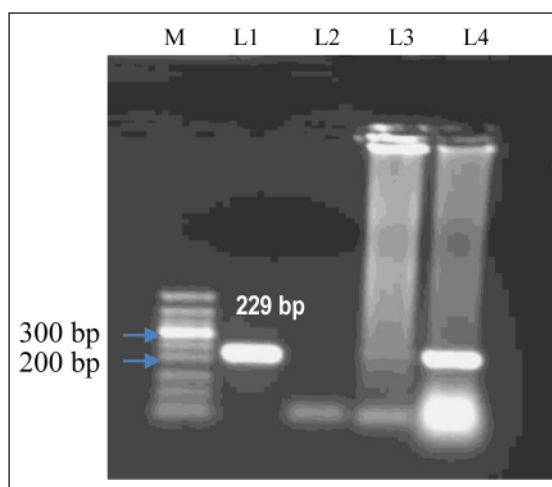


Figure 5. Amplification of DNA from the samples using IS900 primers.

[Lane M – 50 bp ladder, L1 – Positive control (MAP), L2 – Negative control, L3 & L4 – Tissue samples (Intestine)].

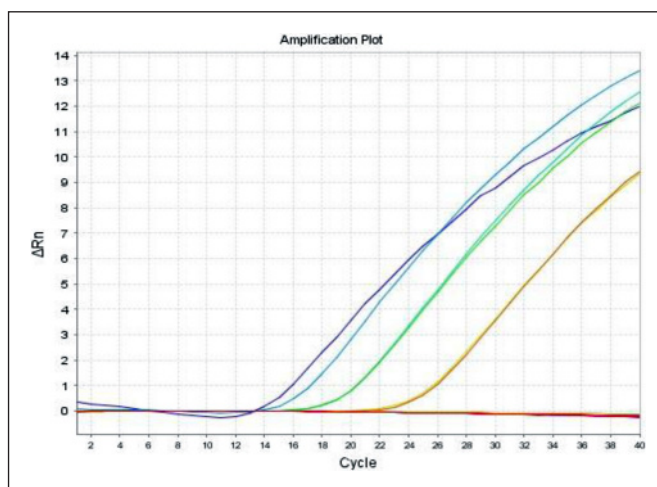


Figure 6. Amplification Plot of MAP DNA in the samples using IS900 TaqMan real-time PCR.

caseous necrosis contained nuclear debris and were surrounded by granulomatous inflammatory reaction in which dense clusters of lymphocytes, epithelioid cells, macrophages and moderate amount of scattered Langan's gaint cells were found, besides few neutrophils and plasma cells (Figure 7). Proliferative type granulomatous reaction were also observed in mediastinal lymph node (Figure 8).

The histopathological lesions of JD in the intestinal mucosa, submucosa and

mesenteric lymph nodes revealed presence of aggregations of large macrophages with abundant granular cytoplasm, epithelioid cells accompanied by focal or diffuse infiltration composed of lymphocytes, eosinophils and occasional neutrophils. Multinucleate giant cells were also seen in the intestinal mucosa and cortex of the mesenteric lymph nodes (Figure 9). Diffuse granulomatous inflammation showing atrophy of villi and a decrease in the number of crypts were also observed (Figure 10).

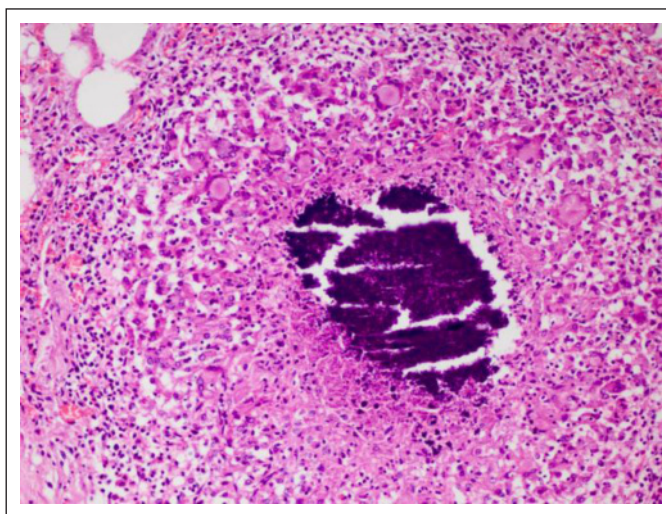


Figure 7. Typical caseo-cacifying granuloma in lung of a cow that died of TB. H&E x 20 X.

