RESEARCH ARTICLE

Comparative Analysis of Intradermal Tuberculin Test and Polymerase Chain Reaction for Bovine Tuberculosis Detection in Punjab, India

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Abstract

Bovine tuberculosis (bTB) is a zoonotic disease that affects domestic animals, humans, and the wild animals. The purpose of the study was to use the comparative intradermal tuberculin test to diagnose bTB in cattle and to find out the prevalence in unorganized, and organized farm with variation according to age, sex, and breed. Eighty three animals were tested with the use of comparative intradermal tuberculin test across two districts of Punjab, India. Additionally, ante-mortem (nasal swab, n=44) samples were examined using molecular methods. The samples were inoculated on Middlebrook7H10 media after decontamination with 4% NaOH. One sample revealed the presence of acid fast bacilli on microscopic examination. Out of 83 animals examined, 12 were bTB positive reactors. Additionally, gender-wise data analysis revealed higher positivity among cows/heifers (16.17%, 11/68) compared to bulls/bullocks (6.66%, 1/15). Out of the 44 nasal swab samples, 3 samples yielded *Mycobacterium kansasii* isolates on culture which were further confirmed by species-specific PCR. The finding of our study indicates animals of indigenous cattle breed in Ludhiana have a higher prevalence of bTB and variation in breed susceptibility. The presence of non-tuberculous mycobacteria may hamper diagnosing bovine tuberculosis in cattle.

Key words: Bovine, Comparative intradermal tuberculin test, *Mycobacterium kansasii,* Non-tuberculous mycobacteria, PCR.

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Introduction

Bovine tuberculosis is an infectious, chronic, granulomatous disease that affects both human and animal health (LoBue *et al.,* 2010). The disease has impact on both the global and national economies by lowering productive efficiency of 10-25%, decreasing animal fertility, lowering the quality of meat, and increasing mortality (Schiller *et al.,* 2010). The basis for the intradermal skin test is the detection of the delayed hypersensitive reaction, which occurs 72 h following the purified protein derivative (PPD) injection. Bovine tuberculosis needs early diagnosis techniques to be eradicated globally, although it's global prevalence and endemic in India. The intradermal tuberculin test is permitted as the bTB screening test by the European Commission and the OIE (Dubey *et al.,* 2020). Bovine TB has been diagnosed using a variety of techniques, and these methods differ in terms of effectiveness and dependability. The most haematological and biochemical studies that were done to determine their values as diagnostic indicators for TB remained unclear (Javed *et al.,* 2010).

The *Mycobacterium* genus includes about 190 different species, the majority of which are classified as non-tuberculous mycobacteria (NTM) (Daley *et al.,* 2020). NTM can infect both humans and animals and some are saprophytic in nature, free-living organisms in both water and soil (Kankya *et al.,* 2011). NTM are all other mycobacteria that are classified as having the same potential to produce skin disease, lung illness, lymphadenitis, and dispersed disease as tuberculosis. The most common manifestation, lung disease, is considered to be caused by inhaling aerosols (Honda *et al.,* 2018). Although there are numerous NTM species identified, the most often causes of lung infections are *M. kansasii, M. avium* complex (MAC), and *M. abscessus*. The existence of NTM species in cattle and wildlife is challenging to diagnose bovine tuberculosis (Gcebe and Hlokwe, 2017). NTM can have an effect either directly by generating grater severe infections and productivity losses or indirectly by interfering the diagnosis of bTB and paratuberculosis. The milk production

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of TB reactor animals is significantly less than that of non-reactor cows, with differences ranging from 120 kg in the third lactation to 573 kg in the first lactation. Bovine tuberculosis (bTB) causes a variable decline in milk production, which results in huge economic losses (Boland *et al.,* 2010). With this context, we have designed the work to evaluate the implementation of comparative intradermal tuberculin test as a screening method for cattle with respiratory distress and examination of ante-mortem samples using bacteriological and molecular methods to detect NTM from nasal swab.

