Research Article

Comparative Study on Detection of *Mycobacterium bovis* Infection in Bovine Tuberculous Lesions

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Abstract | Bovine tuberculosis (bTB) is an infectious and zoonotic disease. A tentative diagnosis of bovine tuberculosis was made following the macroscopic detection at necropsy of typical lesions. Histopathological examination of the lesion enhances the confidence of the diagnosis but bacteriological isolation of *Mycobacterium bovis* from the lesion is the only way to make a definitive diagnosis. The postmortem investigations were carried out for the detection of tuberculous lesions mainly affected by the workload time and the diligence of the meat inspector conducting the examination. The aim of the study was to determine the trend of occurrence of tuberculous lesions; to detect the tuberculous lesions and etiological agent in tissue samples of the respiratory tract and mesenteric lymph nodes of the slaughtered animals; to detect macroscopic characteristics of *M. bovis* in the observed lesions from slaughtered cattle and buffaloes; to detect *M. bovis* and its antigens/mycobacterial fragments through immune-histochemical (IHC) technique the avidin-biotin complex peroxidase (ABC-P) method and to distinguish their possible relationship with the type of lesion. The one-year (January–December 2017) retrospective analysis showed 0.44% culled animals and meat inspection of slaughtered animals (118453) at ring road slaughter house Peshawar showed that 0.099% animals (100) were found with tuberculous lesions in parenchymatous organs with 0.181% cattle (57 of 31459), 0.202% buffaloes (55 of 27227), 0.007% sheep (3of 45441) and 0.014% goats (2 of 14326) at slaughter house. The tuberculous lesions found in cattle and buffaloes were highly significant compared to other animals. Similarly, out of 100 tissue samples histopathological, granulomatous inflammation was evident in 8.7% samples (4 of 46) with tuberculous lesions. The immune-histochemistry (IHC) was positive in 10.9% tissue samples (5 of 46) with tuberculous lesions and in 1.9% of tissue sample (1) without tuberculous lesions. The IHC has the advantages of being robust, cheap and can be used in a routine laboratory for the detection of *M. bovis* and its antigenic fragments in tissue samples. The findings of this study indicated that tuberculosis in large ruminants (cattle and buffaloes) is a neglected zoonotic infection at the animal-human interface in Khyber Pakhtunkhwa, Pakistan which needs to be solved.

Received | February 18, 2024; **Accepted** | November 22, 2024; **Published** | January 20, 2025
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Citation | Ullah, A., F. Hafeez, R. Taj, S. Gul, I. Khan, B. Faheem, M. Ahmad, R. Ullah, A. Ahmad, M. Hanif, A.U. Khan, M.O. Khan, A. Basit, M.I. Khan, S. Khan and M. Islam. 2025. Comparative study on detection of *Mycobacterium bovis* Infection in bovine tuberculous lesions. *Sarhad Journal of Agriculture,* 41(1): 111-125.
DOI | https://dx.doi.org/10.17582/journal.sja/2025/41.1.110.125
Keywords | Tuberculous lesions, Histopathology, IHC, *M. bovis*, ZN staining, Lungs

Introduction

Tuberculosis is a chronic bacterial disease in animals and humans characterized by the progressive development of specific granulomatous lesions of tubercles in affected tissues. The disease affects susceptible hosts of all age groups and is accountable for more deaths throughout the world than any other bacterial diseases ever today (Omer *et al*., 1995). Tuberculosis in cattle and other domestic animals is above all caused by two members of *Mycobacterium tuberculosis* complex (MTC): *M. bovis* and *M. caprae* (Pavlik *et al*., 2002; Prodinger *et al*., 2002; Erler *et al*., 2004). However, occasional occurrence of tuberculosis due to M. tuberculosis species with concurrent tuberculous lesions has been reported in pigs (Popluhar *et al*., 1974; Flesja *et al*., 1978), in cattle (Popluhar *et al*., 1974; Schliesser, 1976; Thoen *et al*., 1981; Pavlik *et al*., 2005), in dogs (Pavlik *et al*., 2003) and other animals (Pavlik, 2006).

The global prevalence of human tuberculosis due to *M. bovis* has been estimated at 3.1% of all human tuberculosis cases, accounting for 2.1% and 9.4% of pulmonary and extra pulmonary TB cases respectively (Cosivi *et al*., 1998). Drinking raw milk is a primary route of *M. bovis* infection in humans; hence the occurrence of human tuberculosis is most commonly extra pulmonary form, particularly in the cervical lymphadenitis form. Kidane *et al*. (2002) indicated that *M. bovis* along with other MTC species were found to be a cause for tuberculous lymphadenitis in humans. According to Enarson (2006) literature review more than 50% of *M. bovis* infection in humans in Europe, USA, Canada, Argentina, Australia and New Zealand has pulmonary localization. This proportion may however, change in other regions of the world especially in Africa. In contrast to this report, cases of pulmonary tuberculosis due to *M. bovis* were also reported (O'Reilly and Daborn, 1995; Kazwala *et al*., 2001).

