Original Research

Descriptive Report of Post-Mortem Diagnosis of Bovine Granulomatous Tubercular Pneumonia through Conventional Histological and Immunohistochemical Techniques

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Abstract

The study was aimed to diagnose the tubercular lesion of lung in bovine in spontaneous cases through histological and immunohistochemical techniques. In a year-long study of bovine pneumonia cases, 9 cases were suspected for granulomatous tubercular lesion. The tissues from the lesions were subjected for histological and immunohistological examination along with acid fast staining to see the presence of bacteria. The tubercle appeared as small to medium size was more pronounced in right cranial lobes in most of the cases. Histologically small to large granulomas with clusters of cells surrounding centrally caseous necrotic area were observed. The caseous necrotic areas had nuclear debris and were surrounded by granulomatous inflammatory reaction in which dense cluster of lymphocytes, epithelioid cell, macrophages and moderate amount of scattered Langhans' giant cell. Staining with Ziehl Neelsen (ZN) stain, 7 cases showed low to high numbers of acid fast bacteria in lung section examined out of 9 cases. Immunohistological (IHC) staining with commercial anti-mycobacterium tuberculosis polyclonal antibody showed positive reaction in Langhan's giant cell, epithelioid cells and macrophages and in caseous necrotic tissues. As compared to ZN staining, the entire 9 samples were found positive for M tuberculosis antigen by IHC. Generally the intensity and number of positivity were much higher in IHC staining than ZN staining for acid fast bacilli. Thus, the present study shows that immunohistochemistry can be used over conventional histopathology and ZN staining in postmortem diagnosis of bovine tuberculosis and can be applied even in cases with small number of tubercle bacilli.

Key words: Bovine Tuberculosis, Granulomatous Pneumonia, Histology, Immunohistochemistry, ZN Staining

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Introduction

Tuberculosis (TB) is a chronic infectious disease of bovine caused by *Mycobacterium tuberculosis* complex which includes M*ycobacterium tuberculosis* and *Mycobacterium bovis*. This infection hinders bovine production and induces severe economic losses to livestock due to death, chronic disease and trade restrictions (Lopez, 2012). Bovine tuberculosis is a chronic granulomatous caseous-necrotising inflammatory process that mainly affects the lungs (Domingo *et al.,* 2014).Conventional diagnosis of tuberculosis is based on macroscopic evaluation at postmortem, detection of organisms in acid fast staining, and histological diagnosis. Considering the limitations in sensitivity and specificity of Ziehl-Neelsen (ZN) staining (Trusov *et al.,* 2009), mycobacterial culture and molecular and serological techniques (Attallah *et al.*, 2003; Dora *et al.*, 2008), histomorphological analysis appears to be the only feasible technique for field diagnosis of TB. Histological diagnosis of TB has long been an important issue in anatomical pathology (Pedersen *et al.,* 2011; Mustafa *et al.,* 2006) since many inflammatory granulomatous morphological characteristics similar to those described for tuberculosis. The alternative rapid diagnosis of tuberculosis is nowadays possible through PCR assay having higher sensitivity and specificity (Meickle *et al.,* 2007; Cardoso *et al.,* 2009). Immunohistochemistry (IHC) technique can be a choice to study the pathomorphological changes as well as it facilitiates mycobacterium detection, in formalin fixed tissue samples even in cases with rare bacilli or its fragments in tissues (Martinez-Burnes *et al.,* 2012). The present study aimed to use histopathological and immunohistopathological techniques along with ZN staining for detection of *Mycobacterium bacilli* in suspected morphological lesions of tuberculosis observed at post mortem in bovine cases.

Materials and Methods

In a year-long study of bovine pneumonia cases at Punjab state, India, out of 112 pneumonic lung examination 9 cases which were suspected for granulomatous tubercular lesion macroscopically were used in this study. The lung sample as well as other visceral organs was fixed in 10% neutral buffered formalin for histopathological examination. Formalin fixed tissue were processed by routine histopathological methods and paraffin embedded, 4-5µ thick sections were stained with routine haematoxylin-eosin and Ziehl-Neelsen method. The maturity of granuloma characterised by formation of fibrous tissue capsule were also assessed by special staining with Masson's trichrome. The presence of acid fast bacilli in the tissue section was assessed by examination of ZN stained slides. Ziehl-Neelsen staining was performed according to the standard protocol. Briefly, tissue specimens were deparaffinized and rinsed with consecutive dilutions of alcohol (96%to 70% ethanol). After heat fixation, specimens were washed with carbol fuchsin for 4 minutes and incubated with HCl. Counterstaining was done using brilliant green for 20s. After rinsing, samples were allowed to dry at room temperature.

