

Diagnosis of Bovine Tuberculosis by Comparative Intradermal Tuberculin Test, Interferon Gamma Assay and *esxB* (CFP-10) PCR in Blood and Lymph Node Aspirates

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Abstract

Bovine tuberculosis (TB) is a chronic debilitating disease of huge economic importance due to loss in production, morbidity and mortality, and has a potential zoonotic threat. TB is endemic in India and has a worldwide prevalence, therefore, needing early diagnostic technique for the eradication of TB globally. Currently, compared to the eradication programme of TB in Medical sector, Veterinary sector is lagging behind though TB is one of the major zoonotic diseases prevalent in dairy animals and wildlife in India. With the “End TB” strategy by WHO in human, parallel measures for early diagnosis and culling has to be followed in case of animals for an overall successful eradication programme. The objective of this study is diagnosis of TB in cattle and buffaloes by using the cell-mediated immune response tests, *i.e.* Comparative Intradermal Tuberculin Test (CITT) and Interferon gamma (IFN- γ) assay, and Polymerase Chain Reaction (PCR) targeting *esxB* gene (CFP-10 protein) and to compare their diagnostic capabilities. This study was carried out in 202 dairy cattle and buffaloes from an organized dairy farm, where almost all of the animals appeared clinically healthy. We found that, the combined use of both CITT and IFN- γ assay lead to more accurate diagnosis of TB, although IFN- γ assay was more specific than CITT. However, *esxB* PCR showed almost similar sensitivity to IFN- γ assay and may be used as a fast alternative method for the diagnosis of bovine TB from blood samples.

Keywords

Bovine TB, CITT, IFN- γ , *esxB* PCR

1. Introduction

TB caused by *Mycobacterium tuberculosis* complex (MTC) comprising of *M. bovis*, *M. caprae* and *M. tuberculosis*, is a major infectious and chronic wasting disease, having a zoonotic potential and a worldwide distribution [1]. TB in milch animals is mainly caused by *M. bovis* [2]. Since 2015, the World Health Organization (WHO) has adopted a new strategy for TB prevention, care and control—The End TB Strategy—with its targets to end the Global TB epidemic by reduction in the incidence rate and death rate of the disease by 90% and 95% respectively in 20 years (2015-2035) compared with level to 2015 by means of improved diagnostics, prevention and control measures [3].

Diagnosis of TB in the early stage is very important for effective prevention and control of the disease. Conventional diagnostic methods like culture and microscopy, though considered as gold standard, is time consuming and requires more than 3 weeks for the visible growth of MTC colony [4], besides, symptoms of TB mostly appears in late or advanced phase of the disease. Therefore, ante-mortem tests of cellular immune response (Tuberculin test and Interferon gamma Assay) and molecular diagnosis is required for the early diagnosis of the disease [2] [5]. Serological test like indirect ELISA can also be used complementarily for screening of anti-TB antibody in a herd [6].

Molecular diagnosis of TB using *esxB* (CFP-10) PCR targeting *esxB* gene present in the RD1 region of the pathogenic Mycobacterial species, is a fast diagnostic tool having higher sensitivity and specificity [7]. Therefore, the present study was aimed at comparative diagnosis of bovine TB in cattle and buffaloes using cell-mediated immune response tests viz. CITT and IFN- γ assay; and molecular diagnosis using *esxB* gene PCR targeting CFP-10 protein.

2. Materials and Methods

2.1. Selection of Animals and Collection of Blood and Lymph Node Aspirate Samples

A total of 202 female milch animals (42 HF-cross cows and 160 Murrah and Nili Ravi buffaloes) from 2 years and above were selected randomly from an organized dairy farm in Ludhiana, Punjab. CITT was performed on all the animals and then blood samples were collected for IFN- γ assay and *esxB* PCR. Lymph node aspirates (n = 15) from the TB reactor animals (positive by either of the two tests viz. CITT or IFN- γ assay) were also collected.

In fact, the sampling size was done irrespective of any criteria of selection and the reason for choosing only female animals for this study was because of the availability of females in large numbers and taking into consideration that female

animals are more important in transmission of the causative organism through milk.