Currently, due to the upsurge of HIV/AIDS infection, the epidemiology of tuberculosis has been greatly affected as many HIV-infected individuals are co-infected with tuberculosis, the incidence of the disease may rise in coming years (Zumla *et al*., 1999; Ayele *et al*., 2004). The occurrence of *M. bovis* in humans, against the background of the soaring HIV/AIDS incidence particularly in eastern and southern Africa, implies that the risk of spillover of zoonotic tuberculosis to rural communities is rapidly increasing (Zinsstag *et al*., 2006). Moreover, Regassa (2005) reported that a higher prevalence of tuberculosis in cattle owned by tuberculous patients was found than in cattle owned by non-tuberculous owners, which suggests the significant role of *M. bovis* in the incidences of human tuberculosis in Ethiopia. Thus, the correlation between the prevalence of *M. bovis* infection in humans and that of local cattle populations highlights the potential threat of this disease for humans (Daborn *et al*., 1996).

Bovine tuberculosis continues to be an important problem in cattle in various countries despite the implementation of eradication campaigns. As a result of these campaigns, initial lesions are most observed, i.e., small primary complexes which usually show few if any mycobacteria in smears or tissue sections stained by the Ziehl-Neelsen (ZN) method (Mcllroy *et al*., 1986). In Spain, tuberculosis due to Mycobacterium bovis is also a significant disease in goats, some flocks showing high infection rates and often sever exudative lesions with cavitation (Perez-Perez *et al*., 1989) in which the number of detectable bacilli is low or nil (Gutierrez and Marin, 1993).

The paucity of detectable bacilli in lesions may be due to immune responses operating in granulomatous inflammation in mycobacteriosis (Ridley, 1983; Young *et al*., 1990), and it is possible that the lesions contain antigens arising from degradation and destruction of bacilli, and also L-forms (Fedoseev *et al*., 1985), neither of which can be detected by the ZN method. A further possibility is the occurrence of granulomatous lesions produced by immuno-complexes in which there is an excess of antibody and few bacilli (Ridley *et al*., 1982). The purpose of the present research work was to investigation the use of an immune-histochemical technique, the avidiu-biotin complex peroxidase (ABC-P) method, to detect *M. bovis*, its antigens and mycobacterial fragments, and to discern their possible association with type of lesion. This technique and another technique called the peroxidase-anti peroxidase (PAP) method are known to be capable of detecting Mycobacterium Para tuberculosis antigens in lesions in which ZN staining gives negative effects (Perez-Perez *et al*., 1989; Navarro *et al*., 1991). Such techniques can give very clear-cut outcome and permit the use of low magnification (Perez-Perez *et al*., 1989; Massone *et al*., 1990; Haines and Clark, 1991; Navarro *et al*., 1991).

In developing countries, animal tuberculosis is still prevalent and is responsible for significant economic loss in animal production and an increase in human health problems and even deaths (Neill *et al*., 1994; Cosivi *et al*., 1998; Pollock and Neill, 2002). According to literature data, approximately 85% of the cattle and 82% of the human population of Africa live in areas where animal tuberculosis is either partly controlled or uncontrolled. In contrast however, only a few African countries have applied disease control measures as part of test and slaughter strategy and consider the disease as notifiable (Cosivi *et al*., 1998). Despite this, in most developing countries pasteurization is not well practiced and therefore, 10% to 15% of human tuberculosis is considered to be caused by *M. bovis* (Ashford *et al*., 2001).

In Pakistan, the endemic nature of tuberculosis in cattle has long been reported and is one of the countries, where tuberculosis is wide spread in both human and cattle population (Javed *et al*., 2006; Akhtar *et al*., 2015). However, the actual prevalence of the disease is yet unknown due to the traditional farming system which causes difficulties in the process of study, inadequate animal health infrastructures and less attention given to *M. bovis* infection. Despite this, the economic impacts and zoonotic importance of the bovine tuberculosis infection are neither well studied nor documented. In Pakistan, bovine TB has been reported in several parts of the state (Javed *et al*., 2006, 2011; Khan *et al*., 2008; Tipu *et al*., 2012).

Detection of bovine tuberculosis in cattle and other susceptible animal species is often made on history, clinical and necropsy findings, tuberculin skin tests and abattoir meat inspections. Definitive diagnosis is made on culture with morphological appearance and biochemical tests (Kent and Kubica, 1985) and molecular biological techniques like PCR (Boddinghaus *et al*., 1990; Cousins *et al*., 1991). In Pakistan, however, due to technical and financial limitations, the new laboratory procedures have not yet been introduced as common diagnostic measures.

In view of increasing zoonotic tuberculosis in several parts of the state (Asad *et al*., 2019, 2020, 2022; Khan *et al*., 2008; Javed *et al*., 2011; Tipu *et al*., 2012). This study was aimed to determine occurrence of tuberculous lesions in in slaughter animals at abattoir; to determine prevalence of the bovine tuberculosis based on histopathology and immunohistochemistry and to detect tuberculous lesions and causal agents from tissue samples of the respiratory tract and mesenteric lymph nodes (LN) of slaughtered cattle in abattoir, district Peshawar, Khyber Pakhtunkhwa (Pakistan).

Materials and Methods

Experimental design and sampling

The present study was carried on slaughter house located at ring road Peshawar, Khyber Pakhtunkhwa, Pakistan to investigate the presence of *Mycobacterium bovis* infection in tissues samples of the respiratory tract and lymph nodes (LN) of cattle and buffaloes through histopathology, Ziehl-Neelsen (ZN) staining and immunohistochemistry (IHC).

