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Molecular Detection of *Mycobacterium bovis* Targeting *esxB* (CFP-10) in Blood Samples and Lymph Node Aspirates by Conventional PCR and qRT-PCR TaqMan Assay

Gursher Singh Sidhu[1], Deepti Narang[1*], Mudit Chandra[1], Sikh Tejinder Singh[2] and Narinder Singh Sharma[1]

[1]Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, INDIA

[2]Department of Animal Genetics and Breeding, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, INDIA

**Corresponding author: D Narang; Email address: deeptivet@rediffmail.com*

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ABSTRACT

Bovine tuberculosis, a chronic infectious disease is caused by an intracellular acid-fast bacilli *Mycobacterium bovis* that affects broad range of mammalian hosts. CFP-10 is a 10 kDa secreted antigen coded by *esxB* gene located on RD1 region of genome and is responsible for virulence of *Mycobacterium bovis*. DNA extraction of blood (n=48) and lymph node aspirates (n=48) was done and extracted DNA was subjected to PCR by targeting *esxB* gene with band size of 302 bp. None of the blood sample and lymph node aspirates was positive for *M. bovis* with *esxB* gene by PCR. The sensitivity of *esxB* was 8 pg/µl by conventional PCR. Among 48 blood samples targeted for *esxB* (CFP-10) gene using In house developed primer probe mix, one sample (2.1%) whose C_T was 34 was considered positive by real-time PCR. Out of 48 animals (lymph node aspirates), four samples (8.3%) whose C_T was between 29-34 were considered positive by real-time PCR. Remaining samples whose C_T values were equal to or greater than 35 were considered negative. The sensitivity of *esxB* was 800 fg/µl by real time PCR. This study indicates the diagnostic potential of *esxB* by using real time PCR TaqMan Assay.

Keywords: *esxB*, CFP-10, *Mycobacterium bovis*, PCR, real time PCR

Bovine tuberculosis (bTB), a chronic debilitating disease is caused by an intracellular acid-fast bacilli *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (MTC). In bovines, tuberculosis (TB) may affect any body tissue, but the lesions are most frequently observed in lymph nodes of head and thorax, lungs, intestines, liver, pleura and peritoneum (OIE, 2009). TB in milch animals is one of the biggest concerns to dairy industry in terms of severe economic losses. Transmission to humans poses a public health problem, as zoonotic TB caused by transmission of *M. bovis* to humans, is clinically identical to infection caused by *M. tuberculosis* (Cosivi *et al*., 1998). Prevalence of TB in cattle also varied between geographical regions. It was much higher in certain dairy farms tested in Northern India (15.76%) as compared to those tested in Western India (0.65-1.85%) (Mukherjee, 2006).

The culture filtrate protein 10 (CFP-10) is a 10 kDa secreted antigen that forms a 1:1 heterodimeric complex with early secretory antigenic protein (ESAT-6) and an important virulence factor coded by *esxB* gene located on RD1 region of *M. tuberculosis* genome (Dikshit *et al*., 2012). Host immune responses are modulated by the complex formed between ESAT-6 and CFP-10. Recombinant ESAT-6:CFP-10 fusion protein is used for differentiation of infections of cattle by *M. bovis* and by *M. avium* subsp. *avium* and *M. avium* subsp. *Paratuberculosis* (Waters *et al*., 2004).

esxB gene coded by CFP-10 (*Rv3875*) was amplified

by using standard polymerase chain reaction (Dikshit *et al*., 2012). Real-time PCR is a very accurate, rapid and sensitive method for detection of mycobacteria, differentiation of mycobacterial species, quantification of mycobacterial load and detection of drug resistance in mycobacterial infection (Parashar *et al*., 2006).

MATERIALS AND METHODS

A total of 48 animals (30 cattle and 18 buffaloes) of 2-8yrs were selected randomly from an organized dairy farm in Ludhiana. Blood samples and lymph node aspirates of all the animals (n=48) were taken and screened for *M. bovis* by PCR and real time PCR targeting *esxB* gene of CFP-10 protein.

DNA extraction from whole blood and lymph node aspirates

Whole blood samples and lymph node aspirates from 48 animals were subjected to DNA extraction using QIAamp DNA blood mini kit (Qiagen).