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The paraffin embedded tissues from all cases were also processed for immunohistochemistry examination. For immunohistochemical staining, 3-4 micron sections were cut on Poly-L-lysine coated microscopic slide. After deparaffinization, section was gradually hydrated with 99 to 70% alcohol, later rinsed with distilled water and PBS. Heat induced epitope retrieval was done with sodium citrate buffer (pH6.0) in easy and enhanced (EZ) retriever system at a temperature of 95^0C for 10 minute and 98^0C for 5 minute. After retrieval and cooling down the tissues, they were rinsed with PBS and specimens were blocked with antiserum. Endogenous peroxidase activity was quenched by dipping the tissue slides in 3% H_2O_2 in methanol for 10 minutes. Antibody was prepared with Tween 20 + Tris buffer at a 1:500 dilution. In the first phase, primary antibody of polyclonal *M. tuberculosis* (ab905, Abcam) was reacted in a humidified chamber at room temperature for 30 min and after rinsing the specimens with PBS, polymer HRP antibody (Bio genex lab, USA) was added. After staining with chromogen, hematoxylin was used as the contrast dye. For each staining, one negative and one positive control were also considered. A brownish colour observed by light microscopy within the tissue sections on sample slide was interpreted as positive IHC reaction.

Results and Discussion

The present study investigated the post mortem diagnosis of 9 cases of bovine tuberculosis from 112 bovine animals showing pneumonia lesions. The diagnosis was based on finding of gross lesions, acid fast staining of lung tissue, histomorphological and immunohistological examination.

Pathological Examination

All the suspected cases of tuberculosis were observed in adult cattle. The tubercle appeared as small to medium size mostly distributed to right cranial lobes as well as to caudal portion of lungs (Fig.1a&b).

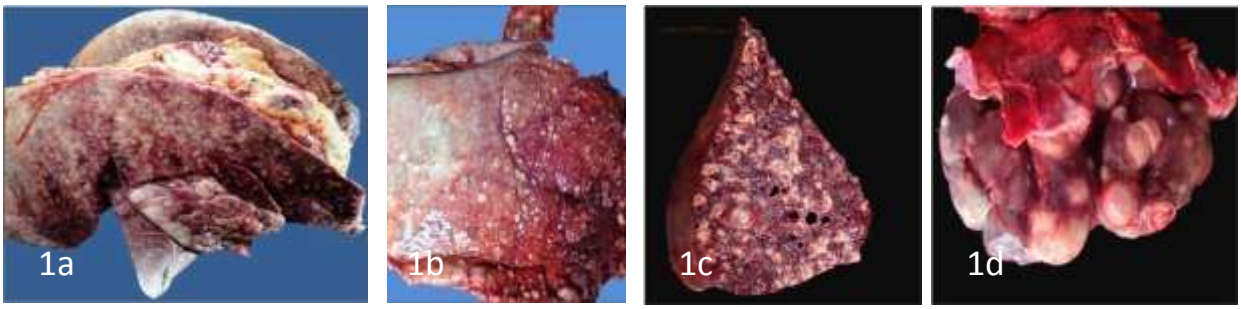

Fig. 1: Gross observation of organs; 1a- Distribution of tubercle lesions in right lung lobes; 1b- Small to medium sized tubercles over lung Parenchyma; 1c- Cut section of lung with dried caseous masses embedded in parenchyma; 1d-Tubercles in Kidney

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The distribution of tubercle was more pronounced in right cranial lobes than others. The pulmonary associated lymphnodes were enlarged with small tubercle in few cases. In natural cases of bovine tuberculosis frequency of tubercular lesion were more pronounced in respiratory organs with involvement of right lung lobes (Menin *et al.*, 2013; Liebana *et al.*, 2008). On contrary to our findings, Vural and Alcigir (2010) recorded most predominant distribution of tubercle of military form in caudal lung lobes on examination of slaughtered cattle. The cut section of tubercle revealed dried caseo-calcerous masses embedded in lung parenchyma mostly (Fig.1c). In few cases, small abscessation were observed resembling tubercle in lungs. The systemic forms of tuberculosis were also observed in two cases revealing tubercle in kidneys (Fig.1d) and spleen besides lungs.

