RESEARCH ARTICLE Indian Journal of Animal Research, Volume 54 Issue 10: 1241-1245 (October 2020)

# Real-Time PCR Detection of *Mycobacterium bovis* in Blood and Lymph Node Aspirates of Bovines Positive in Tuberculosis Screening Tests

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**10.18805/ijar.B-3573**


## ABSTRACT

Bovine tuberculosis is a chronic infectious disease affecting broad range of mammalian hosts. ESAT-6 is a low molecular weight immunodominant protein coded by *esxA* gene located on RD1 region of genome and is responsible for virulence of *Mycobacterium bovis.* Out of 200 animals screened for bovine Tuberculosis (bTB), 38 animals (19%) were found positive for Comparative intradermal tuberculin test (CITT) (32 cattle, 6 buffaloes) and 41 animals were tested positive by IFN-γ assay (29 cattle, 12 buffaloes). DNA extraction of blood (n=200) and lymph node aspirates (n=48) was done. Among 200 blood samples targeted for *esxA* (ESAT-6) gene, three samples (1.5%) whose $C_T$ was between 23-34 were considered positive by real-time PCR. Out of 48 animals (lymph node aspirates) that were positive either by CITT or IFN-γ, one sample (2.08%) whose $C_T$ was between 23-34 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 *esxA* was 8 pg/µl by real time PCR.


**Key words:** CITT, ESAT-6, *esxA*, IFN-γ, *Mycobacterium bovis*.

## INTRODUCTION

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).

Tuberculin skin tests are the international standard tests for ante mortem diagnosis of bTB in cattle herds De la Rua-Domenech *et al*. (2006). Among them, the Comparative intradermal tuberculin test (CITT) involves intra-dermal injections of bovine and avian tuberculin at different sites, usually on the same side of the neck approximately 12-15cm apart and measuring the response three days later. (OIE, 2009). Another test is Gamma interferon assay (IFN-γ) which detects and quantifies the release of cytokine (IFN-γ) from sensitized lymphocytes when whole blood is cultured with bovine and avian tuberculin for 16-24 hrs (Rothel *et al*. 1992). ESAT-6 (*Rv3874*) is a low molecular weight immunodomi--nant protein and an important virulence factor coded by *esxA* gene located on RD1 region of genome which is absent in all vaccine strains of avirulent *M. bovis* BCG, but present in the virulent laboratory and clinical strains of *M. bovis* and *M. tuberculosis*, which was amplified using PCR by Dikshit *et al*., 2012 and by Real time PCR by Rogerson *et al*. (2006).

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**How to cite this article:** Sidhu, G.S., Narang, D., Filia, G., Singh, A., Singh, S.T., Chandra, M. and Sharma, N.S. (2020). Real-Time PCR Detection of *Mycobacterium bovis* in Blood and Lymph Node Aspirates of Bovines Positive in Tuberculosis Screening Tests. Indian Journal of Animal Research. 54(10): 1241-1245.

**Submitted:** 02-02-2019 **Accepted:** 15-05-2020 **Published:** 28-07-2020

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).

Keeping in view the importance of presence of *esxA* in the pathogenic species of members of *Mycobacterium tuberculosis* complex and it's absence in all vaccine strains

of avirulent *M. bovis* BCG, this study was designed to evaluate the diagnostic potential of esxA by Real Time PCR, in blood of bTB suspected cattle and buffaloes and in lymph node aspirates of bTB positive reactors.

## MATERIALS AND METHODS

### Selection of animals

A total of 200 animals (66 cattle and 134 buffaloes) from different age groups (cattle 2-8yrs and buffaloes 2-7 yrs) were selected randomly of which 170 were from an organized dairy farm and 30 were from an unorganized dairy farm in Ludhiana (Punjab), India. Lymph node aspirates were collected from animals found positive in screening tests. All the animals were screened for *M. bovis* by CITT and IFN-γ. The work was conducted after taking permission from Institutional Animal Ethics Committee (IAEC/2015/30-63).

