



Histopathological and Molecular Investigation of Natural Cases of Bovine Tuberculosis Infection in Cattle

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ABSTRACT

Bovine tuberculosis (bTB), a chronic infection in cattle caused by *Mycobacterium tuberculosis/bovis*, that impacts productivity and represents a major public health threat. Although the considerable economic costs and zoonotic risk consequences associated with the disease, accurate estimates of bTB prevalence are lacking in many countries, including India. Therefore, in the current study for collection of tubercular lesions the postmortem examination of 100 cattle was conducted. All major viscera and regional lymph nodes were examined and incised. Histopathology was performed in the cases where gross lesions were suggestive of tuberculosis. PCR was performed on the tissue and faecal samples by using IS6110 insertion sequence, *Mycobacterium tuberculosis/bovis* complex PCR kit. In 12 animals, nodular lesions with caseating mass suggestive of tuberculosis were observed in the lung tissue. All the 12 lung impression smear and only five faecal smear showed acid fast bacilli stained by Ziehl-Neelsen staining. Histologic features comprised a classic granuloma as a characteristic lesion of tuberculosis composed of a central caseous necrosis with mantle of macrophages, lymphocytes, plasma cells, epithelioid macrophages and Langhan's giant cells and were observed in all 12 cases. All the tissue samples and 11 faecal samples were positive for the *Mycobacterium tuberculosis* complex using IS6110 sequence. 8 tissue samples and 4 faecal samples were positive by using *Mycobacterium tuberculosis/bovis* complex PCR kit. It can be concluded that there was good agreement between histopathology, acid fast staining and PCR. It can also be concluded that faecal samples which are easier to collect should be preferred for diagnosis of TB by PCR in cattle.

Keywords: Bovine tuberculosis, Histopathology, Faecal PCR, Tissue PCR, IS6110 insertion sequence, *Mycobacterium tuberculosis/bovis* complex PCR kit

Bovine tuberculosis (BTB) caused by *Mycobacterium bovis* is a chronic, infectious and progressive disease of cattle, other domesticated animals and certain free or captive wildlife species. It is characterized by the formation of non-vascular granulomas known as tubercles which occur most frequently in lungs, lymph nodes, liver, intestine, and kidney (Varshney *et al.*, 2017). The bovine TB is a significant veterinary disease that can spread to humans via ingestion of contaminated milk and contact with infected animals. Bovine tuberculosis is still a major problem in developing countries for wildlife, public health, food safety, and the economy of livestock industries. Animals infected with bTB lose 10-25% of their productive efficiency in terms of milk production,

weight gain, infertility and condemnation of meat (Miller *et al.*, 2002). Eradication of BTB is not easy in developing countries due to close interaction between human and animal and lack of effective control measures. The disease in wildlife still poses a risk to livestock, tourism economy, and wildlife conservation even BTB has been mostly eradicated in the livestock industry of some developed countries through culling of infected animals and milk pasteurization (Mishra *et al.*, 2005). Both officially bovine TB-free (OTF) and non-OTF countries reported