Materials and Methods

The research was conducted on 83 dairy animals from two districts in Ludhiana, Punjab, India, following IAEC approval No. 497/GO/Re/SL/02/ CPCSEA. Animals with a history of respiratory signs (cough, dyspnoea, fever, nasal discharge) in various organized and unorganized dairy farms of these 2 districts of Ludhiana were incorporated in the study. These 83 (68 female and 15 male) animals of various breeds (indigenous 47, crossbred 36) belonged to three different age groups (1.0-2.5, 2.5-5.0, and >5 years).

Intra-Dermal Tuberculin Testing

A comparative intradermal tuberculin test was carried out according to the OIE terrestrial manual (OIE, 2009). Bovine tuberculin protein derivative (PPD) from *M. bovis* culture (strain AN5, 3000 IU), and avian tuberculin PPD from *M. avium* subspecies *avium* culture (strain D4ER, 2500 IU) Prionics (Switzerland) were employed in comparative intradermal tuberculin testing (CITT). Using McClintock tuberculin testing equipment, the test was conducted on middle third of the neck of each animal. Two distinct spots were shaved and cleaned at least 12 cm apart, the thickness of the skin before injection was measured and 0.1 mL each of the avian (PPD-A) and bovine (PPD-B) antigens were injected. After 72 h of injections Vernier calipers were used manually to measure the thickness of the skin. An animal was regarded as bovine reactor, if the skin thickness increased by at least 4 mm as per the OIE guidelines.

Collection of Nasal Swabs and Isolation of *Mycobacterium* spp.

Out of 83 animals tested with CITT, nasal swabs (n=44) were collected in 50 ml sterile PBS falcon tube from cattle with a history of respiratory signs (cough, dyspnoea, fever, nasal discharge). The nasal swab was then placed in a sterile container and transported to the laboratory maintaining a cold chain to ensure sample integrity. The samples were processed using bacteriological and molecular methods immediately upon reaching the laboratory.

The nasal swab samples were properly decontaminated with 4% NaOH before being inoculated onto Middlebrook*7H10* media and Lowenstein-Jensen (LJ media) (HiMedia, India) (Chang *et al*., 2001). In the decontamination process, 2 mL of 4% NaOH was added to the nasal swab in a 15 mL centrifuge tube, achieving an equal volume of NaOH and sample. The tube was then left at room temperature for 15 min to ensure proper decontamination. Following the incubation, the mixture was diluted in 6 mL of PBS (pH =6.8), and the tube was centrifuged at 2300 x g for 20 min at 4°C. After centrifugation the supernatant was removed and then 100 µL was poured on Middlebrook*7H10* media and Lowenstein-Jensen (LJ media) and incubated at 37°C for six weeks until growth was visible on media. First, Ziehl-Neelsen's staining was employed to check for the presence of acid-fast bacilli in the samples.

Molecular Detection of *Mycobacterium* from Nasal Swabs

For PCR, DNA extraction was done using a Blood and Tissue DNA extraction Kit Qiagen catalog no. 69504 (Qiagen) and stored at -20°C until further use. A 25 µl reaction volume containing 12.5 µl of Dream Taq Green PCR Master Mix (Thermo Scientific), 1 µl both of the forward and reverse primers (10 pmol each), 8.5 µl of nuclease-free water, and 2 µl of DNA template was used. Genomic DNA from *Mycobacterium kansasii* (MTCC 3058) (IMTECH, Chandigarh) standard cultures was used as the positive control. The primer details for *hsp65* gene and *M. kansasii* PCR are mentioned in Table 1. The conditions for the PCR targeting hsp65 gene, and *M. kansasii* are provided in Table 2.

Table 1: Details of primer used for *Mycobacterium* genus and *Mycobacterium kansasii*

Organism	Target gene	Primer	Primer sequence	Amplicon size	Reference
Mycobacterium genus	*hsp*65 gene	Forward (TbII) Reverse (Tb12)	5′-ACCAACGATGGTGTGTCCAT- 3′ 5′-CTTGTCGAACCGCATACCCT-3′	439 bp	Shinnick (1987)
M. kansasii	ITS	Forward (ITS) Reverse (ITS)	5′-GCAAAGCCAGACACACTATTG -3′ 5′- AAGAACACGCTACCCGTAGG - 3′	152 bp	Park *et al.* (2000)

Table 2: PCR condition of *Mycobacterium* spp.