Sample collection and transportation

A total of 100 tissue samples suspected for tuberculosis-like lesions were collected from local abattoir. The samples were collected randomly from slaughtered cattle and buffaloes suspected for tuberculosis. All meat inspectors on sample sites were also interviewed to determine whether slaughter house personnel and the butchers could recognize the tuberculosis lesions in the meat they handled. For every sample collected and given a unique number (P.I.D). Sampling was done by the researcher, veterinary officers and meat inspectors at the abattoir based on the observable lesions suspected for bovine tuberculosis. Suspicious lesions were granulomatous in nature, hard and sounded gritty or stony on slicing them and some were even filled with pus. Whole lymph nodes from anatomical sites i.e., liver, lungs and brisket tissues were examined for these lesions and suspected lesions were aseptically collected into sterile 50ml sample tubes. Once in the laboratory, the samples were accessioned (given laboratory numbers) and entered into the computer system and then stored at room temperature until they were processed.

Abattoir meat inspection analysis of tuberculous lesions

During observation of tuberculous lesions of different parts of the carcass like lung, liver, intestine and lymph node (LN), the whole carcass was condemned. While on the other hand, only partial condemnation of organs was undertaken in case of localized tuberculous lesions. During this period, 118453 domestic animals were slaughtered and 31459 cattle, 27227 buffaloes, 45441 sheep and 14326 goats were inspected (Table 1).

Table 1: *Detection of tuberculous lesions in slaughtered animals in local abattoir at ring road district Peshawar, Khyber Pakhtunkhwa, Pakistan.*

Month	Slaughtered animals			Cattle			Buffalo			Sheep			Goat		
	No	TB	%	No	TB	%	No	TB	%	No	TB	%	No	TB	%
January	8447	8	0.095	2524	5	0.198	2411	3	0.124	1598	0	0.000	1914	0	0.000
February	7366	6	0.081	2533	4	0.158	1764	2	0.113	1962	0	0.000	1107	0	0.000
March	8306	9	0.108	2463	6	0.244	2354	3	0.127	2187	0	0.000	1302	0	0.000
April	8163	8	0.098	2589	3	0.116	2193	4	0.182	2293	1	0.044	1088	0	0.000
May	9268	10	0.108	2613	5	0.191	2351	5	0.213	3201	0	0.000	1103	0	0.000
Jun	11135	12	0.108	2703	6	0.222	2400	6	0.250	4947	0	0.000	1085	0	0.000
July	10833	10	0.092	2633	4	0.152	2330	5	0.215	4856	0	0.000	1014	1	0.099
August	11497	12	0.104	2628	5	0.190	2336	6	0.257	5031	1	0.020	1502	0	0.000
September	9586	9	0.094	2322	4	0.172	1818	5	0.275	4375	0	0.000	1071	0	0.000
October	10480	10	0.095	2573	5	0.194	2204	4	0.181	4734	0	0.000	969	1	0.103
November	11239	11	0.098	2827	6	0.212	2469	5	0.203	4883	0	0.000	1060	0	0.000
December	12133	12	0.099	3051	4	0.131	2597	7	0.270	5374	1	0.019	1111	0	0.000
Total	118453	117	0.099	31459	57	0.181	27227	55	0.202	45441	3	0.007	14326	2	0.014

Table 2: *Annual culled animals (%) which came for slaughter, in local abattoir at ring road district Peshawar, Khyber Pakhtunkhwa, Pakistan.*

Month	Slaughtered animals				Culled		Total animals for slaughtering
	M	F	Young	Total No	(N)	%	
January	2753	1951	3743	8447	46	0.54	8493
February	2655	1024	3687	7366	40	0.54	7406
March	3243	1524	3539	8306	32	0.38	8338
April	3367	1155	3641	8163	25	0.31	8188
May	4257	1234	3777	9268	39	0.42	9307
Jun	6253	1698	3185	11135	24	0.22	11160
July	6093	1622	3121	10833	59	0.54	10895
August	5803	1586	4112	11497	57	0.49	11558
September	5063	1436	3088	9586	31	0.32	9618
October	5894	1603	2987	10480	52	0.49	10536
November	6120	1809	3312	11239	54	0.48	11295
December	6698	1934	3502	12133	63	0.52	12197
Total	58199	18576	41694	118453	522	0.44	118991

During the study period, out of 118991 domestic animals, 522 (0.44) animals were unfit for slaughtering on the basis of either they were down or underweight i.e; < 11kg and < 60kg of live body weight for small ruminants (Sheep, Goat) and large ruminants (Cattle, Buffalo), respectively (Table 2).

Abattoir meat inspection

Examination of animals for the detection of tuberculous lesions was undertaken in the local abattoir at ring road, district Peshawar. Detailed examination (inspection, palpation and incision) of different organs were performed for the presence of abscess, cheesy masses and tubercles (Corner *et al.*, 1990). During the routine meat inspection, observation of pathological lesions was marked in the pleural cavity, lungs, liver and intestine. Based on these gross lesion findings a total of 100 tissue samples; 25 lung (bronchial and mediastinal LN), 25 mesenteric LN, 50 parenchymatous organ tissues (25 lung and 25 liver) samples were collected from 100 slaughtered animals.