### Screening of animals by CITT and IFN-γ

The selected animals were subjected to CITT, as per OIE Terrestrial Manual (2009) after blood collection and IFN-γ assay was performed using *Mycobacterium bovis* Gamma Interferon Assay Kit for Cattle (BOVIGAM) procured from Prionics, Switzerland and results were interpreted according to manufacturer's instructions.

### Molecular Characterization by Taq Man Real Time PCR

Whole blood samples from all the 200 animals and lymph node aspirates from animals positive by either of the CMI based tests (CITT and IFN-γ assay) were subjected to DNA extraction using QIAamp DNA blood mini kit (Qiagen). TaqMan real-time PCR assay was done for detection of *M. bovis* by using primer and probe sequence as per the method of Rogerson *et al*. (2006). The sequences for the forward primer, reverse primer and probe, respectively were as follows: ESAT-6: 5′-GTACCAGGGTGTCCAGCAAAA-3′, 5′-CTGCAGCGCGTTGTTCAG-3′ and 5′-GGGACGCCACGG CTACCG-3′. The probe was labelled with the fluorescent reporter dye VIC 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. Specificity of *esxA* (ESAT-6) gene of *M. tuberculosis* was tested by using it on standard DNAs of mycobacterial species (*M. avium, M. kansasii* and *M. smegmatis*) and non-mycobacterial species (*B. abortus* and *P. multocida*). Sensitivity of the *esxA* (ESAT-6) gene was studied by making ten-fold serial dilution of DNA from culture of *M. tuberculosis* (IMTECH, Chandigarh).

## RESULTS AND DISCUSSION

### Screening of animals by CITT and IFN-γ

The antemortem diagnosis of bovine TB is based on intradermal tuberculin testing *in vivo* and detection of release of gamma interferon on stimulation with bovine and avian PPD *in vitro*. CITT is more specific than single intradermal tuberculin test (SID) as it distinguishes infection with *M. bovis* from *M. avium* or *M. avium* subsp. *Paratuberculosis*. In the present study, out of total 200 animals screened for bTB using CITT and IFN-γ release assay, 38 animals (19%) were found positive by CITT (32 cattle and 6 buffaloes). Out of these only 23 animals (19 cattle and 4 buffaloes) showed an exclusively positive reaction to CITT and negative to IFN-γ. Animals that showed an exclusive positive reaction to CITT and negative to IFN-γ may be due to co-infection of the animals with other environmental M*ycobacterium* as also suggested by De la Rua-Domenech *et al*. (2006). Specificity is influenced by sensitisation as a result of exposure to *M. avium*, *M. avium paratuberculosis* and other environmental Mycobacteria (Monaghan *et al*. 1994). In addition, false negative responses are sometimes seen in animals with poor immune responses and in animals that have recently calved.

A total of 41 animals were tested positive (29 cattle and 12 buffaloes) in Gamma Interferon assay. Out of these only 26 animals (16 cattle and 10 buffaloes) showed an exclusive positive reaction to IFN-γ. A total of 15 animals showed a positive response to both IFN-γ and CITT. Animals that showed an exclusive positive reaction to IFN-γ suggests that these animals might be in the early stages of the disease which could not be detected by CITT as also supported by Gormley *et al*. (2006). In cattle, clinical evidence of tuberculosis occurs only when extensive lesions have developed (OIE, 2004). There is growing perception that no single method is sufficient for detecting reactive animals due to complex characteristics of bTB and variable sensitivity of individual test. Therefore, a multidisciplinary approach must be conducted, using various currently available methods.

### Statistical analysis

To compare the degree of agreement between the two tests, Kappa test was applied statistically and the results are given in Table 1. At 95% level of significance, kappa value between CITT and IFN-γ was 0.227, indicating a fair degree of agreement between two tests. So both tests, when used simultaneously increase detection of maximum number of dairy animals.

### Detection of *M. bovis* by TaqMan Real-time PCR

### Sensitivity of Real time PCR for *esxA* (ESAT-6) gene of *M. tuberculosis*

The sensitivity of the *esxA* (ESAT-6) 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 *esxA* (ESAT-6) was upto 8 pg/µl (Table 2 and Fig 1).