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an augment in the proportion of bovine TB positive cattle herds. Incidence and prevalence of BTB are escalating as per report (Gridley, 2006). Proper epidemiological status of bovine tuberculosis in India is lacking but needs to be explored out in order to combat existing BTB situation. The OIE recommends ante-mortem TB diagnostic method is the Intradermal Tuberculin Skin test (TST) which has been used in the eradication of bovine tuberculosis in the developing countries (Gridley *et al.*, 2006). However, TST may give false-positive reactions due to exposure of some animals to environmental mycobacteria and may also give false-negative reactions due to immunosuppression, desensitization towards tuberculin, sub-potent use of tuberculin, and lengthy exposure to a field strain (Gunisha *et al.*, 2000). This test is labor intensive due to revisiting the animal for consecutive three days after tuberculin inoculation and usually impractical for free-ranging wildlife. TST cannot be repeated for at least 60 days because of systemic desensitization caused by the injection of tuberculin. The use of Comparative Intradermal Tuberculin Test (CITT) as a diagnostic test to overcome the problem of false positive reactions of the Single Intradermal test in India is restricted due to unavailability of avian tuberculin (Brahma *et al.*, 2019). *In-vitro* blood IFN- γ assay measures the cell-mediated immune response of *M. bovis* infected animals (Reuss, 1995) with the limitation of non-specific responses in a young animal, high logistic demand (culture start is required within 24 hours after blood collection), the requirement of well-trained personnel (Ramos *et al.*, 2015). A test based on detection of humoral immune response (i.e., antibody production) together with the test based on CMI increase level of pathogen detection can help to control bovine tuberculosis with some limitations (Verma *et al.*, 2014). At present, post-mortem diagnosis based on examination of gross lesions, followed by histopathology and culture, is widely used for surveillance of bovine tuberculosis in animals (Gunisha *et al.*, 2000). Bacteriological culture of clinical samples (i.e., milk, blood, nasal swab, and cattle tissues) is considered as gold standard for bovine tuberculosis diagnosis. But it requires a minimum of 3-6 weeks and presence of viable bacteria (Gunisha *et al.*, 2000). It would be a great advantage for the tuberculosis eradication program if a conclusive diagnosis could be made within a few days, rather than several weeks, after collection of suspected tissues. A number of studies have incorporated the polymerase chain reaction (PCR) into

techniques for detection or identification of mycobacteria in formalin-fixed, paraffin-embedded tissues of humans (Miller *et al.*, 2002). There are very few reports of use of the technique for diagnosis tuberculosis in clinical samples from animals (Leel *et al.*, 2019).

Therefore, the present study reports for detailed investigation regarding the occurrence of bTB by employing various diagnostic platforms viz., pathomorphological changes, Ziehl-Neelsen staining of lung impression and faecal samples smear and polymerase chain reaction (PCR) testing of the bTB suspected cases collected from carcasses of cattle that were subjected to necropsy at Livestock farm Adhartal and organised dairy farms in and around Jabalpur and in the Department of Veterinary Pathology, Veterinary College Jabalpur (M.P.) of India.

MATERIALS AND METHODS

Place of Work

The work was conducted in the Department of Veterinary Pathology, College of Veterinary Science and A.H and the Advanced Tuberculosis Diagnostic Centre for Wild and Domestic Animals, Centre for Wildlife Forensic and Health, N.D.V.S.U., Jabalpur (M.P.).

Animals

Samples were collected from the carcasses of cattle that were subjected to necropsy at Livestock farm Adhartal and organised dairy farms in and around Jabalpur and in the Department of Veterinary Pathology, Veterinary College Jabalpur.

Collection of biological samples

Post-mortem examination of 100 cattle dying during the study period was conducted according to the guidelines for the meat inspection. All major viscera and regional lymph nodes were examined and incised. The location and characteristic of each lesion were recorded.

If the gross findings were indicative of tuberculosis, then the affected tissue was collected and processed for cytological and histo-pathological examination following the method of Gridley, 2006. From the carcass the rectal

scrapping was collected for acid fast staining, DNA extraction and molecular diagnosis.

Isolation of genomic DNA from different biological samples

DNA was isolated from the suspected tissue and faecal samples using DNeasy blood and tissue extraction kit (QIAGEN, GmbH, Hilden, Germany) and DNA-SORB-B extraction kit (SACACE Biotechnology, Italy) according to the manufacturer's instructions.

Evaluation of the quantity and quality of genomic DNA

Quantity and quality of DNA was checked using Nanodrop® spectrophotometer (ND-1000) and agarose gel electrophoresis.

PCR amplification of the genomic DNA

PCR was carried out in a thermal cycler (Gradient Thermal Cycler, Eppendorf, India) using the *Mycobacterium tuberculosis/bovis* complex PCR kit (Sacace Biotechnology, Italy). The procedure and interpretation were done according to the manufacturer instructions.