Identification of *Mycobacterium bovis* by Polymerase Chain Reaction

Identification of *M. bovis* was done by targeting *esxB* gene (*Rv3875*) present on RD1 region of *M. bovis* using specific primers for CFP-10 with band size of about 302 bp (Dikshit *et al*., 2012). Sequence of primers used for *esxB* (CFP-10) was CFP-10F, 5' ATGGCAGAGATGAAGACCGATGCCGCT 3' and CFP-10R, 5' TCAGAAGCCCATTTGCGAGGACAGCGCC 3'. Briefly, a ready to use GoTaq® Green Master Mix, 2X (Promega) (that contains GoTaq® DNA Polymerase, 400µM of each dNTPs, 3mM MgCl2 and two dyes (blue and yellow) were used. A reaction volume of 25 µl was made containing 12.5 µl of GoTaq® Green Master mix, 1 µl of forward primer (10pmol/µl), 1 µl of reverse primer (10pmol/µl), 5.5 µl of nuclease free water and 5 µl of DNA template. DNA was amplified using cycling conditions with denaturation, annealing and extension at 95ºC for 45 sec, 63ºC for 45 sec and 72ºC for 45 sec respectively with 40 amplification cycles. Specificity of *esxB* (CFP-10) gene of *M. tuberculosis* was tested by using it on mycobacterial species (*M. avium, M. kansasi*i and *M. smegmatis*) and non-mycobacterial species (*B. abortus* and *P. multocida*). Sensitivity of the *esxB* (CFP-10) gene was studied by making ten-fold serial dilutions of standard culture *M. tuberculosis* (IMTECH, Chandigarh).

TaqMan real-time PCR assay for the detection of *Mycobacterium bovis*

TaqMan real-time PCR assay was done for detection of *M. bovis* by using In-house developed primer and probe sequence. The sequences for the forward primer, reverse primer and probe, respectively were as follows: CFP-10: 5'- GGCGACCTGAAAACCCAGAT-3', 5'-TGCTTATTGGCTGCTTCTTGGAA-3' and 5'-CCCT GCAACGAACCT-3'. The probe was labelled with the fluorescent reporter dye FAM on the 5' end and the quencher dye NFQ-MGB on the 3' end. A reaction volume of 20 µl was made containing 10 µl of TaqMan master mix (2X), 1 µl of primer-probe mix (20X), 7 µl of nuclease free water and 2 µl of DNA template. Cycling conditions used for real-time PCR were as per the default settings of Applied biosystems Step One Plus Real-Time system.

RESULTS AND DISCUSSION

Detection of *M. bovis* in blood samples by Polymerase Chain Reaction (PCR)

In the present study, the sensitivity of *esxB* targeting CFP-10 protein was assessed by making ten-fold serial dilutions of the known concentration (8 ng/µl) of the standard genomic DNA of *M. tuberculosis*. The detection limit of the *esxB* (CFP-10) was upto 8 pg/µl (Fig. 1).

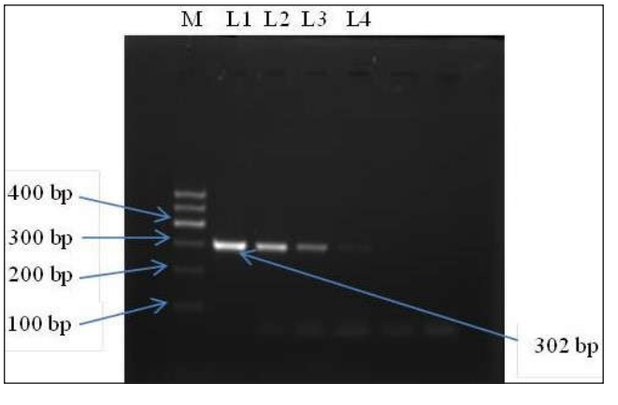

Fig. 1: Sensitivity of PCR for *esxB* (CFP-10) gene of *M. tuberculosis.* M: Ladder Marker(100bp); L1: Undiluted positive control DNA; L2: 10^{-1} dilution; L3: 10^{-2} dilution; L4: 10^{-3} dilution.