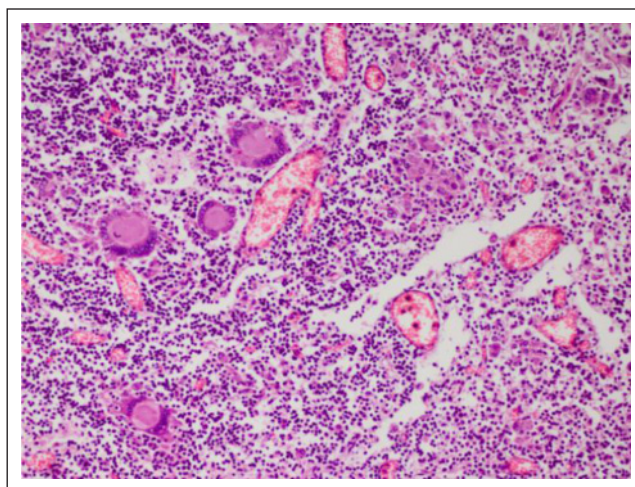


Figure 8. Proliferative type granulomatous reaction in a mediastinal lymph node of a cow that died of TB. H&E x 20 X.

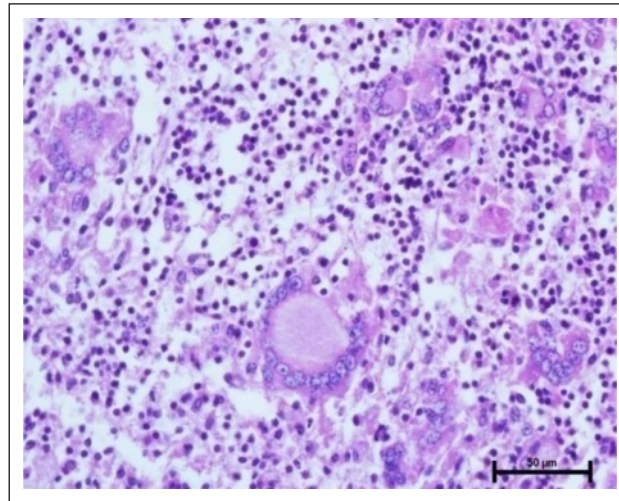


Figure 9. Mesenteric lymph node- JD – Granulomatous inflammation with typical Langhan's type Giant cells. H&E x 40 X.

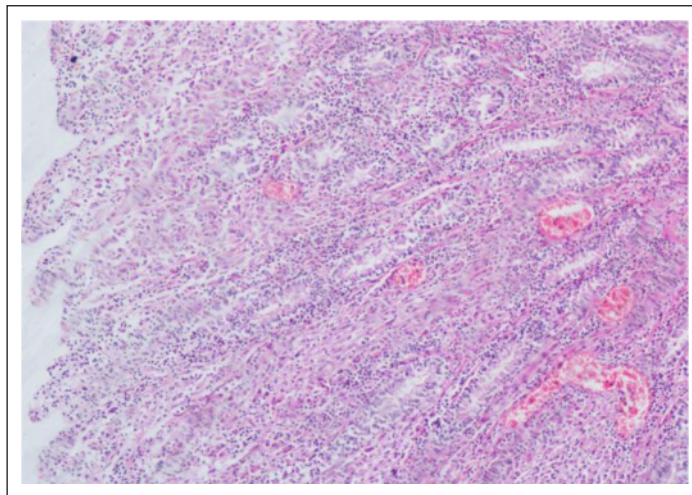


Figure 10. Intestine- JD – Diffuse granulomatous inflammation. H&E x 20X.

After all, the number of animals positive and negative for TB and JD were similar to PCR and real-time PCR, as 9 of 9 and 2 of 2 animals were detected positive for TB and JD by histopathology respectively (Table 4).