The Sacace MTB complex kit used for PCR, after the PCR-mix-1 tubes were prepared and then transfers them into the thermal cycler, only when temperature reached 95°C and the program was started (Table 1).

Result analysis

The analysis of the result was based on the presence of the specific bands of the amplified DNA in agarose gel (2%). The length of the specific amplified DNA fragment was:

- *Mycobacterium tuberculosis* and *M. bovis* – 390 bp.

The amplication of DNA samples was also carried out as per Gunisha *et al.* (2000) in a thermal cycler (Gradient Thermal Cycler, Eppendorf, India) using IS6110 oligonucleotides (Imperial Life Science Pvt. Limited, Gurgaon, India): Upstream primer 5' CCTGCGAGCGTAGGCGTCGG 3' and downstream primer 5' CTCGTCAGCGCCGCTTCGG 3' which code for 123 bp. The amplification of DNA was done in a 50 µl reaction mixture consisting of 5 µl of 10X buffer, 50

µM each of primers, 200 µM of each deoxyribonucleotide triphosphate, Taq DNA polymerase and 50 pg of DNA template. The PCR cyclic parameters were initial denaturation at 94 °C for 5 min. followed by 3 steps profile: Denaturation at 94 °C for 2 min., annealing at 68°C for 2 min. and extension at 72 °C for 2 min., for a total of 30 cycles. Extension time was increased by 5 sec. for each subsequent cycle.

PCR products were subjected to electrophoresis on 2% agarose gel containing ethidium bromide (at the rate of 0.5 µg/1ml of gel solution) with 100 bp DNA ladder (GeneRuler™, Fermentas) at 80V for 70 min in 0.5X Tris-borate EDTA buffer. PCR products were visualized under UV transilluminator gel documentation system (Alpha Innotech, JH India Ltd.).

Demonstration of acid fast bacilli

Demonstration of acid fast bacilli was done in the faecal samples and impression smears from suspected tuberculous lesions by using the Ziehl - Neelsen staining. Faecal smear were prepared and fixed by using few drops of methanol. In the same manner, lung impression smears obtained from the post mortem examination of cattle suspected for tuberculosis were stained by this method. For this, staining procedure ZN staining kit manufactured by Hi-media was used as described in their guidelines.

Gross and microscopic pathology

The gross appearance of lung samples was documented and the whole lung was collected from cases of postmortem and fixed in 10% normal saline. Representative tissues with tuberculous lesions from each lung sample were embedded in paraffin. Sections were cut at 4-5 µm thickness and stained with Haematoxylin and Eosin.

RESULTS AND DISCUSSION

Out of the 100 animals, in 12 cases nodular lesions with casseating mass suggestive of tuberculosis were observed in the lung tissue. Several small sized nodules of 2-6 mm in diameter were observed in lungs. They were scattered in all the seven lobes of lung. These nodules were small, whitish yellow in colour and protruded from the organ. There was caseation and mineralization on cutting. In all



the 12 animals, there was enlargement and caseation of mediastinal and bronchial lymph nodes also. In two cases, tubercles were also observed in the pleura and the ribs (Fig. 1). Only in two animals, generalised lesions with involvement of liver, spleen and mesentery along with lungs and regional lymph nodes were noticed. In these animals, the peritoneum was studded with small sized nodules. Spleen had nodules of 2 cm in size while liver had small sized numerous nodules.

