SHORT COMMUNICATION

Detection of *Mycobacterium* Species by Targeting *esxA*, *esxB* and *espC* genes in Cattle and Buffaloes

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Abstract

Bovine tuberculosis (bTB) is a chronic disease having a potential zoonotic threat and has a huge economic importance due to morbidity, mortality and production losses. bTB is endemic in India and has a worldwide prevalence, therefore there is a need for early diagnostic technique for the eradication of bTB globally. The objective of this study was to diagnose bTB by targeting *esxA*, *esxB* and *espC* genes in the blood of cattle and buffaloes. A total of 223 blood samples were randomly collected from cattle and buffaloes with a history of respiratory distress. The samples were subjected to conventional PCR targeting *esxA*, *esxB* and *espC* genes. A total of 12 samples out of 223 blood samples were found positive for *esxA, esxB* and *espC* by conventional PCR. Blood sample can be used for molecular detection of *Mycobacterium tuberculosis* organisms by targeting *esxA, esxB* and *espC* genes.

Key Words: Bovine tuberculosis**,** *Mycobacterium tuberculosis complex*, Polymerase Chain Reaction.

Ind J Vet Sci and Biotech (2023): 10.48165/ijvsbt.19.1.25

Introduction

Bovine tuberculosis (bTB), a chronic disease of animals is caused by *Mycobacterium bovis* (*M. bovis*) which is closely related to the bacteria causing human and avian tuberculosis. For the purpose of diagnosing tuberculosis (TB), the purified protein derivative (PPD), comprising both mycobacterial secreted and somatic proteins, has been used. This PPD is used in single intradermal (SID) or double intradermal (DID) tuberculin skin testing. In addition to skin testing, polymerase chain reaction (PCR) targeting immunodominant and highly immunogenic *esxB* [10-kDa culture filtrate antigen (CFP-10)] and *esxB* [6-kDa early secretory antigen (ESAT-6)], respectively can also be used for diagnosis of *Mycobacterium tuberculosis complex* (Millington *et al*., 2011 and Brahma *et al.,*2019). Both these proteins are potent T-cell antigens which are observed in 70% of TB patients which has led to their use as diagnostic reagents for TB in both humans and animals. Both latent and active TB infections can be effectively differentiated by *espC*, and T-cell responses to *espC* are very specific (93%) for *M. tuberculosis* infection (Millington *et al*., 2011). These antigens can also be used to distinguish between BCG vaccinated animals and NTM sensitized animals. This RD1 region contains genes for nine proteins (Rv3871-Rv3879c) which are involved in pathogenesis of TB. Among these, the genes Rv3874 and Rv3875 code for two proteins CFP-10 and ESAT-6, respectively. The expression of these two genes is co-ordinately regulated and both are found at low levels in *M.tuberculosis* and *M. bovis* culture supernatants (Millington *et al*., 2011). Real-time PCR assay has been used to detect *M. bovis* in blood samples. The sensitivity and specificity of real-time PCR was found to be 25.81% and 94.73% respectively (Kaya *et al.,* 2015). Cezar *et al.* (2016) detected *M. bovis* using TaqMan real-time PCR by targeting RD4 region and found one (0.25%) milk sample and 8 (2%) blood samples positive out of 401 samples. Hence keeping in mind the above facts the present study was designed to detect *Mycobacterium* species by targeting *esxA, esxB* and *espC* genes in blood of cattle and buffaloes.

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How to cite this article: Singh, S., Narang, D., Chandra, M., Singh, R., & Singh, J. (2023). Detection of Mycobacterium Species by Targeting esxA, esxB and espC genes in Cattle and Buffaloes. Ind J Vet Sci and Biotech. 19(1), 109-111.

Source of support: Nil

Conflict of interest: None

Submitted: 28/07/2022 **Accepted:** 20/10/2022 **Published:** 10/01/2023

Materials And Methods

Ethical statement: The approval of research protocol was obtained by IAEC (Registration no. *497/GO/Re/SL/01/CPCSEA*).

Selection of Animals: The study included different organised and unorganised, dairy farms across districts of

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Punjab. Animals (cattle and buffaloes), mainly with history of respiratory distress from age group 1-8 years were selected randomly from herds. A total of 223 animals (214 cattle and 9 buffaloes) were included in the study.

DNA extraction from whole blood: Whole blood samples from 223 animals were subjected to DNA extraction using QIAamp DNA blood mini kit (Qiagen, Germany) as per instructions of the manufacturer. DNA was stored at -20˚C till further use.