### Specificity of Real time PCR for *esxA* (ESAT-6) gene of *M. tuberculosis*

In the present study, specificity of *esxA* gene 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 2). None of the organisms other than *M. tuberculosis* showed amplification which clearly indicates the specificity of *esxA* gene of *M. tuberculosis* in qRT-PCR.

### *esxA* (ESAT-6) *M. bovis* specific TaqMan real-time

PCR TaqMan real time PCR assay measures the amount of target DNA produced during each cycle of an amplification reaction in a real-time format. Thus, the system is able to quantify the amount of target DNA in contrast to the conventional PCR, which measures only the end-point values with qualitative results. Each sample was run in duplicate. The sensitivity of the detection of *esxA* (ESAT-6) in Real time PCR was upto 8 pg/µl. 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

**Table 1:** TB results with CITT and IFN-γ.

| Intra-dermal Test Result | No. of Animals with IFN-γ positive | No. of Animals with IFN-γ negative | Total No. of Animals |
|---|---|---|---|
| Positive | 15 | 23 | 38 |
| Negative | 26 | 136 | 162 |
| Total | 41 | 159 | 200 |

**Table 2:** $C_T$ values against serial dilutions of standard *M. tuber-culosis* DNA (8ng/µl).

| Dilutions of standard DNA | Concentration of DNA (ng/µl) | $C_T$ Value Using TaqMan assay |
|---|---|---|
| Neat | 8 | 20.4 |
| 1:10 | 0.8 | 26.8 |
| 1:100 | 0.08 | 30.7 |
| 1:1000 | 0.008 ng/ µl ~8 pg/ µl | 34.8 |