Condition	*Mycobacterium* genus (*hsp65)*	*M. kansasii*
Initial Denaturation	94oC for 3 min	94oC for 5 min
Denaturation	94oC for 1 min	94oC for 1 min
Annealing	56°C for 45 sec	60°C for 1 min
Extension	72°C for 1 min	72°C for 1 min
Final Extension	72°C for 10 min	72°C for 10 min
No. of cycle	45	30

Results and Discussion

A total of 83 animals were tested, out of which 12 (14.4%) were found positive reactors by CITT. The animals tested from different herds and areas were split up into three groups according to their breed, age, and gender. Among a total of 47 indigenous and 36 crossbred cattle tested, 7 and 5 animals were identified to be bTB-positive reactors across three different age groups indicating prevalence of 14.8% and 13.8%, respectively. On the basis of gender-wise data analysis the bTB results showed that the positivity rate was higher in cows/heifers (16.17%, 11/68) than in bulls/bullocks (6.66%, 1/15). Additionally, cattle positive for bTB were found across all age categories but greater susceptibility to bTB infection existed in animals older than 5 years of age compared to younger ones. Out of 44 nasal swab samples stained by Ziehl-Neelsen's staining and examined under the microscope, one sample showed the presence of acid fast bacilli (Fig. 1). 18 out of 44 nasal swab samples tested positive for *M. kansasii* (152 bp) by species-specific PCR (Fig. 2), and 3 samples yielded *M. kansasii* isolates (Fig. 3) on culturing which were further confirmed by species-specific PCR. Three nasal swabs isolates were tested positive for *hsp65* gene (439 bp) indicative of *Mycobacterium* genus (Fig. 4).

In total, 12 animals were found positive for bTB, out of which 10 and 2 were from organized and unorganized farm systems with a prevalence rate of 12% and 2.4%, respectively. In conjunction with our study, Das *et al.* (2018) also reported the highest prevalence from organized farms (25.4%) than unorganized farm (3.2%). Many workers in India have stated that organized farms have significantly higher bTB prevalence. In a previous study, which involved a small cohort of dairy animals of India's states, high prevalence rates of bTB were found to range from 14.31 to 34.42% (Thakur *et al.,* 2010). Mukherjee (2006) observed the bTB prevalence at 0.65-1.85% (Western India), and 15.76% (Northern India) in two dairy herds. Prevalence rates in southern Indian states like Tamil Nadu and Karnataka were 34.58% and 30-35%, respectively (Dhinakaran *et al.,* 1991). Breed type, screening procedures, agricultural practices, and contact between animals from the same or separate herds using shared facilities, and grazing regions could all contribute to the broad range in bTB frequency (Ameni *et al.,* 2011). Our study shows that prevalence of bTB-positive reactors was higher in indigenous cattle than crossbred cattle. Because majority of the indigenous cattle tested were from gaushalas, where disease dissemination is more likely because of the close confinement of the animals. Our findings contradict those of Vordermeier *et al.* (2012) and Das *et al.* (2018), who found that crossbreds were more susceptible to tuberculosis.

The higher prevalence of bTB in female animals (16.17%, 11/68) than in males (6.66%, 1/15) concurred with Das *et al.* (2018), who also reported the higher prevalence in female (25.8%) than in male (7.3%) animals. As previously reported, a female's longer stay in the same herd may be the cause of her higher positive rate (Dinka and Duressa, 2011). In humans, the risk of tuberculosis is primarily age-dependent. There are few data available on the age-dependent risks in cattle. Furthermore in the current study, incidences of bTB positive found across all age categories but with greater susceptibility in animals older than 5 years concurred with observations of Thakur *et al.* (2010), Brooks-Pollock *et al.* (2013) and Moiane *et al.* (2014).