2.2. Time and Place of Work

The present study was carried out during the year 2015-2016, at Department of Microbiology, COVS, GADVASU, Ludhiana, Punjab, India.

2.3. Variables

In this study, the variables to be tested/compared are CITT and IFN- γ assay for cell-mediated immune response and *esxB* PCR for molecular diagnosis of TB in cattle and buffaloes.

2.4. Comparative Intradermal Tuberculin Test (CITT)

The selected animals were subjected to CITT, as per OIE [2]. Bovine tuberculin PPD from culture of *M. bovis* (strain AN5, 3000 IU) and avian tuberculin PPD from culture of *M. avium* subspecies *avium* (strain D4ER, 2500 IU), obtained from Prionics (Netherlands), were used for CITT. Two 2 × 2 square inch areas were shaved at the middle third of left side of neck approximately 12 - 15 cm apart. The zero hour skin thickness was measured with the help of a vernier caliper. The bovine and the avian tuberculin PPD (0.1 ml each) were injected intra-dermally; the bovine PPD being injected in the caudal shaved area. The correction of the injection was confirmed by palpation of a small pea-like swelling at the site. Inflammatory responses were recorded 72 hours post injection. The observations were made on the basis of hot, pain and swelling at the site of injection. Animals were considered positive if the increase in skin thickness at the bovine site of injection was more than 4 mm greater than the reaction shown at the site of the avian injection. The reaction was recorded as negative, if no or ≤1 mm difference in the increase in skinfold thickness was observed. Difference between 1 - 4 mm was considered as doubtful.

2.5. Bovine Interferon Gamma (IFN- γ) Assay

This test was performed using *M. bovis* Gamma Interferon Assay Kit for Cattle (BOVIGAM, Prionics, Switzerland). BOVIGAM is a rapid *in-vitro* blood based assay of cell-mediated immune response to *M. bovis* purified protein derivative (PPD). A minimum volume of 5 ml of blood from jugular veins of each animal was collected in commercially available sterile 10 ml heparinized tubes. Three 1.5 ml aliquots of heparinized blood from each animal were dispensed into wells of 24-well tissue culture plate, to which 100 μ l each of PBS as nil antigen control (pH-7.2), avian and bovine PPD (Prionics, Netherlands) were added aseptically into the wells containing heparinized blood. The antigens were mixed thoroughly into the aliquoted blood and then incubated in a humidified atmosphere for 16 - 24 hours at 37°C. The plasma was then collected and assayed for IFN- γ production in duplicate. Optical densities were measured on an ELISA plate reader (Multiskan, MTX Lab Systems, Inc., USA) at 450 nm filter with a 620 nm reference filter. Animal was considered positive when mean OD of bovine PPD

minus mean OD of avian PPD and mean OD of bovine PPD minus mean OD of nil antigen was ≥ 0.1 . Animal was considered negative when mean OD of bovine PPD minus mean OD of avian PPD and mean OD of bovine PPD minus mean OD of nil antigen was < 0.1 .

2.6. DNA Extraction

Initially, buffy coat was obtained from 1 ml of whole blood samples by centrifugation at 10,000 rpm for 10 min. The buffy coat and lymph node aspirates were subjected to DNA extraction using QIAamp DNA blood mini kit (Qiagen). DNA was stored in -20°C till further use.

2.7. *esxB* (CFP-10) PCR

PCR targeting *esxB* gene (Rv3875) present on RD1 region of MTC encoding CFP-10 protein was done for confirmation of TB. The sequences of *esxB* primer pair were: Forward 5'ATGGCAGAGATGAAGACCGATGCCGCT3' and Reverse-5'TCAGAAGCCCATTTGCGAGGACAGCGCC3' with an expected band size of 302 bp [7]. PCR conditions were performed as per Brahma *et al.* [8]. Briefly, a ready to use GoTaq® Green Master Mix, 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), 2.5 μl of nuclease free water and 8 μl of DNA template. Along with sample DNA, a known positive control DNA from *M. tuberculosis* culture (IMTECH, Chandigarh) was also amplified. Thermal cycling was performed in research thermal cycler (Eppendorf, Germany) with 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 sec for 40 cycles and final extension at 72°C for 10 min for 1 cycle. PCR products were run by 1.5% agarose gel electrophoresis and visualized in gel-documentation system (Alpha Innotech, San Leandro, CA). The sensitivity of *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* and specificity of *esxB* primers were tested against other non-tuberculous mycobacterial species [*M. avium*, *M. kansasii*, *M. fortuitum* and *M. smegmatis* (Microbiologics)] and non-mycobacterial species (*B. abortus*, *P. multocida* and *E. coli*).