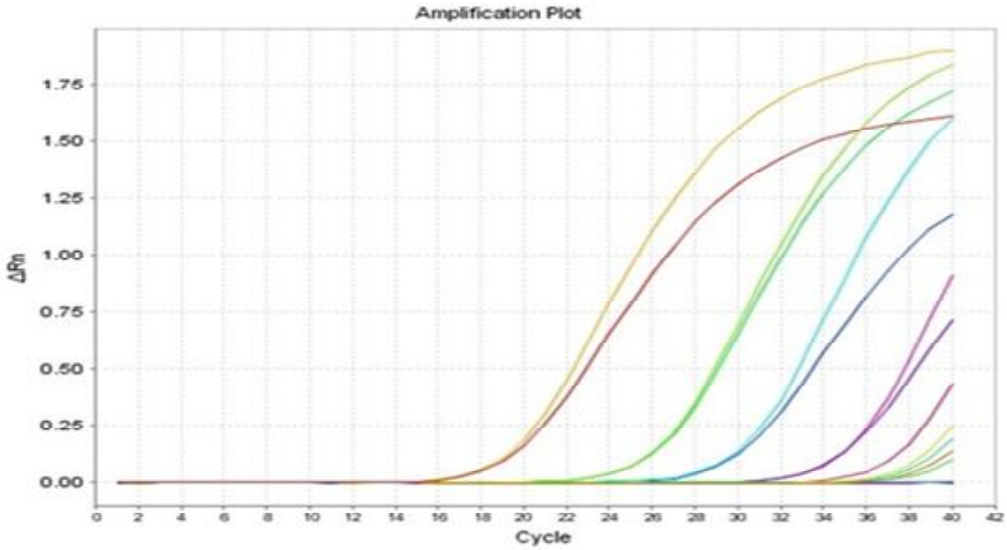


**Fig 1:** Amplification plot showing sensitivity of *esxA* (ESAT-6) gene of *M. tuberculosis* by qRT-PCR.

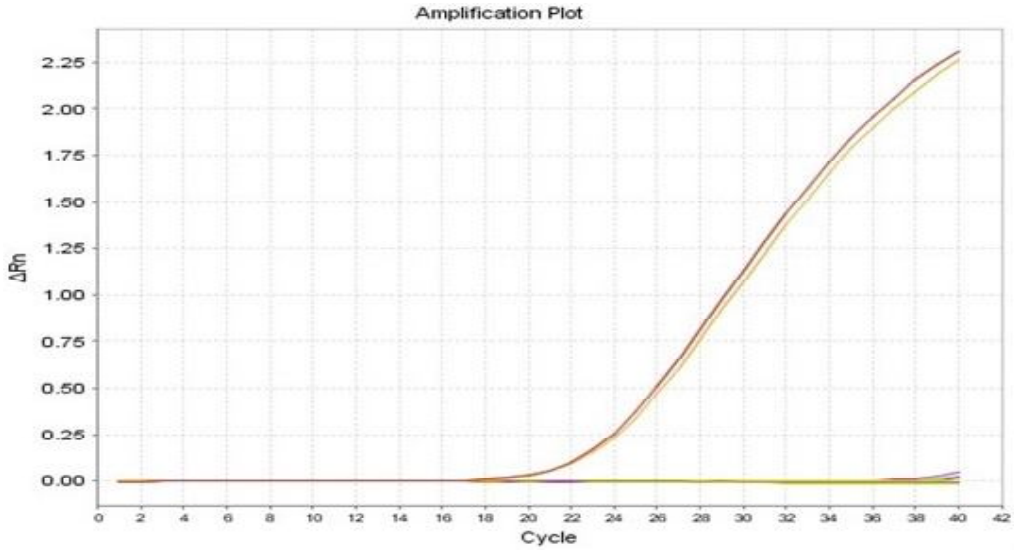


**Fig 2:** Amplification plot showing specificity of *esxA* (ESAT-6) gene of *M. tuberculosis* by qRT-PCR.

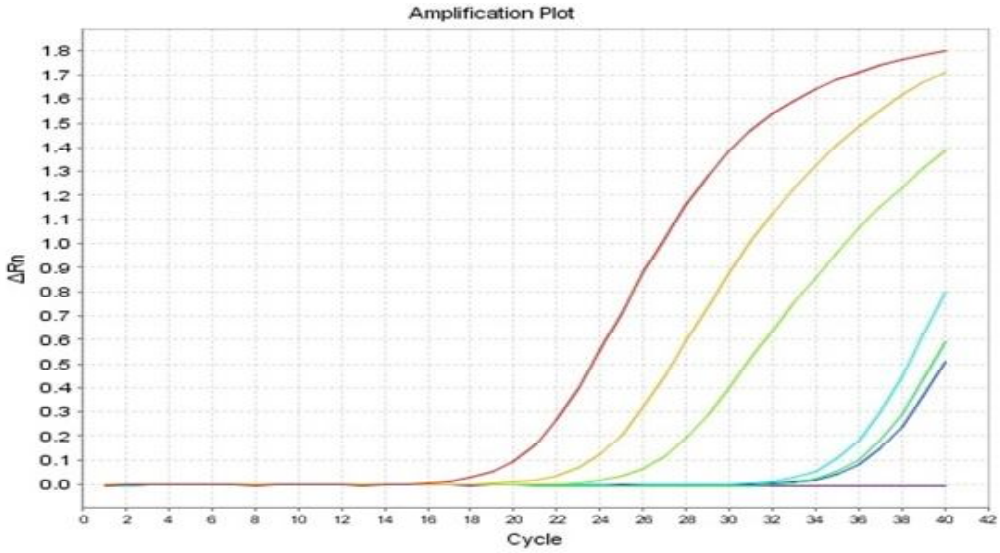


**Fig 3:** Amplification Plot of *M. bovis* DNA in blood samples targeting *esxA* (ESAT-6) gene using qRT-PCR TaqMan Assay.