Specificity of *esxB* gene targeting CFP-10 protein of *M. tuberculosis* was tested by using it on other mycobacterial species (*M. avium, M. kansasii* and *M. smegmatis*) and non-mycobacterial species (*B. abortus* and *P. multocida*). None of the organisms other than *M. tuberculosis* showed amplification which clearly indicates the specificity of *esxB* gene of *M. tuberculosis* in PCR (Fig. 2).

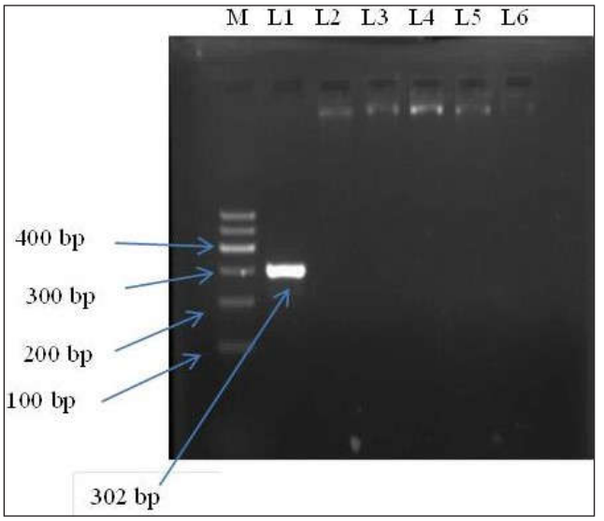

Fig. 2: Specificity of PCR for *esxB* (CFP-10) gene of *M. Tuberculosis*. M: Ladder Marker(100bp); L1: Positive control *M. tuberculosis*; L2: *M. avium*; L3: *M. kansasii*; L4: *M. smegmatis*; L5: *B. abortus*; L6: *P. multocida*.

In the present study, cycling conditions were standardized using the standard culture of *M. tuberculosis* and PCR amplification yielded specific amplicon of 302 bp by *esxB* (CFP-10) gene. None of the 48 blood samples were positive for *M. bovis* with *esxB* (CFP-10) gene by PCR. It might be due to the low concentration of DNA (even less than 8 pg/µl) in clinical samples which remains undetected by PCR or the DNA template per section is very low (Gomez-Laguna *et al*., 2010; Marchetti *et al*., 1998). Along with sample DNA, a known positive control DNA from *Mycobacterium tuberculosis* culture (IMTECH), Chandigarh was also amplified.

Detection of *M. bovis* in Lymph node aspirates by Polymerase Chain Reaction (PCR)

Lymph node aspirates were taken from the animals that were positive either by CITT or IFN-γ. None of the 48 lymph node aspirates were found positive for *M. bovis* with *esxB* (CFP-10) gene by PCR. Along with sample DNA, a known positive control DNA from *Mycobacterium tuberculosis* culture (IMTECH) was also amplified.

Detection of *Mycobacterium bovis* in blood samples and lymph node aspirates by TaqMan Real-time PCR

In the present study, the sensitivity of *esxB* targeting CFP-10 protein was assessed by making ten-fold serial dilutions of the known concentration (8 ng/µl) of the standard genomic DNA of *M. tuberculosis*. The detection limit of the *esxB* (CFP-10) was upto 800 fg/µl by TaqMan real time assay which corresponded to a C_T of 34.1 as shown in Table 1 and Fig. 3.

Table 1: C_T values against serial dilutions of standard *M. tuberculosis* DNA (8ng/µl) (CFP-10)

Dilutions of standard DNA	Concentration of DNA (ng/µl)	C_T Value Using TaqMan assay
Neat	8	19.9
1:10	0.8	22.8
1:100	0.08	26.9
1:1000	0.008 ng/µl ~8 pg/µl	30.5
1:10000	0.0008 ng/µl ~0.8 pg/µl ~800 fg/µl	34.1