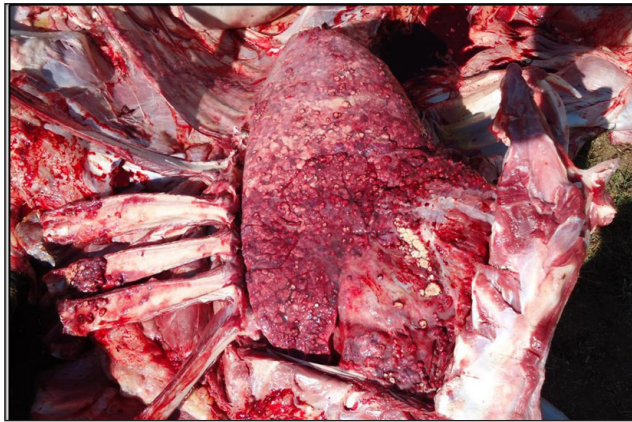


Fig. 1: Thoracic cavity of cross bred cow with tubercles in lungs and ribs

All 12 lung impression smear and only five faecal smear showed acid fast bacilli. The number of acid-fast bacilli was high in all lung smears. Impression smears were also prepared from enlarged pre-scapular lymph nodes observed only in two animals. In both the cases acid fast bacilli could be demonstrated. They were mainly observed free in the caseous debris within macrophage cytoplasm and also in Langhan's type multinucleated giant cells.

Histologic features comprised a classic granuloma as a characteristic lesion of tuberculosis composed of a central caseous necrosis with mantle of macrophages, lymphocytes, plasma cells, epithelioid macrophages and Langhan's giant cells and were observed in all 12 cases. All the lesions were characterized by a thick fibrous capsule surrounding irregular multicentric granulomas with multiple necrotic cores (Fig. 2). Necrotic cores contained foci of dystrophic mineralization.

Microscopic evaluation of all affected lymph nodes revealed caseous necrosis in the centre with varying degrees of calcification (Fig. 3). A layer of inflammatory

cells, consisting of lymphocytes, macrophages, epithelioid cells and Langhan's giant cells, surrounded this necrotic area. These inflammatory cells were surrounded by an extensive layer of fibrous connective tissue.

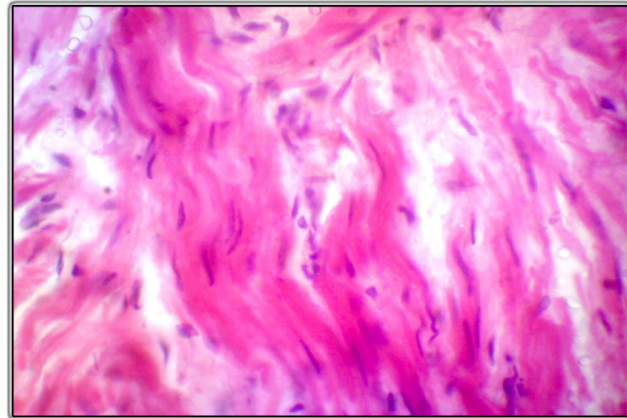


Fig. 2: Microscopic section of cattle lung granuloma with cellular infiltration and fibrous encapsulation (H&E $\times 400$)

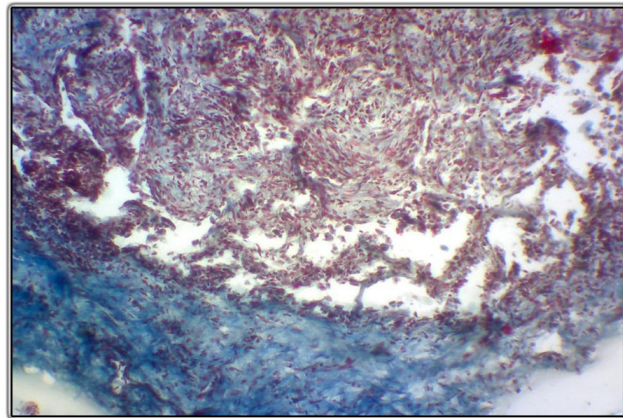


Fig. 3: Microscopic section of the cattle lung granuloma with fibrous encapsulation stained blue (Masson Trichrome $\times 100$)

In the early nineteenth century, tubercle bacilli were considered to occur in the faeces only in pulmonary or abdominal tuberculosis, due to ingestion of the infected sputum or to ulceration of the intestinal tract. However, Reuss, 1995 reported that the proportion of cattle heavily infected with *M. bovis* that are excreting the organism in their faeces is typically 10 per cent, but it can be as high as 80 per cent. This is likely to be much reduced for cattle in the early stages of infection, but in the absence of further information, all infected cattle should be considered as

potential excretors. The testing at long intervals will increase the risk of significant *M. bovis* contamination of slurry, by allowing the disease to progress to a mature stage, where the muco-ciliary escalator can convey bacilli from the respiratory to the gastrointestinal tract.