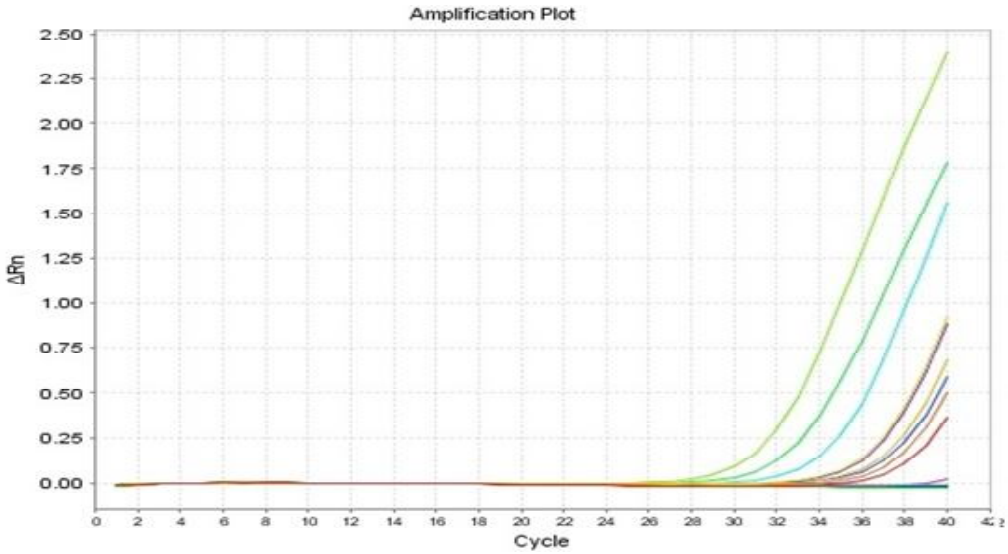


**Fig 4:** Amplification Plot of *M. bovis* DNA in lymph node aspirates targeting *esxA* (ESAT-6) gene using qRT-PCR TaqMan Assay.

IS6110 gene in tissue samples by Thacker *et al*. (2011). None of the organisms other than *M. tuberculosis* showed amplification which clearly indicates the specificity of *esxA* gene of *M. tuberculosis* in qRT-PCR. In the present study, among 200 blood samples targeted for *esxA* (ESAT-6) gene, three samples (1.5%) whose $C_T$ was between 23-34 were considered positive by real-time PCR. Remaining samples whose $C_T$ values were equal to or greater than 35 were considered negative (Table 3). Out of three samples which were positive by blood real-time PCR for ESAT-6, two of the samples were also positive by IFN-γ and one sample was positive by both CITT and IFN-γ (Fig 3).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).

Out of 48 animals (lymph node aspirates) that were positive either by CITT or IFN-γ, one sample (2.08%) whose $C_T$ was 30 was considered positive by real-time PCR (Fig 4).

**Table 3:** *M. bovis* specific TaqMan RT-PCR results in blood and lymph node aspirates.

| $C_T$ values | ESAT-6 | |
|---|---|---|
| | Blood real-time PCR | Lymph node aspirate real-time PCR |
| 23-25 | 2 | - |
| 26-28 | 1 | - |
| 29-31 | - | - |
| 32-34 | - | 1 |
| 35-39 | 13 | 24 |

Remaining samples whose $C_T$ values were equal to or greater than 35 were considered negative based on the sensitivity testing as shown in Table 2. One lymph node aspirate sample that was positive by real-time PCR for ESAT-6 was also positive by both CITT and IFN-γ.

## CONCLUSION

The results of the present study indicate that *esxA* gene targeting ESAT-6 protein can be used in the diagnosis of *M. bovis* by real time PCR in blood samples and lymph node aspirates. The sensitivity of *esxA* was 8pg/µl by real time PCR. None of the blood samples which were negative for CMI responses by CITT and IFN-γ gave a positive reaction to *esxA* indicating that these animals are not exposed to Mycobacterial species. The specificity of *esxA* 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. This study indicates the diagnostic potential of *esxA* by using real time PCR TaqMan Assay. The use of ESAT-6 proteins need to be compared *vis a vis* tuberculin PPD.

## Conflicts of Interest

There is no conflict of interest.

## ACKNOWLEDGEMENT

The authors are thankful to DBT for providing the necessary funds under the scheme “Evaluation of diagnostic assays for quicker diagnosis of Mycobacterial infections in cattle and buffaloes” (BT/PR5776/MED/30/928/2012) and the Director of Research, GADVASU, Ludhiana for providing the necessary facilities.

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