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Diagnosis of Mycobacterium Tuberculosis Complex in Buffaloes using Antemortem Techniques

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Abstract

Bovine tuberculosis is an important and chronic mycobacterial disease of cattle and buffaloes. However very few reports are available on the prevalence of bovine tuberculosis in buffaloes. The present study was thus conducted to detect Mycobacterium tuberculosis complex (MTC) in buffaloes from an organized dairy farm. Comparative intradermal tuberculin test (CITT), Interferon gamma assays (IFN-γ) and Polymerase Chain Reaction (PCR) was used to detect the diseased animals. Out of 188 buffaloes tested by CITT, 17 (9.04%) were positive reactors. Out of these 17 animals, 9 (4.7%) buffaloes were positive by IFN-γ assay. By IS6110 PCR, 17 (9.04%) were positive for MTC. Thus, the study showed that PCR was more sensitive and specific diagnostic technique for tuberculosis. The study provides additional knowledge in the field of tuberculosis in buffaloes and may help in development of strategies for control of the disease.

Keywords: Buffalo, Bovine Tuberculosis, Comparative Intradermal Tuberculin Test, Interferon Gamma Assay, IS6110PCR

Introduction

Bovine tuberculosis (bTB) is an infectious, chronic, debilitating contagious disease caused by the pathogenic mycobacterial species of *Mycobacterium tuberculosis* complex (MTC). MTC comprises of *M. bovis*, *M. tuberculosis*, *M. africanum*, *M. canneti*, *M. microti*, *M. pinnipedii* and *M. caprae* (Thoen *et al*., 2009). Bovine tuberculosis is characterised by granulomas of the lungs and their draining lymph nodes. The organism infects over 50 million cattle world-wide that results in economic losses of over $3 billion annually. Economic losses from TB in India amount to Rs. 12,000 crore annually. Bovine tuberculosis is endemic in Indian dairy cattle and the overall prevalence rate is 5.38 % (Sharma *et al*., 2013) due to lack of disease control programmes which may help to eradicate this disease. Various diagnostic methods have been used in diagnosis of bTB such as post-mortem techniques, bacterial culture and antemortem techniques [single intra-dermal (SID), comparative intra-dermal tuberculin test (CITT), enzyme linked immunosorbant assay (ELISA), gamma interferon (IFN-γ) assay and molecular techniques (Strain *et al*., 2011).

In India, few reports are available on the detection of bTB in buffaloes. Therefore, the present study was designed to investigate the presence of bTB in buffaloes from an organized farm using the ante-mortem techniques.

Materials and Methods

Ethical Approval

The permission for testing of the animals and sampling was duly approved by Institutional Animal Ethics Committee GADVASU, Ludhiana (IAEC vide order no: GADVASU/2019/IAEC/49/19 dated 29/4/2019).

Study Population

A total of 188 buffaloes (Murrah breed, female) aged>2 years from an organized dairy farm of Ludhiana, Punjab, India were subjected to CITT, IFN- γ and PCR.

Comparative Intradermal Tuberculin Test (CITT)

Comparative Intradermal Tuberculin Test (CITT) was performed by using bovine tuberculin PPD (30000 IU/ml) and avian tuberculin PPD (25000 IU/ml), obtained from Prionics (Switzerland), as per OIE Terrestrial Manual (2009). Briefly two 2 x 2 square inch area was shaved on the left side of neck of the animal approximately 12-15 cm apart. Bovine and avian tuberculin PPD (0.1ml each) were injected intradermally (OIE, 2009). Inflammatory responses were recorded 72 hrs post injection with the help of a vernier caliper. Animals were considered positive if the increase in skin thickness at the bovine site of injection was 4 mm or 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 skin fold thickness was observed. Difference in thickness between 1-4mm was considered as inconclusive.