Immunohistochemical detection of TB and JD

IHC using specific polyclonal and monoclonal antibodies (Table 2) against TB and JD detected mycobacterial antigen extracellularly in caseous areas as well as

intracellularly in macrophages and giant cells (Figure 11 & 12 for TB) (Figure 13 & 14 for JD). Therefore, similar to PCR, real-time PCR and histopathology, all the 9 (56.25%) TB positive animals and 2 (22.22%) JD positive animals were found positive for anti-TB antibodies in lung and mediastinal lymph node samples and anti-JD antibodies in intestine and mesenteric lymph node samples by IHC respectively (Table 4). Thus, the sensitivity and specificity of both histopathology and IHC in detecting the TB

Table 4. Summary of the results of in-house multiplex PCR, PCR, real-time PCR, histopathology and IHC tests

	Multiplex PCR			PCR			Real-time PCR			Histopathology		IHC		
	<i>M. bovis</i>	MAP	<i>M. smegmatis</i>	ESAT-6	CFP-10	IS900	ESAT-6	CFP-10	IS900	TB	JD	ESAT-6	CFP-10	Anti-JD
Tissue samples	Lung, Mediastinal LN, Intestine and Mesenteric LN			Lung, Mediastinal LN		Intestine and Mesenteric LN	Lung, Mediastinal LN		Intestine and Mesenteric LN	Lung, Mediastinal LN	Intestine and Mesenteric LN	Lung, Mediastinal LN		Intestine and Mesenteric LN
No. of Positive cases	4	2	–	9	9	2	9	9	2	9	2	9	9	2
No. of Negative cases	12	4	16	7	7	4	7	7	4	7	4	7	7	4
Total no. of cases	16	6	16	16	16	6	16	16	6	16	6	16	16	6

LN = Lymph node

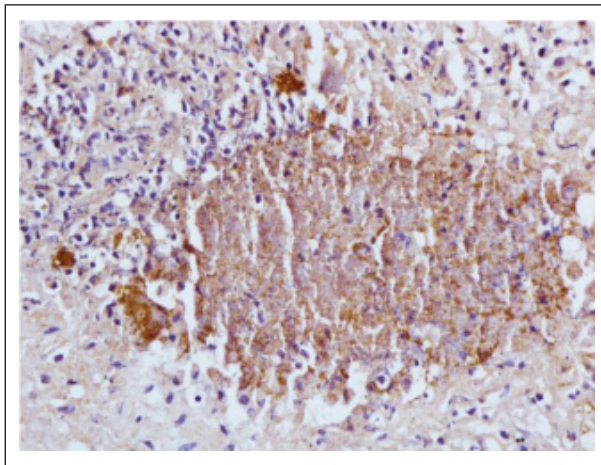


Figure 11. Lungs- ESAT 6 – Positive reactivity (Poly) Polymer HRP staining x 40 X.

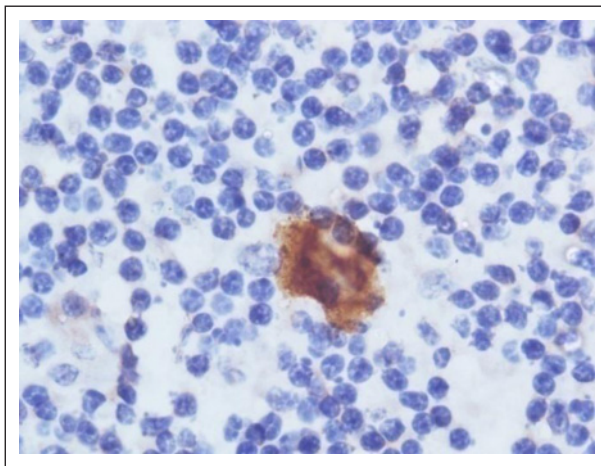


Figure 12. Mediastinal lymph node- CFP-10 – Positive reactivity (Poly) Polymer HRP staining x 100 X.