Sample processing and tissue preparation

After collection of samples, fat and other tissues were

trimmed from LN and organs for histopathological examinations. Samples were then preserved in 10% buffered neutral formalin (NBF) in a screwed glass container, close d tightly and kept under room temperature until examination. After transportation of samples to the laboratory of veterinary clinical pathology, University of Agriculture Peshawar, Khyber Pakhtunkhwa Pakistan, all formalin fixed samples were dehydrated, embedded in paraffin wax and sectioned on microtome at a thickness of 4 µm. Histological samples were stained by hematoxylin and eosin (HE) stain. Finding of granulomatous inflammation was taken as positive and other findings such as pyogranulomas and abscesses that can imitate macroscopically tuberculous nodules were taken as suspected results. The presence of Acid-fast bacteria (AFB) was examined by Ziehl-Neelsen (ZN) staining.

Histopathological examination

A total of 100 tuberculous lesions/tissue samples were collected and stored in 10% Neutral Buffered Formalin (NBF). The tissue samples were processed for histopathological examination as per standard protocol. After fixation, tissues were prepared for light microcopy evaluation by paraffin embedding technique called Formaline-Fixed, Paraffin Embedding Technique (FFET). The formalin fixed tissue samples were processed for tissue sectioning, staining and detail histopathology. At the end, slides were mounted with a cover slip by placing a drop of DPX. Slides were studied under 10X and 40X for recording detailed histopathological changes (Wesonga *et al*., 2004). The mycobacterium was recognized as red color bacillus (*Mycobacterium tuberculosis*) with blue background (Bancroft and Gamble, 2007; Awad *et al*., 2009; Ankle and Joshi, 2011).

Immunohistochemistry (IHC)

In this research work, IHC technique was used for detection of Mycobacterial antigens (proteins) in tissue sections with the help of Avidin-Biotin Complex method (ABC-method) (Figure 2). Tissues used for Immunohistochemistry were lungs and/ bronchial and mediastinal lymph nodes. The samples were taken from the lesions together with normal adjacent tissue. For immunohistochemistry, the paraffin embedded tissues sections of lungs and lymph nodes were processed. The tissues were subjected to IHC for chromogenic detection of antigen in the cell and tissue as per standard protocol adopted by Gutierrez and Marin (1993) and Dominique *et al.* (2000).

Procedure

Each sample was fixed in 10% neutral buffered formalin and processed by the standard paraffin was technique. The samples with heavy calcification were decalcified with formic acid-Na citrate solution before they were embedded in wax. Three sections of 4µm thickness were obtained from each sample and were mounted on glass slides. One section was stained with ZN and one with haematoxylin and eosin (HE), and the third section was subjected to the ABC-P method. In the ZN method, the staining with carbo-fuchsin at 60° was prolonged 1hr to maximize the sensitivity.

For immunohistochemistry, in the ABC-P technique, the sections were dewaxed with xylene and hydrated in water. The endogenous peroxidase activity was inhibited by immersing the slides in H_2O_2, 0.3% in absolute methanol for 30 minutes. After washing in Tris-buffered saline (TBS) having pH 7.5, they were incubated with 0.1 g of trypsin and 0.1 g of calcium chloride in 100 ml of Tris buffer for 40 minutes at 37 °C (trypsin-calcium chloride solution) having pH 7.8. The sections were blocked for 30 minutes with normal goat serum. The goat serum was removed from the slides, and the sections were incubated with a *M. bovis* polyclonal antiserum i.e; the slides were then incubated for 12-18 hrs at room temperature with primary antibody of rabbit IgG anti-BCG serum (Dakopatts) diluted 1 in 5000 in TBS with bovine albumin. Next, the slides were incubated under the same conditions, first with secondary antibody biotinylated goat anti-rabbit IgG (R.T.U-ready to use) for 30 min and then with ABC-P for 40 min, according to the manufacturer's instructor (Vectastain ABC kit, Vector Laboratory). The primary antibody was detected by ABC-Peroxidase detection kit. Then brown color was developed by a final incubation with diaminobenzidine (DAB) 0.05% in TBS with 0.3ml or hydrogen peroxide 3% in distilled water for 3-5 min at room temperature. These slides were counterstained with hematoxylin, dehydrated and mounted. Tissues samples with and without *M.bovis*were always used as positive and negative controls for the ABC-P and ZN staining (Gutierrez and Marin, 1993; Dominique *et al*., 2000).

Statistical analysis

The collected data was subjected to the statistical analysis using the version 20 Statistical Package (Zar,

2004; Steel *et al*., 1997). All values at p<0.05 were considered as significant. Also, the prevalence rates were calculated and presented in form of percentages (%).

Results and Discussion

The overall prevalence results of abattoir meat inspection, postmortem investigation, tuberculous lesions, histopathology and immunohistochemistry were documented.

Tuberculous lesions

The meat inspection analysis showed that among the total of 118453 slaughtered animals,0.099% were found with tuberculous lesions during the study period (Table 1). Out of 100 tuberculous lesions, 57(0.181%) of 31459 cattle, 55(0.202%) of 27227 buffaloes, 3(0.007%) of 45441 sheep and 2(0.014%) of 14326 goats. Firm creamy whitish nodular focal lesions have been predominantly observed in the lung cavity and associated LN and very rarely in mesenteric LN and liver organs.

Frequencies of tuberculous lesions in small ruminants (sheep and goats) were significantly lower (P < 0.01) than in large ruminants (cattle and buffaloes) (Table 1) and in these species, tuberculous lesions were usually observed in the digestive tracts. The maximum detection rates of tuberculous lesions were clearly recorded in large ruminants (cattle and buffalo).