Gamma Interferon Assay

This test was performed using commercially available BIONOTE TB-feron ELISA kit as per the manufacturer's protocol. It is a rapid *in-vitro* blood-based assay of cell mediated immune response to *M. bovis* PPD. Prior to the conduction of CITT, blood collection was carried out for IFN-γ assay. Briefly minimum volume of 5 ml of blood from each animal was collected into heparin tube and gently mixed and transported to the laboratory at ambient temperature and stimulated with bovine and avian TB stimulation antigen (100 µl each) for 16-24 hours at 37°C. The plasma was then collected and assayed for IFN-γ production in duplicate using a commercially available kit and optical densities were measured on an ELISA plate reader at 450 nm. The mean OD of control, avian PPD and bovine PPD were recorded and compared for each animal and interpreted as-

Positive= OD Bovine PPD-OD Control ≥ 0.1; and
OD Bovine PPD- OD Avian PPD ≥ 0.1

Negative= OD Bovine PPD-OD Control <0.1; and
OD Bovine PPD-OD Avian PPD <0.1

Detection of MTC from Blood Samples

Extraction of DNA was carried out from blood sample using the QIAamp Blood and tissue DNA Kit following the manufacturer's protocol. The DNA was stored at -20˚C till further use. In the present study, IS*6110* PCR was employed by using specific primers (INS1/INS2) to amplify an insertion sequence IS*6110* of MTC directly from blood sample. PCR amplification yielded specific products of 245bp in size by INS1/INS2 primers as per Filia *et al.* (2016) with some modifications. PCR reaction was performed in a total 25 µl volume, containing 13 µl of Taq Master mix, 1 µl of forward primer INS1 (10pmol/µl) 5'- CGTGAGGGCATCGAGGTGGC-3', 1 µl of reverse primer INS2 (10pmol/µl) 5'-GCGTAGGCGTCGGTGACAAA-3' and 10 µl of DNA template Along with sample DNA, a known positive control DNA (obtained from ADRC) and negative control were also amplified. Thermal cycling was performed in T. Gradient Thermo cycler (Biometra, Germany) and cycling conditions were as follows i.e., an initial denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min. and PCR products were analyzed by agarose gel electrophoresis using 1.5% agarose gel and visualized in gel documentation system (Bio-Rad, CA).

Results and Discussion

Diagnosis of bovine tuberculosis in live animals can be performed using multiple approaches such as cell mediated immune response, isolation and molecular techniques. In the present study, out of 188 buffaloes tested by CITT, 17 (9.04%) were positive reactors, 15 (7.9%) animals were inconclusive and 156 (82.9%) were negative reactors. Further nine (4.7%) buffaloes were positive by IFN- γ assay. The buffaloes which were positive for IFN- γ assay were also positive reactors for CITT (Table 1). The possible reason for the animals to be positive by CITT but negative by IFN- γ assay may be that these animals might have been infected by the causative agent for a long time and IFN- γ assay detects the infection in early stage only. Nine (4.7%) animals positive by IFN- γ and CITT may indicate that the tuberculosis in these animals was caused by *M. bovis.* Ahir *et al.* (2016) reported 5% buffaloes being positive reactors by CITT and 12% by IFN- γ assay. The low number of IFN-γ positive buffaloes indicates chronic stage of disease. IFN-γ assay is regarded as a means for the early identification of *M. bovis* infected animals (Gormley *et al*., 2006).

Table 1: Cell mediated immune response using CITT and IFN-γ

CITT	**Buffaloes**		
	IFN- γ	**IFN- γ**	**Total**
	+ve	**-ve**	
CITT +ve	9	8	17
CITT -ve	0	156	156
Inconclusive	0	15	15
	Total		188

In India, few reports are available regarding the presence of bTB in buffaloes and was-reported to be 6.39% (Lall *et al*., 1969), 4.37% (Joshi *et al*., 1976), 5.91% (Dabade *et al*., 2017) and 13.7% (22/160) (Brahma *et al*., 2019). Srinivasan *et al*. (2018) performed meta-anlaysis of bTB in Indian cattle and buffaloes and reported 4.3% and 16.0% prevalence of bTB in buffaloes by random effect (RE) and fixed effect (FE) model of analysis respectively. By CITT, the prevalence of bTB were reported mainly from Pakistan in Nilli ravi-breed of buffaloes as 5.48% (Javed *et al*., 2006), 4.37% (Akhter *et al*., 1992), 1.7% (Ifrahim, 2001) and 4.16% (Malhi *et al*., 2018). Hossain *et al.* 2012 recorded the prevalence of bovine tuberculosis as 4.08% in buffaloes from Bangladesh. Cataluna and Gordoncillo (2006) found 38% of the water buffaloes to react positively with tuberculin test. Variations in the prevalence of tuberculosis in buffaloes from different parts of the world may be due to different geography, climate and management practices.