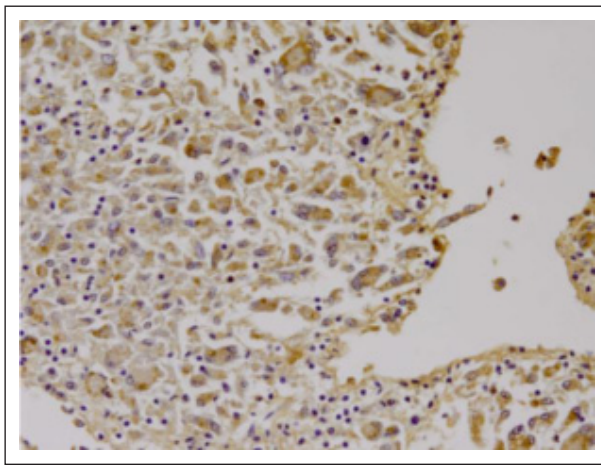


Figure 13. Intestine- JD – Positive reactivity Polymer HRP staining x 40 X.

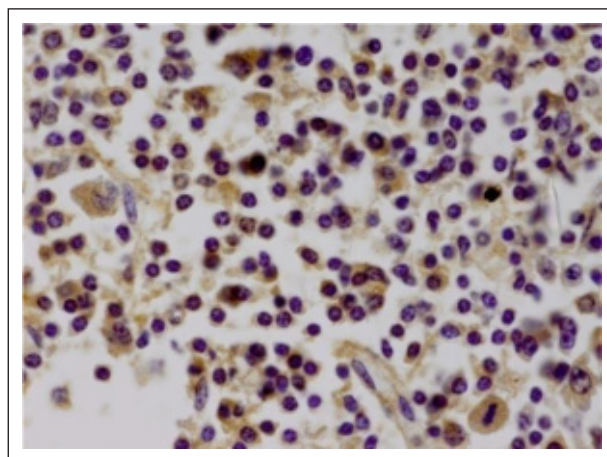


Figure 14. Mesenteric lymph node- JD – Positive reactivity
Polymer HRP staining x 100 X.

and JD granulomatous lesions were found to be 100%.

DISCUSSION

TB is characterized by progressive development of granulomatous lesions or tubercles in affected tissues and organs, mostly in the respiratory tract and associated lymph nodes (Cassidy, 2006; Liebana *et al.*, 2008) and presence of histopathological lesions: caseous necrosis, mineralization, epithelioid cells, multinucleated giant cells and macrophages (OIE, 2004). JD is characterized by granulomatous enteritis and lymphadenitis with mononuclear infiltration of macrophages, lymphocytes, plasmatic and epithelioid cells, Langhans-type giant cells and necrotic areas may also be observed (González *et al.*, 2005). However, histopathology often cannot differentiate between the lesions caused by MTC, NTM and other granulomatous diseases (Purohit *et al.*, 2007). Therefore, molecular methods, especially PCR, real-time PCR and multiplex PCR are the most promising methods for the rapid and specific diagnosis of TB and JD (Serrano-Moreno *et al.*, 2008; Figueiredo *et al.*, 2010; Dugassa and Demisie, 2014).

Multiplex PCR can differentiate different species in a single reaction. Our in-house designed multiplex PCR detected *M. bovis* and MAP in tissue samples, though *M.*

smegmatis was not detected in any of the tissue samples indicating that there was no *M. smegmatis* infection among the test tissue samples. 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 (Shah *et al.*, 2002; Bakshi *et al.*, 2005). A genus specific multiplex PCR targeting the *hsp65* (Mycobacterial genus specific), CFP-10 (MTC specific) and 16s-23s ITS (*M. avium* complex specific) had analytical sensitivity of 10 fg (3-4 cells) of Mycobacterial DNA (Gopinath and Singh, 2009).