Following faecal deposition at pasture, *M. bovis* survival depends on the amount of sunlight and the thickness of the deposit. Typically, the faeces will remain infective for up to six months when deposited in winter but only one to two months in the summer (Mitserslich and Marth, 1984).

Now it is established that, a significant proportion, probably between 10 and 40 per cent of cattle that are infected with *M. bovis*, excrete the organism in their faeces. In a study carried out by Srivastava *et al.*, 2008, only 3 of 40 (7.5%) isolates of *M. bovis* could be isolated from rectal pinch followed by and faecal specimens (1/40) (2.5%). Cadmus *et al.* (2011) demonstrated *M. tuberculosis* and *M. africanum* in stools from children.

Thus, our finding of positive cases of acid fast bacilli in the faecal impression smear which possibly included non-tuberculous bacilli also in faecal sample is in agreement with those of earlier workers.

In several studies, histologic examination was mainly applied in addition to acid fast staining to maximize the identification of *M. bovis* infected cattle (Shitaye *et al.*, 2006 and Varshney *et al.*, 2017) and for the evaluation of lesions evolution in experimental studies of pathogenesis (Palmer, 2007). A study on the accuracy of histopathological techniques was carried out on limited number of samples from cattle with classical histology features of tuberculosis by Virieux, 2006. The accuracy of histopathologic techniques on large number of samples is available only in cervids (Gormley and Corner, 2018). Varello *et al.*, 2008 claimed that histopathology demonstrated high sensitivity (93.4%) and specificity (92.3%), while ZN sensitivity and specificity were respectively 33.9 and 100 per cent.

All the tissue samples were positive for the *Mycobacterium tuberculosis* complex using IS6110 sequence (Fig. 4) and eight cases were found to be positive for *Mycobacterium tuberculosis/bovis* kit (Fig. 5). Faecal samples were also collected these cases, 11 cases were positive by using IS6110 sequences and four samples were positive by using *Mycobacterium tuberculosis/bovis* complex PCR kit.

PCR was evaluated by using the fragment of DNA of 123

bp belonging to insertion sequence IS6110 in the faecal and tissue samples of the 100 cattle for its sensitivity and specificity to detect *M. tuberculosis* genome.

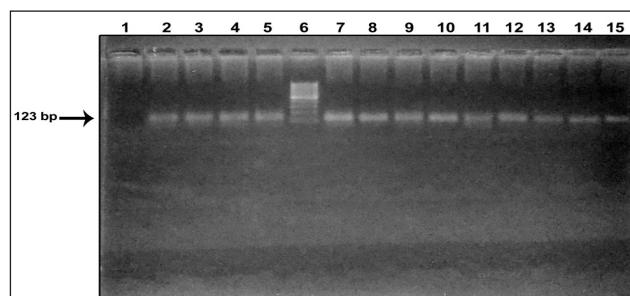


Fig. 4: PCR amplification of genomic DNA 123 bp product using IS6110 insertion sequence from cattle lung tissue samples

Lane 1 – Negative control.