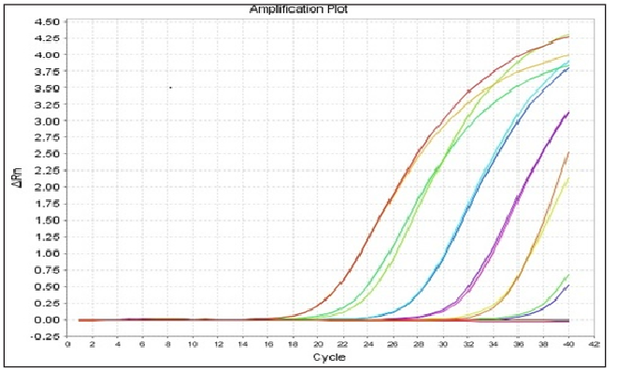

Fig. 3: Amplification plot showing sensitivity of *esxB* (CFP-10) gene of *M. tuberculosis* by qRT-PCR

Specificity of *esxB* gene targeting CFP-10 protein of *M. tuberculosis* was tested by using different mycobacterial species (*M. avium, M. kansasii* and *M. smegmatis*) and

non-mycobacterial species (*B. abortus* and *P. multocida*) (Fig. 4). None of the organisms other than *M. tuberculosis* showed amplification which clearly indicates the specificity of *esxB* gene of *M. tuberculosis* in qRT-PCR.

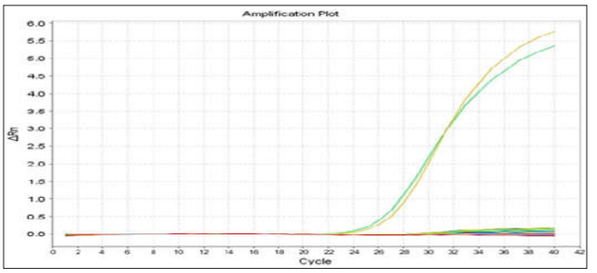

Fig. 4: Amplification plot showing specificity of *esxB* (CFP-10) gene of *M. tuberculosis* by qRT-PCR

In the present study, among 48 blood samples targeted for *esxB* (CFP-10) gene, one sample whose C_T was 34 was considered positive by real-time PCR (Fig. 5). Remaining samples whose C_T values were equal to or greater than 35 were considered negative (Table 2). Out of 48 lymph node aspirates targeted for *esxB* (CFP-10) gene, four samples whose C_T was between 29-34 were considered positive by real-time PCR (Fig. 6). Remaining samples whose C_T values were equal to or greater than 35 were considered negative (Table 2) based on the sensitivity testing as shown in Table 1.

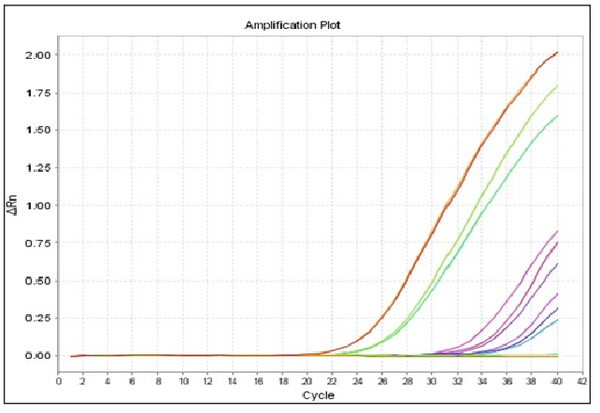

Fig. 5: Amplification Plot of *M. bovis* DNA in blood samples targeting *esxB* (CFP-10) gene using RT-PCR TaqMan Assay

In cattle, clinical evidence of tuberculosis occurs only when extensive lesions have developed (OIE, 2004). Conventional laboratory diagnosis of TB relies mainly on isolation, though gold standard is time consuming and acid-fast staining is low in sensitivity and does not identify the species of Mycobacterium causing the disease so interpretations of the results of conventional methods are highly subjective and prone to errors (Parsons *et al*., 2002). Therefore, a multidisciplinary approach must be conducted, using various currently available methods (Medeiros *et al*., 2010).