Further in the present study, for confirmation of the bTB in buffaloes, IS*6110* PCR was employed by using specific primers (INS1/INS2) to amplify an insertion sequence IS*6110* for MTC with an amplified product of 245bp. MTC was detected in 17 (9.04%) out of total 188 animals (Figure 1). These 17 animals were also positive for CITT (Table 2) and nine animals positive for IFN-γ test (Table 3). This interferon gamma assay positive animals were considered to be affected by *M bovis* since the IFN-γ test was specific for the organism.

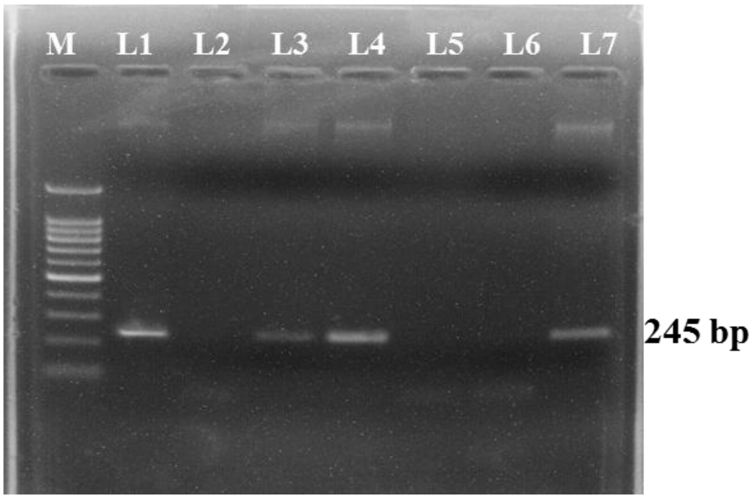

Figure 1: Agarose gel electrophoresis of the amplified IS*6110* gene of MTC using INS1/INS2 primers (size of product 245bp) M: DNA 100bp ladder L1: Positive control, L2: Negative control and L3-L7: samples

Table 2: Animals positive for Mycobacterium tuberculosis complex by PCR and CITT

PCR	**Buffaloes**		
	PCR +ve	**PCR -ve**	**Total**
CITT +ve	17	0	17
CITT -ve	0	156	156
Inconclusive	0	15	15
	Total		188

Table 3: Animals positive for Mycobacterium tuberculosis complex by PCR and IFN- γ

PCR	**Buffaloes**		**Total**
	IFN-γ (+ve)	**IFN-γ (-ve)**	
PCR +ve	9	8	17
PCR -ve	0	156	156
	Total		188

Detection of MTC in early stage is important since these organisms can cause a serious threat to animals. Several alternative approaches have been attempted for the rapid and specific diagnosis of TB, but molecular methods, especially PCR assays, are the most promising (Serrano-Moreno *et al*., 2008, Figueiredo *et al*., 2009). Araujo *et al.* (2014) detected MTC in bovine and bubaline tissues through nested-PCR and author reported that, out of 40.7% of the CITT positive animals, 22.5% were positive for MTC both in the culture and the nested-PCR. In the present study, the buffaloes might have got the infection from cattle since these animals have a history of bTB. Usually, respiratory route is the primary source of infection in buffaloes but contaminated ground and water with naso-oral excretions containing mycobacteria may assist in spreading of the disease (Mitchel *et al*., 2007)

Conclusion

The present study reports the comparative evaluation of diagnostic modalities for bTB in dairy buffaloes. PCR proved to be very sensitive and specific diagnostic tool as compared to other tests used in the study.

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Conflict of Interests

There is no conflict of interest.

Publisher Disclaimer

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