Patho-morphological examination

The meat inspection investigation carried out during the study period identified 46% of 100 inspected tuberculous lesions (PL stages 2 and 3). Tuberculous lesions were observed in 29 (49.15 %) of 59 lung LN and in 9 (42.85%) of 21 mesenteric LN (PL stages 2 and 3). Similarly, tuberculous lesions were observed in the parenchymatous tissues which include 5 (38.46%) of the lung (PL stage 2) and in 3 (42.85%) of the liver organs (PL stage 2) (Table 2).

Histo-pathological examination

Hematoxylin-eosin (HE) staining: From the 100 histopathologically investigated tissue sections, 19(35.2%) acute lymphadenitis and reactive hyperplasia and 22 (47.8%) pyogranulomas were observed in lung and mesenteric lymph nodes including parenchymatous tissues i.e., lung and liver tissues. Caseation, lipomatosis and abscess were each found in 3 (3%) mesenteric LN. Chronic focal purulent inflammatory reactions due to parasites were evident in 2 (2%) tissues. The typical tuberculous lesions granulomas with calcification and multinucleated giant cells (of Langhan type) were identified in 3 (5.1%) lung and 1(4.8%) mesenteric lymph node. Statistically significant (P < 0.01) differences were observed between tuberculous lesions of lung and mesenteric lymph node (Table 3).

Ziehl-Neelsen (ZN) staining

Microscopically, mycobacteria have been detected in 2(3.4%) and 1(4.8%) in lung and mesenteric lymph node respectively while 1(7.7%) in parenchymatous tissue (lung) among all examined tissue samples stained by ZN staining technique (Table 3).

Immunohistochemistry (IHC)

Tuberculous lesions occurred in some tissue samples. Minor lesions (i.e; primary complexes) which spread characteristically as miliary tubercles which sometimes became confluent. They are formed by the usual components of granulomatous inflammation having central necrosis with calcification surrounded by macrophages, epitheloid cells, lymphocytes, plasma cells, neutrophils and langhans giant cells. In lymph nodes, granulomas showed surrounding fibrous capsules of variable thickness while in general, there was no capsule in the pulmonary foci, the most typical feature being the association of the small granulomas with bronchioles.

The more extensive lesions typically showed areas of necrosis. There was a clear separation between healthy and damaged tissue, which generally consisted of many macrophages and giant cells. Out of 6 positive tissue samples, only 1.9% (1/54) tissue sample was without tuberculous lesions while on the other hand, 10.9% (5/46) samples were with tuberculous lesions showed greater areas of necrosis surrounded by a thinner band of cellular inflammation consisting of neutrophils, lymphocytes, plasma cells, macrophages and few if any giant cells (Table 3).

Detection of mycobacteria by ZN and ABC-P methods

The mycobacteria stained by ZN method was appeared as isolated organisms (Figure 1). The positivity was noted as brownish circles or granules with IHC and with a more in size than acid fast bacilli stained by ZN method. By both methods, positivity was located either in the cytoplasm of phagocytic cells, usually

giant cells and sometimes macrophages or in necrotic areas. In lesions with a low degree of positivity, acid fast bacilli or their antigens were observed only in giant cells and in areas of necrosis. The ABC-P-stained slides generally showed a much clear and more striking positivity than that observed with ZN method (Table 3).

Areas of necrosis having sporadic ill-defined bacilli as observed by ZN staining, were seen to contain many more organisms or mycobacterial debris by ABC-P. Further, some necrotic areas were negative with the ZN method were positive by ABC-P. In the sections with many mycobacteria, the organisms could be observed in neutrophils located on the periphery of the necrosis, mainly by the ABC-P method. In some tissue samples positive with ABC-P but negative with ZN, the positivity was observed in areas of necrosis and in necrobiotic neutrophils. This feature was seen mainly in sever lesions.

With ABC-P technique, positivity was noted in 6 tissue samples. Out of 6 positive tissue samples, only 1.9% (1/54) tissue sample was without tuberculous lesions while on the other hand, 10.9% (5/46) samples were with tuberculous lesions showed greater areas of necrosis surrounded by a thinner band of cellular inflammation consisting of neutrophils, lymphocytes, plasma cells, macrophages and few if any giant cells whereas with the help of ZN stain, microscopically, mycobacteria have been detected in 2 (3.4%) and 1(4.8%) in lung and mesenteric lymph node respectively while 1 (7.7%) in parenchymatous tissue (lung) among all examined tissue samples stained by ZN staining technique (Table 3).

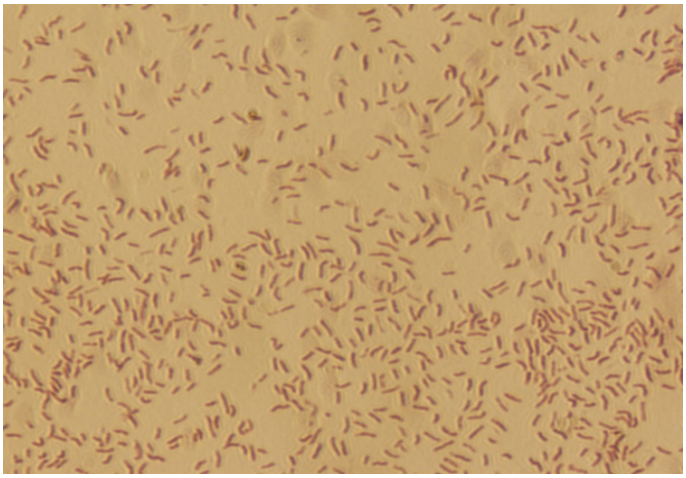

Figure 1: *Ziehl-Neelsen staining of sputum samples of abattoir workers showing acid-fast.*