The OIE and the European Commission both recognize the intradermal tuberculin test as the main method of tuberculosis screening in bovines (Bezos *et al.,* 2014). Confirmatory testing for bTB is still difficult, because the currently accessible diagnostic tests have low sensitivity and specificity levels, and not a single test can accurately identify bTB at all stages of infection (Coad *et al.,* 2013). The CITT is more precise than the single intradermal testing (SID) test (Dubey *et al.,* 2020). The finding of our study indicates the nasal swabs collected from bTB positive reactor animals contain more *M. kansasii*. Senanayake *et al.* (2016) also identified *M. vaccae,* and *M. kansasii* in respiratory samples along with other NTM from sputum samples. In a related investigation, sputum samples contained *M. kansasii, M. smegmatis, M. intracellulare,* and other NTM were identified (Yu *et al.,* 2014). Nour-Neamatollahie *et al.* (2017) carried out a similar investigation in TB suspect patients utilizing several clinical samples (bronchial lavage, sputum, and skin samples), in which the most of NTM was found along with *M. tuberculosis* and *M. bovis*. The most common *Mycobacterium* species was *M. kansasii* was 45.4% (5/11) with NTM lung disease. In view of endemicity and zoonotic potential of tuberculosis, routine screening should be done, and the farm should put the required preventative and control measures in place.

Conclusions

The prevalence of bTB reactors was 14.4% (12/83) in two different districts of Punjab. Based on this study, it can be concluded that organized farms have a higher frequency of bTB, with a higher incidence in females and indigenous cattle breed in Ludhiana, Punjab. *M. kansasii* is the most predominant of non-tuberculous mycobacteria (NTM) found in nasal swabs of bTB positive reactor animals by comparative (avian and bovine strain) intradermal tuberculin testing, The finding indicates PCR as a good screening test of the nasal swabs samples for the detection of NTM. The presence of NTM may affect the results of tuberculin test in bovines.

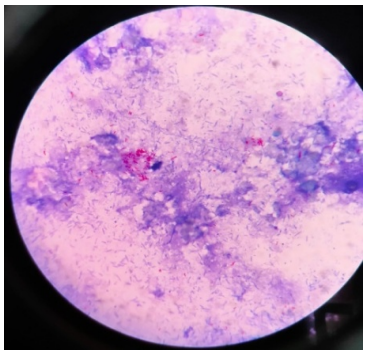

Fig. 1: Clumps of acid fast bacilli from nasal swabs samples

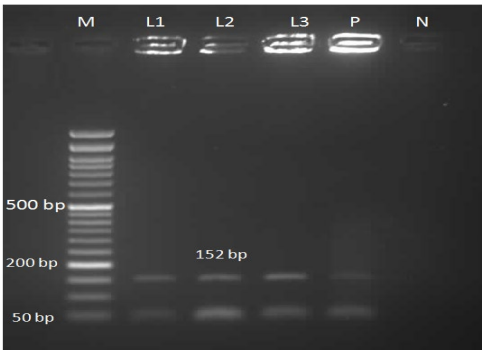

Fig. 2: Agarose gel electrophoresis showing an amplicon of 152 bp (*M. kansasii*) from nasal swabs: Lane M: 50 bp DNA ladder, Lane P: Positive control (MTCC 3058, *M. kansasii),* Lane N: negative control, Lane L1-L3: Positive for *M. kansasii*

Fig. 3: Growth of mycobacterial cultures of nasal swabs on Lowenstein-Jensen media

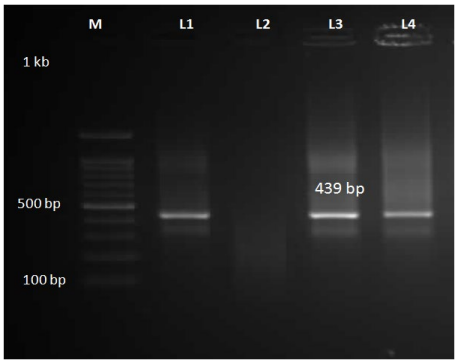

Fig. 4: Agarose gel electrophoresis showing an amplicon of 439 bp (*hsp65* gene) from nasal swabs samples: Lane M: 100 bp DNA ladder, Lane L1 and L3-L4: Positive for *hsp65* gene.

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