Histopathological Examination

Histological examination in the present study revealed small to large tuberculous granulomas which were formed by clusters of cell surrounding centrally caseous necrotic area (Fig. 2a). There were perivascular and peribronchiolar accumulations of resident epithelioid macrophages, neutrophils and lymphocytes located in the parenchyma, close to major airways and blood vessels. The caseous necrosis contained nuclear debris and were surrounded by granulomatous inflammatory reaction in which dense cluster of lymphocytes, epithelioid cell, macrophages and moderate amount of scattered Langhans' giant cell were found (Fig. 2b). There were also presence of few neutrophils and plasma cell. In some cases caseous necrosis developed at centre of tubercle and appears as amorphous eosinophilic material with necrotic cell debris and central mineralization (Fig. 2c). The lesions were suggestive of progressive development of tubercles grown over time (Neill *et al.*, 2001). Domingo *et al.* (2014) described the typical gross lesion of tuberculosis as a tubercle, which is a circumscribed yellowish granulomatous inflammatory nodule approximately 2–20mm in diameter that is more or less encapsulated by connective tissue and often contains central caseous necrosis and mineralization. A presumptive diagnosis of bovine tuberculosis can be made if the tissue has the following characteristic histological lesion: caseous necrosis, mineralization, epithelioid cells, multinucleated giant cell and macrophages (OIE, 2004). Similar changes were also recorded in the present study to describe the lesion as tubercular granuloma. Histopathologic examination is a reliable tool for rapid diagnosis and permits identification of typical mycobacterial lesions and its differentiation from other causes (Varello *et al.*, 2008). Formation of connective tissue capsule by proliferation of fibroblast and apposition of the pre-existing fibrous tissue of the interlobular septa, interspersed with lymphocytes was observed in the tubercle (Fig. 2d) were probably more mature form and very chronic lesion (Marshall *et al.*, 1996). The chronic lesion was also characterised by presence of large caseous necrotic lesion with mineralization in centre and fibrotic were observed in lung as well as in mediastinal lymph node. Lymphnode showed typical well organized granulomas in all of the cases (Fig.

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2e). The granulomas of lymph node were found to show lesions with necrosis and mineralization. Interstitial pneumonia was also observed in some areas of lung section. In two cases, ganuloma were observed with abundant neutrophilic infiltration without the presence of langhans giant cell. The granulomas were covered with fibrous tissue capsule giving the appearance of tubercle granuloma. Similar histological lesions were also observed in kidneys (Fig. 2f) and pulmonary lymph node showing tubercular lesions grossly.