Table 3: *Examination of tissue samples of cattle and buffaloes slaughtered in local abattoir, Peshawar, Khyber Pakhtunkhwa, Pakistan.*

Examined samples	Total No.	Gross pathological lesions (GPL)				Hematoxylin-Eosin (HE) staining[2]		Ziehl-Neelsen (ZN) staining		Immunohisto-chemistry (IHC)	
		stages[1]	No	%	±	%	+	%	+	%	+	%
Lung LN[3]	59	0	11	18.6	0	0.0	0	0.0	0	0.0	0	0.0
		1	19	32.2	16	27.1	0	0.0	0	0.0	0	0.0
		2	13	22.0	13	22.0	0	0.0	0	0.0	0	0.0
		3	16	27.1	0	0.0	3	5.1	2	3.4	2	3.4
Mesenteric LN[3]	21	0	10	47.6	0	0.0	0	0.0	0	0.0	0	0.0
		1	2	9.5	2	9.5	0	0.0	0	0.0	0	0.0
		2	5	23.8	3	14.3	0	0.0	0	0.0	0	0.0
		3	4	19.0	0	0.0	1	4.8	1	4.8	2	9.5
Lung	13	0	7	53.8	0	0.0	0	0.0	0	0.0	0	0.0
		1	1	7.7	1	7.7	0	0.0	0	0.0	0	0.0
		2	5	38.5	4	30.8	0	0.0	0	0.0	0	0.0
		3	0	0.0	0	0.0	0	0.0	1	7.7	1	7.7
Liver	7	0	4	57.1	0	0.0	0	0.0	0	0.0	0	0.0
		1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
		2	3	42.9	2	28.6	0	0.0	0	0.0	0	0.0
		3	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Total	100	0 to 1	54	54.0	19	35.2	0	0.0	0	0.0	1	1.9
		2 to 3	46	46.0	22	47.8	4	8.7	4	8.7	5	10.9

[1]Stages of gross pathological lesions (GPL): 0 (tissues with no lesions), 1 (lymphadenopathy or non-specific lesion in the tissue of parenchymatous organs), 2 (caseation), and 3 (calcification). [2]Histological examination after the hematoxylin-eosin staining: ± (tissues with lesions like pyogranulomas, hyperplasia, lymphadenitis and abscess), + (granulomatous inflammation). [3]Lymph Nodes.

Links Researchers

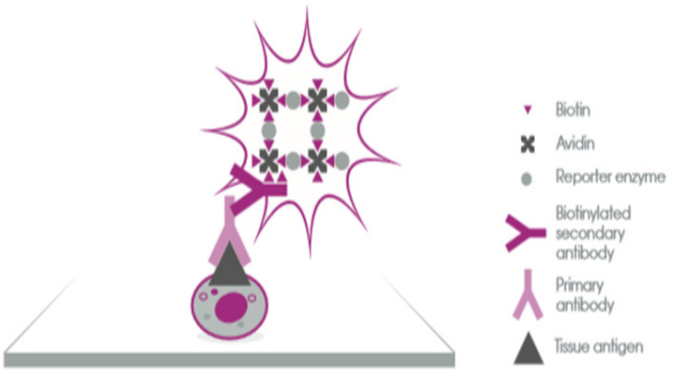

Figure 2: *Avidin-Biotin Complex (ABC) method (https://vectorlabs.com/browse/abc-avidin-biotin-complex-kits).*

Tuberculous lesions

The Peshawar abattoir is the largest abattoir found in the Khyber Pakhtunkhwa, Pakistan and delivers daily beef requirements to the metropolitan. In the abattoir, cattle, buffalo, goats and sheep, coming from different directions of the city, are slaughtered most often here. In this study, tuberculous lesions were detected in 100 (0.099%) examined animals in which cattle and buffalo were predominantly found with tuberculous lesions than other slaughtered animals and also the rate of positive incidences has been found to be low considering the quantity of slaughtered animals. This result strengthen our suggestion that in most cases post mortem investigations depend on the time, work load and the attentiveness of the inspector conducting the examination for the detection of bovine tuberculous lesions (Corner *et al.*, 1990). In abattoirs, the recognition of tuberculous lesions can be affected due to early infection, parasites, non-specific reactions and due to infection other than *M. bovis* (Corner, 1994) and other irregularities of abattoir meat inspections (Edwards *et al.*, 1997). However, the presence of tuberculous lesions confirms that bTB is an existing problem in Khyber Pakhtunkhwa, Pakistan which needs to be solved.

Pathomorphological examination

The abattoir meat inspection investigation revealed tuberculous lesions in 46% of the total number of inspected animals (Pathological Lesion (PL) stages 2 and 3). This was higher than previous findings by various researchers where lower prevalence rates have been reported (Nsengawa and Otaru, 1987; Radostits *et al.*, 2000; Asseged *et al.*, 2004). Similarly, our results were found higher than the results reported by Shitaye *et al.*, (2006) and Ameni and Wudie (2003) where the prevalence rate was 3.5 and 5.2%, respectively. In our study (Table 2) the tuberculous lesions were detected predominantly in the lung and associated LN which was consistent with the previous findings (Corner *et al.*, 1990; Whipple *et al.*, 1996; Asseged *et al.*, 2004).