Identification of *Mycobacterium tuberculosis* complex by Polymerase Chain Reaction: Identification of *Mycobacterium tuberculosis* complex was done by targeting *esxA* (Rv3874), *esxB* (Rv3875) and *espC* (Rv3615c) using a specific pair of primers, ESAT-6, CFP-10 and Rv3615c, the expected band size was about 61 bp, 302 bp and 50 bp respectively. Sequences of primers of *esxA* (ESAT-6) were forward 5′GTACCAGGGTGTCCAGCAAAA3′ and reverse 5′TGCAGCGCGTTGTTCAG3′ (Rogerson *et al.,* 2006), *esxB* (CFP-10) were forward 5′ATGGCAGAGATGAAGACCGATGCCGCT3′ and reverse 5′TCAGAAGCCCATTTGCGAGGACAGCGCC3′ (Dikshit *et al.,* 2012) and *espC* (Rv3615c) were forward 5′CG*GCTAGC*GC CACCA TGACGGAAAACTTGACCGTCCAG3′and reverse 5′GCCGATCGCGGTGGTACTGCCTTTTGAACTGGCAGGTC 3′ (Hunt *et al.,*2012). PCR was performed by targeting these proteins with some modifications suggested in these studies. A ready to use Master Mix *i.e.,* GoTaq® Green, 2X (Promega) was used. A reaction volume of 25 µL was made containing 12.5 µL of GoTaq® Green Master mix, 1 µL of forward primer (10 pmol/µL), 1 µL of reverse primer (10 pmol/µL), 5.5 µL of nuclease free water and 5 µL of DNA template. Along with sample DNA, a known positive control DNA from *Mycobacterium tuberculosis* culture (IMTECH, Chandigarh) was also amplified. Thermal cycling was performed in research thermal cycler and PCR products were run by agrose gel electrophoresis (1.5 % agrose gel prepared in 1X TBE buffer containing 0.5µg/mL of ethidium bromide) and visualized in gel-documentation system. Thermocycling parameters for *esxA* (ESAT-6) were initial denaturation of 10 min at 95˚C for 1 cycle, denaturation, annealing and extension at 94˚C, 65˚C and 72˚C respectively for 1 min for 35 cycles and final extension at 72˚C for 10 min for 1 cycle. Thermocycling parameters for *esxB* (CFP-10) were initial denaturation of 10 min at 95˚C for 1 cycle, denaturation, annealing and extension at 95˚C, 63˚C and 72˚C respectively for 45 s for 40 cycles and final extension at 72˚C for 10 min for 1 cycle. Thermocycling parameters for *espC* (Rv3615c) were initial denaturation of 2 min at 94˚C for 1 cycle, denaturation, annealing and extension at 94˚C, 54˚C and 72˚C respectively for 30 s, 30 s and 1 min for 30 cycles and final extension at 72˚C for 8 min for 1 cycle.

Results and Discussion

Detection of *Mycobacterium tuberculosis* complex in blood samples by Polymerase Chain Reaction (PCR)

In the present study, overall 5.38 % prevalence of ESAT-6 (*esxA*), CFP-10 (*esxB*) and Rv3615c (*espC*) was recorded. All the 12 samples were positive with respect to the primers with amplicon size of 61 bp, 302 bp and 50 bp respectively. Out of these positive samples, 4 were from higher age group animals i.e.>4.5 years while the rest 8 were of < 4.5 years. 5 animals were from larger herds while 7 were from medium herds. Results of the gel electrophoresis of the samples are shown in figures 1, 2 and 3 respectively.

The presence of these antigenic targets in 12 out of the 223 blood samples of bTB suspected animals indicates the fact that there is incidence of hematogenous dissemination and that it is worthwhile to depend on the blood PCR assays based on multi copy target sequences for rapid diagnosis of tuberculosis by using blood as a convenient clinical specimen.