Conventional and real-time PCR targeting ESAT-6 and CFP-10 are potential diagnostic tools for TB (Rogerson *et al.*, 2006; Dikshit *et al.*, 2012). In this study, both the primers (ESAT-6 and CFP-10) in both conventional and TaqMan real-time PCR detected same number of positive and negative cases indicating the same sensitivity and specificity for TB diagnosis. There are also reports of IS6110 PCR using T4/T5 and INS1/INS2 primers specific for MTC detecting positive in 75% cases and IS6110 real-time PCR using IS6110_T primers confirmed 37.5% samples positive for *M. bovis*, which indicated increased specificity of the real-time PCR over conventional IS6110 PCR (Phom *et al.*, 2016). The sensitivity of detection of MAP by conventional IS900 PCR was as low as

1 pg/μl of MAP DNA (Mobius *et al.*, 2008). In our study, we could detect even lower quantity of MAP DNA i.e. upto 30 fg/μl using the IS900 PCR. TaqMan real-time PCR targeting IS6110 gene detected 5 pg/μl of *M. bovis* specific DNA or even smaller quantities in tissue samples (Thacker *et al.*, 2011). The IS900 TaqMan assay can identify as low as 1 organism in pure culture (Kim *et al.*, 2002) and even as low as 5 pg of MAP specific DNA per assay (Khare *et al.*, 2004). Quantitative 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 (Lazaro *et al.*, 2005). In our study, the IS900 TaqMan real-time PCR had detection limit as low as 3 fg/μl of MAP genomic DNA. Further, real-time PCR is faster and ten times more sensitive compared to conventional PCR. However, the results obtained from both conventional and real-time PCR have shown 100% agreement, for which the most probable cause may be the advanced clinical stage of TB and JD in the affected animals, making the diagnosis more easier and accurate.

Histopathological studies revealed both well-organized and poorly-organized granulomas in the lungs and mediastinal lymph nodes of affected animals (Phom *et al.*, 2016). The sensitivity, specificity, positive and negative predictive value of classical TB histopathology were 92, 37, 60 and 81% respectively considering IS6110 PCR as the gold standard (Mustafa *et al.*, 2006). The histopathological lesion of JD is characterized by the presence of aggregations of epithelioid cells, accompanied by focal or diffuse infiltration composed of lymphocytes, eosinophils and occasional neutrophils, multinucleate giant cells in the intestinal mucosa and cortex of the mesenteric lymph nodes. The diffuse granulomatous inflammation of the intestinal mucosa is associated with atrophy of villi and a decrease in the number of crypts (González *et al.*, 2005; Dugassa and Demisie, 2014). Thus, histopathological findings for TB and JD in our study were in agreement with the above studies.

IHC using specific polyclonal antibody against *M. bovis* detected mycobacterial antigen extracellularly in caseous areas as well as intracellularly in macrophages and giant cells (Phom *et al.*, 2016). IHC of lymph node biopsies using anti-MPT64 (secreted MTC specific antigen) had over all sensitivity, specificity, positive and negative predictive values of 90–92%, 83–97%, 86–98% and 85–88% respectively (Purohit *et al.*, 2007; Mustafa *et al.*, 2006). Considering IS6110 PCR as the gold standard, an agreement of 87% between the PCR and IHC was found (Mustafa *et al.*, 2006). However, Goel and Budhwara (2007) found that the sensitivity and specificity of IHC was 100%. Similarly, there was 100% agreement between the PCR, real-time PCR, histopathology and IHC in our study.

CONCLUSION

The in-house multiplex PCR could differentiate Mycobacterial infections caused by *M. bovis*, MAP and *M. smegmatis*, though a large-scale study is required to determine whether this in-house multiplex PCR assay is adequate for TB and JD control program. The combination of PCR, multiplex-PCR, real-time PCR, histopathology and IHC techniques successfully diagnosed TB and JD from post-mortem tissue samples of cattle and buffaloes, with 100% sensitivity and specificity, though the PCR and real-time PCR are rapid method for the specific diagnosis of TB and JD compared to histopathology and IHC which are slow, laborious and time consuming method. After all, the increased ratio of TB to JD cases (9:2) at PM may be due to high mortality rate of TB infected animals than the JD. TB infection may increase the susceptibility of the animal to JD or vice versa and co-infection can occur with both the diseases. Considering the endemicity and zoonotic potential of TB and JD, there should be regular screening and appropriate preventive and control measures should be carried out in the farm.

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