However, observation of tuberculous lesions in 9 (42.85%) mesenteric LN may also imply that the occurrence of intestinal tuberculosis might have been more frequent than assumed. Although gross lesions were observed in the lungs, typical TB lesions were not detected histopathologically. In most cases, tuberculous lesions in beef cattle and buffaloes in particular may not always be observed, therefore the presence of tuberculous lesions or absence of visible lesions may not often lead to *M. bovis* detection as isolation of *M. bovis* has been confirmed from non-visible lesions (de Kantor *et al.*, 1987; Corner, 1994; Whipple *et al.*, 1996). The presence of caseous and/or calcified lesions and even lesions resembling tuberculous lesions may not always found to be of mycobacterial origin. In contrast, they can be caused by any other intracellular organisms or parasites that could mislead a veterinarian to consider the non-tuberculous cattle or buffaloes as being tuberculous.

However, during examination of mycobacterial infections, care should be given because of the tuberculous lesions caused by species of mycobacteria not belonging to MTC (i.e., *M. fortuitum* and *M. farcinogenes*) have been also isolated from cattle and human in Chad (Diguimbaye-Djaibe *et al.*, 2006a, b). Despite the irregularities and some casual problems we faced during inspection in abattoirs; these findings strengthens our suggestion that abattoir meat inspection is the utmost obligator y and fundamental step in detection of tuberculous cattle and buffaloes and/or in diagnosing of bovine tuberculosis in Khyber Pakhtunkhwa, Pakistan, where other diagnostic options are limited.

Histopathological examination

In our histopathological examination, a reactive hyperplasia of follicles (35.2%), acute lymphadenitis and non-specific reactions with pyogranulomas (47.8%) were evident in most sections of LN samples. In some tissue samples parasitic c ysts have also been observed. The presence of reactive hyperplasia of lymphatic follicles represents generally the response of the immune system to any antigenic stimulation. The pyogranulomas were composed of central necrosis with the presence of degenerated and/or decayed

neutrophilic and eosinophilic granulocytes.

Granulomatous lesions typical to bovine tuberculosis, manifesting granulomas with central necrosis surrounded by multinucleated cells of Langerhans cells, were observed only in 3 (5.1%) lung and 1(4.8%) mesenteric lymph node of the 100 tissue samples in the LN which have had gross lesions. A similar result was reported by Shitaye *et al.* (2006) in which granulomatous lesions were observed in 3 (4.3%) of the 69 tissue samples in the LN with gross lesions. Consistent to this finding, Whipple *et al.* (1996) described that histologically, manifestation of typical granulomatous lesions in tissues with gross lesions was evident. These granulomas were presented by a central caseation and focal calcifications (Rhyan and Saari, 1995). Around the necrotic mass a margin formed by large macrophage cells with light cytoplasm was also evident.

Ziehl-Neelsen (ZN) staining

Among all examined tissue samples, mycobacteria were detected in 2 (3.4%) in lung and 1(4.8%) mesenteric lymph node while 1(7.7%) in lung samples stained by ZN staining technique. These results were consistent with a previous report (Gracey, 1986; Shitaye *et al.*, 2006). This result justifies that viable mycobacteria may be present in less quantity in tuberculous lesions. Even viable mycobacteria may not be present in calcified and hardened lesions or the lesions were caused by infection with other intracellular causal agents and/or parasites. *M. bovis* are often low in bovine specimens and they can be visualized by ZN only if a limited quantity (at least 5×10^4 mycobacteria/ml) of materials is present (Quinn *et al.*, 1994). Despite this, the results of ZN staining may also be affected by the sample taking technique during smear preparation as mycobacteria are not be evenly distributed in the tissue sample. Berk *et al.* (1996) described that out of 18 PCR positive samples fixed with formaldehyde solution, ZN staining was positive only in three samples and a positive acid fast bacilli staining in a formalin fixed tissue may be most likely low. However, sometimes lesions determined to be tuberculous upon examination of HE-stained section with no AFB may be regarded as suggestive of tuberculosis (Thoen *et al.*, 1995).

Immunohistochemistry (IHC)

The ABC-P technique detected *M.bovis* in tissue sections of tuberculous lesions. Similar results have been described for other mycobacteria with the ABC-P (Perez-Perez *et al.*, 1989; Massone *et al.*, 1990; Gutierrez and Marin, 1993), indirect immunoperoxidase (Kobayashi *et al.*, 1989) and PAP (Navarro *et al.*, 1991) methods. This result showed the potential value of the ABC-P technique for the detection of mycobacterial antigens in ZN negative sections as founded by Perez-Perez *et al.* (1989) and Gutierrez and Marin (1993) in an investigation of ovine paratuberculosis and bovine and caprine tuberculous lesions, respectively. The anti-BCG serum used in this research showed a good degree of positivity of *M. bovis* in different tissue sections. It is well known that *M. bovis* and *M. paratuberculosis* share common antigens (Gunnarson and Fodstad, 1979; Kobayashi *et al.*, 1989), as do *M. bovis*, BCG and other mycobacteria (Harboe *et al.*, 1979). The specificity of the technique might be improved by the use of monoclonal antibodies.