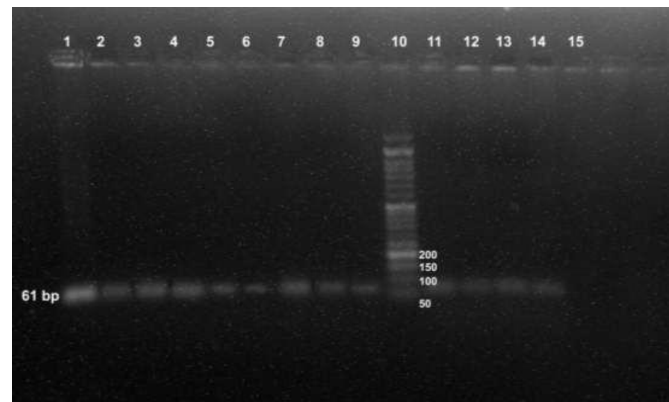

Fig. 1: Detection of esxA (ESAT-6) in blood samples of bTB suspected animals

Lane 1 : Positive control
Lane 10 : 50 bp ladder
Lane 15 : Negative control Lanes 2-9 and 11-14: Positive samples

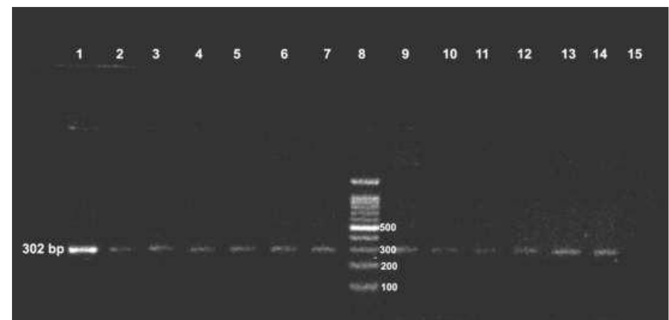

Fig. 2: Detection of esxB (CFP-10) in blood samples of bTB suspected animals

Lane 1 : Positive control
Lane 8 : 100 bp ladder
Lane 15 : Negative control Lanes 2-7 and 9-14 : Positive samples

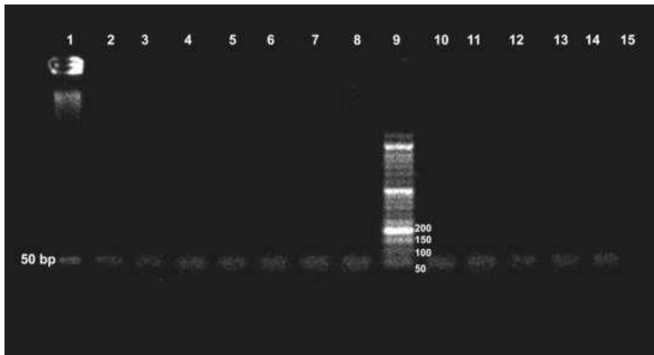

Fig. 3: Detection of espC (Rv3615c) in blood samples of bTB suspected animals

Lane 1 : Positive control
Lane 9 : 50 bp ladder
Lane 15 : Negative control Lanes 2-8 and 10-14 : Positive samples

Several alternative approaches have been attempted for the rapid and specific diagnosis of tuberculosis, but molecular methods, especially Polymerase Chain Reaction (PCR) assays, are the most promising (Serrano-Moreno *et al.,*2008 ; Figueiredo *et al.,* 2010). PCR techniques offer high sensitivity and have been successfully used for diagnosing bTB in several types of naturally infected organic materials such as tissue, blood and nasal exudates (Gomez-Laguna *et al.,* 2010).

In order to detect the presence of M. *tuberculosis* and M. *bovis*, PCR amplification of the genes *esxA* and *esxB,* which target the proteins ESAT-6 and CFP-10, respectively, in pathogenic *Mycobacterial* species, can be utilised **(**Pinxteren *et al*., 2000**).** Dikshit *et al.*(2012) reported the amplification of *esxA* and *esxB* targeting ESAT-6 and CFP-10 protein by PCR in DNA extracted from the standard culture of *M. bovis*. Such findings support the hypothesis that the escape of tubercle bacilli from alveolar spaces to the bloodstream may be more frequent in case of immune-competent patients than previously thought (Scordo *et al*., 2016).

Conclusion

It can be concluded that blood sample can be used for ante-mortem detection of bTB using PCR. However, a negative PCR assay does not rule out the disease. Prevalence was greater in animals of higher age group and across larger herds.

Acknowledgement

The authors are thankful to DBT for providing the necessary funds under the scheme **"**Mycobacterial Diseases in Animal Network (MyDAN**)** programme in cattle and buffaloes" (Registration no. *497/GO/Re/SL/01/CPCSEA*) and the Director of Research, GADVASU, Ludhiana for providing the necessary facilities.

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