2.8. Statistical Analysis

The proportions of the animals that were positive by either CITT or IFN- γ assay were calculated. Kappa test was applied to compare the degree of agreement between the two tests at 95% level of significance.

3. Results

3.1. CITT and IFN- γ Assay

In this study, out of total 202 animals screened for TB, 40 animals (19.80%) were found to be positive by CITT (18 cattle and 22 buffaloes). Out of these only 30

animals (13 cattle and 17 buffaloes) showed an exclusively positive reaction to CITT by showing an increase in the thickness > 4 mm and a negative response to IFN- γ .

The numbers of animals positive by IFN- γ assay were 17 (7 cattle and 10 buffaloes), out of which only 7 animals (2 cattle and 5 buffaloes) showed an exclusively positive reaction to IFN- γ and a negative response to CITT. However, out of all animals, only 10 showed a positive response to both CITT and IFN- γ assay.

Based on the results of CITT and IFN- γ assay, the animals were divided into four groups, as given in **Table 1**. Group 4 was considered as the control group for further comparison of various observations. Considering the results of TB positive animals by either one or both the tests, the incidence of TB recorded in cattle (47.62%) was almost three times more than that of buffaloes (16.88%).

3.2. *esxB* (CFP-10) Gene PCR

The detection limit of the *esxB* (CFP-10) PCR was upto 8 pg/ μ l of pure culture *M. tuberculosis* DNA. None of the organisms other than *M. tuberculosis* showed amplification which clearly indicates the specificity of *esxB* gene only for the pathogenic Mycobacterial species viz. *M. tuberculosis* and *M. bovis*.

Overall, 13 (6.44%) out of 202 animals were found to be positive for TB by *esxB* (CFP-10) gene PCR. (**Figure 1**) in blood samples, where 10 of 13 were positive by both CITT and IFN- γ assay in common and the rest 3 of 13 were IFN- γ positive but CITT negative. In contrast, *esxB* PCR from the lymph node aspirates of 15 animals revealed only 1 animal to be positive for TB.

3.3. Statistical Analysis

The proportions of the animals that were positive by either CITT or IFN- γ assay are given in **Table 2**. At 95% level of significance, kappa value between CITT and IFN- γ assay was 0.264, indicating a fair degree of agreement between the two tests.

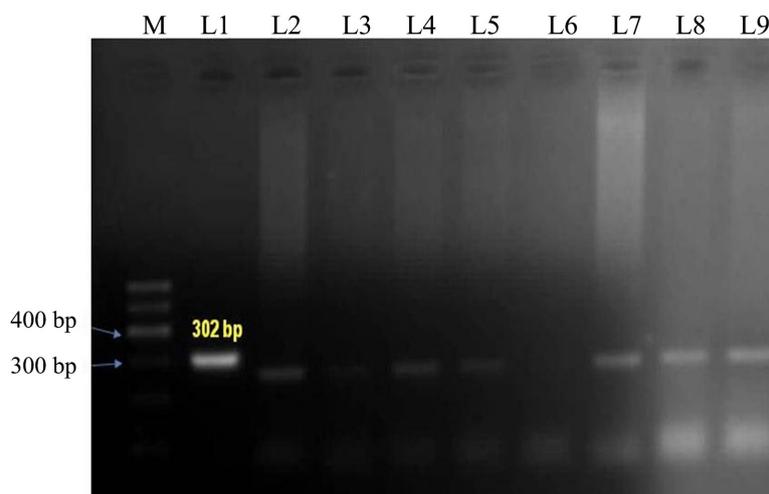


Figure 1. Amplification of DNA from the samples using *esxB* (CFP-10) primers [Lane M-100 bp ladder, L1-Positive control (*M. tuberculosis*), L2-L9-Blood samples].