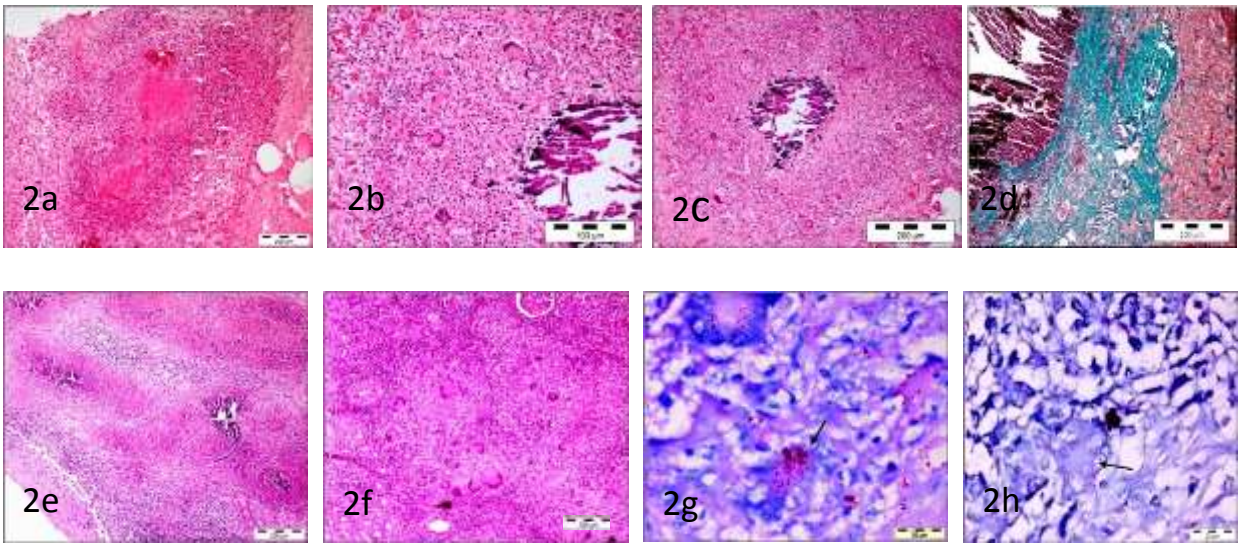

Fig. 2: Histological observation; 2a. Lung-Tuberculus granuloma with cluster of cell centrally caseous necrotic area (H&E 10X); 2b.Lung-Granulomatous reaction with lymphocytes, epithelioid cells, macrophages and Langhan's giant cell (H&E 20X); 2c.Lung-Tuberculus granuloma in lung with centrally calcified and caseous necrosis (H&E 10X); 2d. Formation of thick fibrous tissue capsule over tubercle in lung (MST 10X); 2e. Pulmonary lymphnode-multiple tuberculus granuloma (H&E 10X); 2f. Kidney-granuloma showing lymphoid cell, Langhans giant cell (H&E 10X); 2g. High numbers of acid fast bacilli in Langhan's giant cell, epithelioid cell and in macrophages (ZN 100X); 2h.Low numbers of acid fast bacilli in lung section (ZN 100X)

Infiltrating cell observed in the tissues samples included macrophages, multinucleated giant cell, lymphocytes, neutrophils, epithelioid cell and fibroblast. The macrophages and lymphocytes are recruited across the endothelium and migrated through infected tissue and thus aggregated to form granulomas. Macrophages and T-cell play key role in the immune containment of mycobacterium. Other immune cells like neutrophils are associated with increased susceptibility to *M. tuberculosis* infection (Keller *et al.*, 2006) indicating that neutrophils may play a more important role during *M. tuberculosis* infection.

Moreover, in the tissues, lymphocytes were observed along with the macrophages, epithelioid cells and giant cells. Macrophages differentiated in to epithelioid cell during chronic cytokine stimulation. These epithelioid cells fused to form typical multinucleated giant cell. Further, some mycobacterium, persist in the infected lung due to blocking of phagolysosomal fusion and acidification of infected phagosome by tuberculous organism, leading to chronic antigenic stimulation and T cell accumulation around

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macrophages. The close apposition of lymphocytes and macrophages is necessary for the activation of macrophages to kill *M. tuberculosis* (Kumar *et al.*, 2013).

Detection of Acid Fast Bacilli

The tissue section of lung were stained with modified ZN stain revealed low to high numbers of acid fast bacilli settled in caseous necrotic debris, Langhans giant cells as well as in epithelioid cell and macrophages in lung and lymph node (Fig. 2g). Staining with ZN, 7 out of 9 cases showed low to high presence of acid fast bacteria in lung section (Fig. 2h). Within the macrophages these slow growing mycobacteria adapted for survival and this capacity to persist in the face of a potent cellular response underlies the chronic inflammatory reaction of the host (Saunder and Britton, 2007). The fastest and simplest methods of confirming a mycobacterial infection is staining suspected samples with ZN stain to demonstrate the acid fast bacilli under the microscope (WHO, 2008). However, the apparent absence of acid fast bacteria in histological slides with typical tuberculous lesions is not sufficient to rule out the morphological diagnosis of tuberculosis (Domingo *et al.*, 2014).