The ABC-P method was more sensitive than the ZN method. It detected a greater number of positive samples, by means of a more visually striking reaction, which made possible the use of comparable low magnifications, thus saving effort and time. Similar results with other mycobacteria were obtained by (Kobayashi *et al.*, 1989; Perez-Perez *et al.*, 1989; Navarro *et al.*, 1991; Massone *et al.*, 1990; Gutierrez and Marin, 1993). This difference in sensitivity may be because the ZN method detects only perfect organisms whereas IHC method detect mycobacterial antigens, fragments, living or dead organisms even with defective cell walls. The very nature of ABC-P method, it may also amplify the size of the organisms (Haines and Clark, 1991; Kobayashi *et al.*, 1989) and changes morphology (Meador *et al.*, 1986; Perez-Perez *et al.*, 1989).

The number of observed mycobacteria was small in most of the cattle and buffalo, and the organisms were located in the giant cell or in areas of necrosis, as described by Mcllroy *et al.* (1986). There appeared to be a direct relationship between numbers of mycobacteria and the severity of the lesion. Out of 46 samples, only four (4) of the (8.7%) being ZN positive, these four being equally distributed between mild and sever cases; the number of positive results was increased considerable by means of the ABC-P method. Possibly, the immunological response of the cattle identifies more mycobacteria in tissue samples as compared to ZN staining; so, than lesions

can be intense but contain few mycobacteria, as in the borderline-tuberculoid group in human leprosy caused by *mycobacteria leprae* (Ridley, 1983; Young *et al*., 1990). It may also be relevant that cattle/buffaloes came to abattoir from herds with only individual cases of tuberculosis. However, the positivity was higher by ABC-P than by ZN, most probably due to antigens from degraded bacilli were also recorded.

The presence of bacilli in neutrophils has been recorded as a feature of early tuberculous lesions (Pritchard, 1988). In the present study, the neutrophils were found mainly in sever lesions. Some authors have shown in vitro that human neutrophils are capable of killing *M. tuberculosis* (Brown *et al*., 1987). However, it has been pointed out that neutrophils cannot kill *M. bovis* efficiently, though they can remove dead bacilli or fragments (Pritchard, 1988). Both possibilities might explain the increased positivity observed in neutrophils and adjacent areas of necrosis in slides stained by the ABC-P technique due the ability of the immune-histochemical technique but not the ZN method for the purpose to detect antigens or debris of bacilli. In conclusion, the results suggested that the ABC-P technique could be used with advantage on samples from tuberculous lesions which are negative or only slightly positive with the ZN technique.

Conclusions and Recommendations

The endemic nature of *M. bovis* infection has long been reported in Khyber Pakhtunkhwa, Pakistan. Recent studies showed the presence of *M. bovis* infection in different tissue samples indicating the significance of tuberculosis in large ruminant population. In addition, some other potential risk factors that favors the spreading of *M. bovis* infection included the existing Pakistani culture of eating raw meat and drinking raw milk; the very common close contact of animals with humans in rural areas and; the prevailing low standard of hygienic status in the production farms.

It is concluded that the widespread lesions in different organs of the infected animals and the septicemic nature of the disease suggest Mycobacterium bovis as the causative agent of bTB. Immunohistochemistry with ABC-P method confirmed the detection of *Mycobacterium bovis* antigen in different tissues including germinal centers of lymph nodes, alveolar macrophages and intercellular tissue spaces of lungs. An appropriate and immediate control program against bovine tuberculosis infection should be designed. Equally it is highly important to integrate research and disease surveillance programs in between the medical and veterinary institutions as it helps and simplifies the designing of feasible control program to minimize zoonotic threat of bovine tuberculosis across the province Khyber Pakhtunkhwa, Pakistan.

However, it is concluded from the results that the ABC-P technique could be used with advantage on samples from tuberculous lesions which are negative or only slightly positive with the ZN technique. The immunohistochemistry (IHC) was efficient in detecting the presence of mycobacterium tuberculosis complex (MTC) members that enables us to make a definitive diagnosis of infectious diseases.

Acknowledgments

The authors are thankful to the technical and supporting staff of local abattoir and veterinary clinical pathology laboratory, University of Agriculture Peshawar, Khyber Pakhthunkhwa Pakistan for their support during the research trial.

Novelty Statement

The research and experimental work on the subject title is original and new in the field of veterinary pathology in Khyber Pakhtunkhwa, Pakistan.

Author's Contribution

Asad Ullah: Investigation, conceptualization, writing-original draft preparation.
Faizan Hafeez: Data curation.
Raheela Taj: Project administration.
Shumaila Gul: Methodology.
Imad Khan: Supervision.
Brekhna Faheem and Mansoor Ahmad: Validation.
Rafiq Ullah and Ashfaq Ahmad: Visualization.
Muhammad Hanif: Data curation.
Aziz Ullah Khan and Muhammad Owais Khan: Formal analysis.
Abdul Basit and Muhammad Idrees Khan: Resources.
Shakirullah Khan: Software.
Muneeb Islam: Writing-review and editing.

Ethical approval

This research study was duly approved by the ethical review committee, Agriculture University Peshawar, Khyber Pakhtunkhwa, Pakistan.

Conflict of interest

The authors have declared no conflict of interest.

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