Table 1. Grouping of cattle and buffaloes based on the results of CITT and IFN- γ Assay.

Group No.	Criteria	Number of Cattle (%)	Number of Buffaloes (%)	Total no. of cattle and buffaloes (%)
Group 1	Animals tested positive for TB by both the tests <i>i.e.</i> CITT and IFN- γ Assay	5 (11.90%)	5 (3.13%)	10 (4.95%)
Group 2	Animals tested positive for TB by CITT but tested negative by IFN- γ Assay	13 (30.95%)	17 (10.63%)	30 (14.85%)
Group 3	Animals tested negative for TB by CITT but tested positive by IFN- γ Assay	2 (4.76%)	5 (3.13%)	7 (3.47%)
Group 4	Animals tested negative by both CITT and IFN- γ Assay	22 (52.38%)	133 (83.13%)	155 (76.73%)
Total TB positive Animals	Number of animals tested TB positive with one or both the tests	20 (47.62%)	27 (16.88%)	47 (23.27%)

Table 2. Proportions of animals positive or negative by CITT and INF- γ Assay.

Intra-dermal Test Result	No. of Animals with IFN- γ +ve	No. of Animals with IFN- γ -ve	Total No. of Animals
CITT +ve	10	30	40
CITT -ve	7	155	162
Total	17	185	202

4. Discussions

In this study, the incidence of TB recorded in cattle (47.62%) either by one or both the tests were almost three times more than that of buffaloes (16.88%). Probably the cattle which were mostly HF-cross may be more susceptible to the disease due to production and environmental stress, compared to the local indigenous buffaloes, similar findings have also been reported by Das *et al.* [9] After all, the overall prevalence rate of TB in the farm was 23.27%, which is higher than the overall prevalence rate of TB in the state (Punjab) *i.e.* 5.38% [10]. However, based on a random-effects meta-regression model analysis, Srinivasan *et al.* [11], revealed a pooled prevalence estimate of 7.3% indicating that there may be an estimated 21.8 million infected cattle in India.

Numerous studies have been carried out in the last decades to evaluate sensitivity and specificity of the intradermal test and IFN- γ assay in cattle under different epidemiological situations using different antigens [12] [13] [14]. Since both the tests measure the cell-mediated T-cell response, there is to be expected a considerable overlap (~80%) between the animals that respond to these tests [15]. In our study, no significant difference has been observed between the sensitivity and specificity of CITT and IFN- γ assay, similar to the findings by Ameni *et al.* [16]. However, the sensitivity of the IFN- γ assay was less than the CITT

whereas specificity of IFN- γ assay was greater than the CITT in our study. The IFN- γ assay has a sensitivity of 84% that is comparable to, but lower than, the observed sensitivity of the CITT (90%), whereas, the specificity of the IFN- γ assay (97%) is lower than that of the CITT (99.9%) [15]. When used in parallel with *i.e.* at the same time as the tuberculin test, the combined tests give a sensitivity of over 90% - 95% [5] [15] [17] [18], which is also supported by the findings in our study as well as Ahir *et al.* [19]

The probable reason of our findings, that is, 30 CITT positive animals testing negative for IFN- γ assay may be due to co-infection of the animals with an environmental mycobacterium or anergic situation of infected animals, as recorded by other workers [13] [20]. In contrast, 7 CITT negative animals which were tested positive by IFN- γ assay suggested that these animals might be in the early stage of the disease that couldn't be determined by CITT, as the same has been recorded by Gormley *et al.* [5], Strain *et al.* [21] Good *et al.* [15] also reported subpopulations of *M. bovis*-infected cattle which give a positive reaction to the IFN- γ assay and not to the tuberculin test and vice versa. However, animals which tested positive for IFN- γ assay and negative for intra-dermal tuberculin test were subsequently converted to tuberculin positive and posed an increased risk to the other cattle [20].