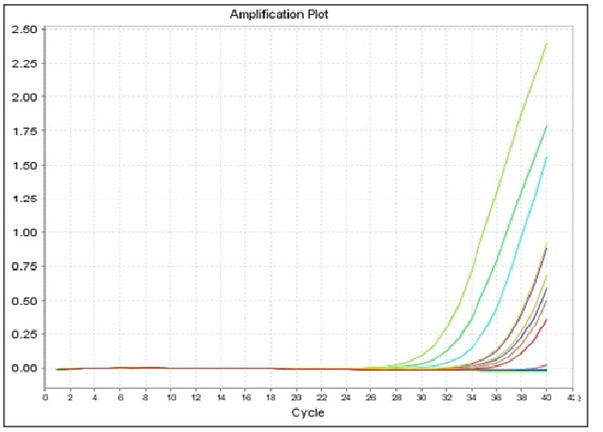

Fig. 6: Amplification Plot of *M. bovis* DNA in lymph node aspirates targeting *esxB* (CFP-10) gene using qRT-PCR TaqMan Assay

Table 2: *M. bovis* specific TaqMan RT-PCR results in blood and lymph node aspirates

C_T values	CFP-10	
	Blood real-time PCR	Lymph node aspirate real-time PCR
23-25	—	—
26-28	—	—
29-31	—	1
32-34	1	3
35-39	14	19

esxB was amplified by targeting CFP-10 protein by PCR in DNA extracted of the standard culture of *M. tuberculosis* (Dikshit *et al*., 2012). All the blood samples and lymph node aspirates were found negative for *M. bovis* with *esxB* (CFP-10) gene which might be due to the low concentration of DNA (even less than 8 pg/µl) in clinical samples

which remains undetected by PCR (Gomez-Laguna *et al*., 2010; Marchetti *et al*., 1998) or the sample may contain some PCR inhibitors that may hinder the PCR assay (Chakravorty *et al*., 2005). Several alternative approaches have been attempted for the rapid and specific diagnosis of tuberculosis, but molecular methods, especially Polymerase Chain Reaction (PCR) is the most promising (Figueiredo *et al*., 2010; Serrano-Moreno *et al*., 2008). MTC was detected in formalin-fixed, paraffin-embedded tissues by Polymerase Chain Reaction using the primers IS6110 in 92 of 99 (93%) tuberculosis cases (Miller *et al*., 2002). Detection of *M. bovis* in blood and tissue samples of cattle was done using the JB21-JB22 primer pair (Zali *et al*., 2014) and also using IS6110 insertion sequences (Bassessar *et al*., 2014).

The detection limit of the *esxB* (CFP-10) was upto 800 fg/µl by TaqMan real time assay which corresponded to a C_T of 34.1 as shown in Table 1 and Fig. 3, so in accordance with it one blood sample and four lymph node aspirates were found positive by Real-time PCR whose C_T was in range 29-34. Similarly *M. bovis* was detected in lymph node samples of cattle by using TB1 primers by Real-time PCR. 13 (76%) out of the 17 samples were giving positive amplification curves which were interpreted according to the presence of fluorescence FAM curve with the threshold line. Real-time PCR detected 9 out of 50 blood samples to be positive for *M. bovis* (Helmy *et al*., 2015). TaqMan real-time PCR detected *M. bovis* by targeting RD4 region and found 8 (2%) blood samples positive out of 401 samples (Cezar *et al*., 2016). The TaqMan method detects as low as 5 pg/µl of *M. bovis* specific DNA and has the potential to detect even smaller quantities. A similar level of template DNA was detected by TaqMan assay targeting IS6110 gene in tissue samples (Thacker *et al*., 2011).

The sensitivity of real time PCR was ten times more of the conventional PCR. This indicates that there was less than 8 pg/µl of DNA in the samples which were undetectable in conventional PCR but were detected by real time PCR TaqMan assay.

CONCLUSION

The In-house developed *esxB* (CFP-10) primer-probe for real time PCR TaqMan Assay can be used in the diagnosis of *M. bovis* in blood samples and lymph node aspirates. The sensitivity of *esxB* was 8 pg/µl and 800 fg/µl by conventional PCR and real time PCR respectively. The specificity of *esxB* was shown as it was present only in *M. tuberculosis* and *M. bovis* and not in some of the other Mycobacterial species as well as in other non-mycobacterial species (both in conventional PCR and real time PCR). This study indicates the diagnostic potential of *esxB* by using real time PCR TaqMan Assay.

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