Lane 2 – Positive control

Lane 6 – Molecular weight marker (100 bp)

Lane 3 to 5, 7 to 15 – Amplified 123 bp fragment corresponding to insertion sequence IS6110

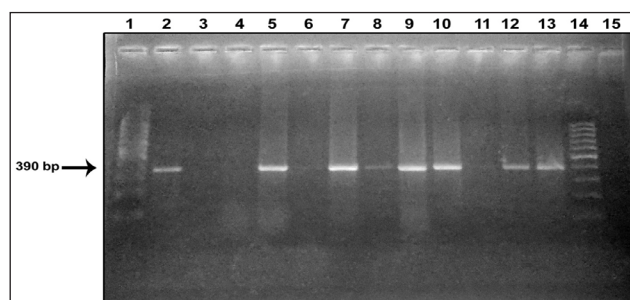


Fig. 5: PCR amplification of genomic DNA 390 bp product using *Mycobacterium tuberculosis/bovis* complex PCR kit from cattle lung tissues

Lane 3 – Negative control

Lane 2 – Positive control

Lane 1, 14 – Molecular weight markers (100 bp)

Lane 5, 7-10, 12-13 – Amplified 390 bp fragment corresponding to *Mycobacterium tuberculosis/bovis* complex PCR kit

The primers used for the PCR detect a specific insertion sequence (IS6110) that is very specific for *M. tuberculosis*, not being found in other mycobacteria or other organisms (Balamurugan *et al.*, 2006). Therefore, its detection confers great specificity. Multiple copies of the IS6110 sequence are normally considered to be present in *M. tuberculosis* making it an attractive target sequence for PCR-based diagnosis (Chauhan *et al.*, 2007).



Gunisha *et al.*, 2000 found that PCR using 123 bp fragment of DNA belonging to IS6110 is specific up to 95.6 per cent for diagnosing tuberculosis. However, in a recent study, 11 per cent of mycobacterial isolates from different parts of India lacked the IS6110 element (Singh *et al.*, 2004). Future fecal PCR testing for TB may therefore have to include a second gene specific for *M. tuberculosis* to cover the IS6110 negative strains. Thus, our results are in accordance with the findings of earlier workers and establish the utility of PCR for diagnosing bovine tuberculosis.

Wolf *et al.* (2008) on the basis of their studies on pulmonary tuberculosis in children concluded that if sensitivity can be increased, stool PCR would be a rapid, non-invasive, and relatively bio-secure initial test for children with suspected pulmonary tuberculosis. Similar observations were made by Sheen *et al.* (2009) who also postulated that the stool PCR is a sensitive, specific, rapid and relative bio-secure technique for the diagnosis of pulmonary tuberculosis and should be considered when sputum samples are unavailable. Albrecht *et al.* (2009) found that the overall sensitivity was similar for PCR (13%) and culture (12%). PCR and culture had similar sensitivity when stratified by microscopic results. They had concluded that the PCR and culture of stool specimens have similar sensitivities for the diagnosis of pulmonary tuberculosis.

PCR in faecal and tissue samples from 12 necropsy cases of cattle was also carried out in a thermal cycler using the *M. tuberculosis/bovis* complex PCR kit. The *M. tuberculosis/bovis* complex PCR Kit constitutes a ready-to-use system for the detection of DNA of all members of the *M. tuberculosis* complex (*M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. microti*, *M. pinnipedii*) using polymerase chain reaction.

Nahar *et al.* (2011) using the commercial PCR kit observed that PCR is a sensitive screening assay for the detection of *Mycobacterium bovis* DNA in lymph nodes of cattle (Taylor *et al.*, 2007). PCR can generally be used to diagnose bovine TB in field conditions.

We agree with the observation of Bermudez *et al.* (2010) that the availability of numerous commercial kits for DNA extraction from tissues and body fluids has made sample preparation simple as PCR increases the rate of detection and will be a useful tool for control programs.

CONCLUSION

In the present study there was good agreement between histopathology, acid fast staining and PCR suggesting that histopathological examination is a reliable tool for rapid diagnosis in countries where active tuberculosis eradication programs allow the prompt identification and elimination of reactor cattle. It can also be concluded that faecal samples which are easier to collect should be preferred for diagnosis of TB by PCR in cattle.

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Competing interests

Authors declare that they have no Competing interests.

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