At 95% level of significance, kappa value between CITT and IFN- γ assay was 0.264, indicating a fair degree of agreement between the two tests. So both the tests, when used simultaneously increase the accuracy of detection of TB positive dairy animals, similar to the findings by Gormley *et al.* [5], Ahir *et al.* [19]

The sensitivity (detection limit) of the *esxB* (CFP-10) PCR was up to 8 pg/ μ l of pure culture *M. tuberculosis* DNA [8]. There are reports of PCR from blood and tissue samples of cattle, using JB21 and JB22 primers specific for *M. bovis*, detecting as low as 10 fg/ μ l of purified *M. bovis* DNA [22] [23].

Several PCR systems have been developed for the detection of TB viz. PCR amplification of *esxA* and *esxB* genes targeting ESAT-6 and CFP-10 proteins respectively, present in pathogenic Mycobacterial species, can be used for confirmation of *M. tuberculosis* as well as *M. bovis* [7] [24]. Although the presence of ESAT-6 and CFP-10 has also been detected in other mycobacterial species and further studies of their extent of amino acid sequence similarities are required [25]. Besides, *M. tuberculosis*, as well as *M. bovis*, can also be detected by PCR targeting IS6110 insertion sequence [2] [26]. *M. bovis* in cattle lymph nodes were detected by PCR using TB1 and TB2 primers targeting gene that codes for MPB70 protein [27].

In our study, the 3 PCR positive animals that were IFN- γ positive but CITT negative may be in their early stage of TB infection. Besides, the probability that most of the TB reactor animals positive by CITT and IFN- γ assay failed to be detected by PCR may be due to low concentration of DNA (even less than 8 pg/ μ l) in clinical samples which remained undetected by PCR, besides *M. bovis* DNA may not be present in the clinical samples as the animal may not be in the

stage of bacteraemia. Therefore, information on *M. bovis* or *M. tuberculosis* bacteraemia and time of dissemination in blood stream need to be further explored for proper sampling time. However, compared to lymph node aspirate PCR which detected only 1 TB positive out of 15 animals, blood PCR had better sensitivity as the later detected TB positive in more numbers (13 out of 47) of either CITT and IFN- γ positive animals.

After all, there were certain limitations in this study, as no test is 100% sensitive and 100% specific [28] and no single test can diagnose bovine TB at all stages of infection [29] [30]. CITT has many limitations including difficulties in administration and interpretation of results, need for a second step visit, low degree of standardization and imperfect test accuracy [13]. The sensitivity of the test is affected by the potency and dose of tuberculin administered, desensitization, deliberate interference, post-partum immune-suppression and observer variation. Specificity is influenced by sensitisation as a result of exposure to *M. avium*, *M. avium paratuberculosis* and other environmental mycobacteria [31]. Because intradermal tuberculin testing elevates the production of IFN- γ by lymphocytes of cattle that have had prior exposure to *M. bovis* antigens [32], there is the potential for multiple injections of tuberculin to increase the production of IFN- γ , resulting in higher optical density (OD) values of the IFN- γ assay and thus, for animals subsequently being classified falsely as positive [33]. Besides, TB is a chronic granulomatous disease, therefore, diagnosis of TB from blood samples may be done especially during the stage of bacteraemia. After all, the difficulty in lymph node aspirate sampling from cattle and buffaloes were an inevitable drawback in this study. So, the accurate sense of the lymph node aspirate PCR cannot be ascertained from the present study as the numbers of samples are insufficient to make a correct judgement.

5. Conclusion

From this study, we can conclude that diagnosis of bovine TB can be done in early stage in live animals with cell-mediated immune response based tests (CITT and IFN- γ assay) and blood PCR especially during the stage of bacteraemia. Combined use of both CITT and IFN- γ assay lead to more accurate screening of TB, though IFN- γ assay was more specific than CITT. However, *esxB* (CFP-10) PCR can also be used as a fast and easy alternative method for the laboratory diagnosis of bovine TB. Early diagnosis of TB can lead to quick segregation of infected animals, restrict transmission and help in eradication of bovine TB from the country. In fact, equal importance must be given for eradication of TB from the dairy animals to make the WHO's "End TB strategy" a 100% success.

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Ethical Approval

This study was approved by Animal Ethics Committee of Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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