Immunohistochemical Detection

Immunohistological (IHC) staining used with commercial anti *Mycobacterium tuberculosis* polyclonal antibody developed in rabbit showed brownish colour positive reaction in Langhan's giant cell, epithelioid macrophages and in caseous necrotic tissues (Fig. 3a). As compared to ZN staining the entire 9 samples found positive for *M. tuberculosis* antigen by IHC. Purohit *et al.* (2007) used IHC for detection of anti *M. tuberculosis* antigen in tubercular granuloma in cattle. The positive staining were coarse granular/or bacilli in giant cell as well as in macrophages. The antigens were determined generally in cytoplasm and/or outside of macrophages (Fig. 3b.). In this study, epithelioid cells in the periphery of granuloma were stained more positively than the epithelioid cells at the center of granuloma or adjacent to the necrotic zone. Phom *et al.* (2016) found that epithelioid cells outside the necrotic zone showed greater positivity than cells within the necrotic area. The positive IHC staining similar to lung also observed in lymph node granuloma as well as in kidney granuloma. However, there was no IHC positive reaction observed in kidney, which showed granulomatous lesion histologically. The positive tissue section with and without addition of *Mycobacterium tuberculosis* antibody in IHC staining showed positive and negative reaction respectively are shown in Fig. 3c&d.

A total of 9 numbers of cases showed positive reaction immunohistologically including the 7 ZN positive cases. Generally the intensity and number of positivity were much higher in IHC staining than ZN staining for acid fast bacilli. Goel and Budhwar (2007) reported 64–100% sensitivity for IHC and zero to 44% sensitivity for ZN staining in determining tubercle bacilli in tissue section.

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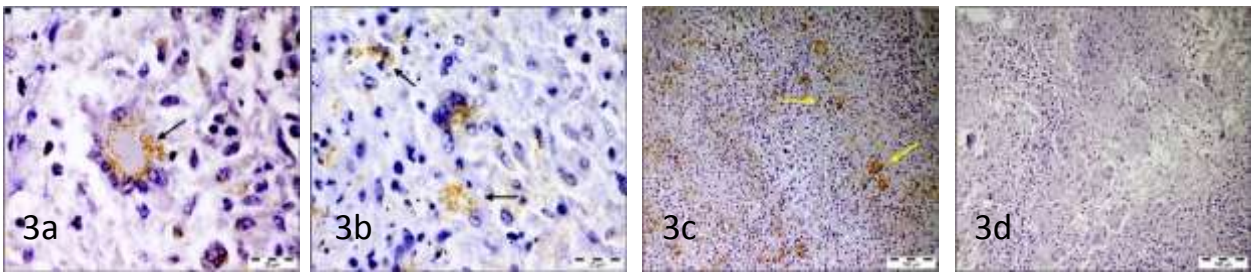

Fig. 3: Immunohistological observation; 3a. Lung-IHC staining of *Mycobacterium organism* in giant cell (100X); 3b. Lymph node-IHC staining of *Mycobacterium spp.* in giant cell, macrophages (100X); 3c. IHC reaction of *Mycobacterium spp.* in epithelioid cell, giant cell and macrophages (10X); 3d. Negative control for *Mycobacterium* antigen in tissue section (IHC 10X).

An explanation for ZN low sensitivity may be a low survival rate of mycobacteria in the environment of the central caseation (Cassidy, 2006) or loss of bacterial structure owing to immune responses operating in granulomatous inflammation in mycobacteriosis (Gutierrez and Garcia, 1993). For this reason, the detection of acid fast bacilli using ZN method needs to be reconsidered with other diagnostic methods like immunohistochemistry (Watrelot-Virieux *et al.,* 2006). Gutierrez and Garcia (1993) have compared ZN and IHC for detection of *M. bovis* in bovine and caprine tuberculosis lesion and showed IHC to be more sensitive than the ZN method. However, lower specificity of IHC may occur due to cross reactivity of polyclonal anti *M. bovis* antibody with various microorganism has been observed by Arrese and Pierard (1998).

Conclusion

The present immunohistological findings were in accordance to Goel and Budhwar (2007) describing small bacillary fragments and antigenic dust in tissue sections. Ziehl-Neelsen staining has low sensitivity and requires the presence of intact bacilli. Logani *et al.* (1999) demonstrated that IHC staining with pAbBCG yields positive results even in tissues with 10 bacilli per slide in human TB. Thus, the present study suggests that immunohistochemistry can be used over conventional histopathology and ZN staining with small number of tubercle bacilli for any reason.

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