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OVINE PARATUBERCULOSIS-A HISTOPATHOLOGICAL STUDY FROM PAKISTAN

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ABSTRACT

This study was conducted to elucidate the histopathological depiction of ovine paratuberculosis. Tissue samples were randomly collected from 47 sheep slaughtered at two municipal abattoirs of district Jhang, Pakistan. The tissue samples were inspected for the presence of *Mycobacterium avium subspecies paratuberculosis* (MAP) by means of acid-fast staining and gross/histopathological examination. Indirect ELISA was also performed for the confirmation of paratuberculosis. Intestinal pathological lesions were observed in 04.12% animals. While only 03.77% of mesenteric lymph nodes (MLN) were associated with gross lesions. Acid-fast staining of tissue hard pressed smears were positive for MAP in 12.76% intestinal and 10.63% MLN tissue samples. Similarly Ziehl-Neelsen (ZN) staining of the histopathological tissue sections of MAP positive smear samples reflected the occurrence of acid-fast bacilli in 100% intestinal as well as lymph nodes samples. This indicated the superiority of intestinal samples over mesenteric lymph nodes and hence tissue section could be considered to be a comparatively better preparation for the analysis of paratuberculosis with ZN staining technique. ELISA confirmed 10.63% samples positive for MAP. It was concluded from the study that infection of MAP could be precisely identified by histopathology and indirect ELISA, which tends to be unlikely if merely based upon acid-fast staining and gross examination.

Keywords: Sheep; Paratuberculosis; Histopathology; ELISA.

INTRODUCTION

Ruminant Paratuberculosis (Johne's disease) is characterized by chronic diarrhea, progressive debilitation and poor response to therapy. The disease has a worldwide prevalence and it has been reported to affect domestic, wild and zoo ruminants (Buergelt and Ginn 2000; Hope *et al.* 2000; Kruze *et al*. 2006**)**. The etiological agent is an acid-fast bacillus, known as *Mycobacterium avium subsp. paratuberculosis* (MAP) that has also been suspected for causing regional ileitis or Crohn's disease (CD) in humans (Erume *et al.* 2001; Kaevska and Hruska 2010; Sarman and Gopinath 2011). Paratuberculosis in animals exists in two distinct forms; the lepromatous or multibacillary in which the macrophages are stuffed with bacilli (Biplab *et al.* 2010) and the paucibacillary in which lymphocytes are predominantly present with or without bacilli in intestinal mucosa (Clark *et al.* 2010). The affected animals discharge large quantities of the microorganism in their milk and feces thus contaminating the pasture and farm premises (Hulten *et al.* 2001). As yet, the disease is regarded as incurable **(**Jones, 1989; Maxie *et al.* 2007; Singh *et al.* 2011). A wide array of procedures and laboratory tests ranging from conventional methods like skin sensitivity test, Ziehl-Neelsen (ZN) stained smears, and histopathological analysis of terminal ileum and mesenteric lymph nodes (Buergelt and Ginn 2000) have been employed for its diagnosis. Histological examination is a reliable indicator (Kurade *et al.* 2004; Sikandar *et al.* 2012) and culturing technique is deemed as gold standard for the diagnosis of paratuberculosis (Erume *et al.* 2001; Huntley *et al.* 2005).

This study was accomplished to assess the efficacy of histological examination, ZN staining and indirect ELISA for the diagnosis of multibacillary form of ovine paratuberculosis in Jhang (Pakistan).

MATERIALS AND METHODS

For the sake of expediency, the study was divided into gross pathology, histopathology and serology.

A. Sampling and gross pathology: For the random collection of intestinal samples along with mesenteric lymph nodes, the abattoirs situated in Jhang city and Bagh Wala Mohallah, Jhang, Punjab, Pakistan were visited three to four times per week for a period of four years (October, 2007 to October, 2011). On the basis of gross pathology, a careful ante-mortem assessment was routinely performed to opt for the animals assumed for having Johne's disease (JD). Slaughtering and opening of such animals was followed by the removal of intestines from each carcass. Only those intestines were selected for further analyses that were showing thickened walls particularly around the ileocecal junction.

The most striking gross lesions were observed in terms of mucosal corrugations and chronic lymphadenitis of associated lymph nodes (MLNs) in the selected

intestines. Total four impression smears were prepared from the selected intestinal mucosal sites where the corrugations were most prominent. ZN staining method as recommended by Cappuccino and Sherman (2008) was used for the staining of intestinal impression smears (ISs). The acid fast bacilli were seen as bright/ rose red rods with a blue background under oil immersion lens. Positive samples were preferred for further processing.

B. Histopathology: The excessive mesenteric attachments were trimmed off both from the intestines and MLNs. Later on, the samples were cut down into suitable segments. Fixing was carried out using 10% neutral buffered formalin. Samples were subjected to further processing steps that included dehydration, clearing, embedding, sectioning of 0.5-0.7 µm thickness and routine staining (following the method suggested by Bancroft *et al.* 2007). The ZN staining protocol developed by Huntley *et al.* (2005) was adopted with some modification during the heating step. Heating was performed by exposure to flame of a spirit lamp for 10-15 seconds, whereas, Huntley and his co-workers followed a steam protocol for 1 hour. Data collected during the study were analyzed by applying descriptive statistical techniques (Zar, 2003).

C. Serology: Blood samples (5-8 ml per animal) were collected aseptically from the jugular veins of 47 animals. Only those samples were subjected to further processesing which manifested the gross lesions of JD. Further processising was carried out in terms of centrifugation at 4000 rpm for 5 min for the extraction of sera. Later on ELISA kit method was utilized for the detection of antibodies against MAP in ovine serum samples.

As per manufacturer's instructions,{Serelisa™ M. Para TB Ab Mono Indirect, Synbiotics Johnin ELISA Kit, Cat No. ASPTB3 (2 Plates), Symbiotic Europe SAS, 2, rue Alexander Fleming, F-69367 Lyon, Cedex 07, France}, the spare conjugate was eliminated through a washing step. The complex-bound enzyme was revealed by the addition of a substrate that was turned into a colored product and optical densities were measured after the stoppage of reaction. Indirect immunoenzymatic procedure was employed with slight modification. The threshold values obtained from positive control were compared with optical densities to validate the presence of antibodies against MAP.

RESULTS

A. Sampling and Gross pathology: A total of 1140 thin, emaciated animals were examined, out of which only 47 (04.12%) manifested gross intestinal lesions and only 43 animals (03.77%) revealed pathological lesions in lymph nodes. The intestinal walls were found to be disproportionately thickened at various locations predominantly near the ileo-caecal junction. Intestinal thickening and mucosal corrugations were observed in all 47 samples; however, 06 (12.76 %) out of these samples provided the most typical lesions. Opening of such intestinal segments divulged corrugations of multiple degrees that retained their contour even after being stretched. Intestinal impression smears of the later (06 animals) were found as positive for the incidence of acid fast bacilli and later on, their tissue sections were cautiously examined in the laboratory.

Table. 1. Assessment of Acid-fast Ziehl Neelsen staining of smears and sections from intestines and mesenteric lymphnodes.

ORGANS	INTESTINE		Mesenteric LN	
Total suspected positive Animals	**Impression Smears**	**Tissue Sections**	**Impression Smears**	**Tissue Sections**
47	06	06	05	05
+ve Impression Smears	06/47		05/47	
+ve Tissue Sections		06/06		05/05
Percentage	12.76	100	10.63	100
TotalImpression Smears	11/94(11.70%)			
Total Tissue Sections	11/11(100%)			

LN = lymph node, +ve = Positive -ve = negative

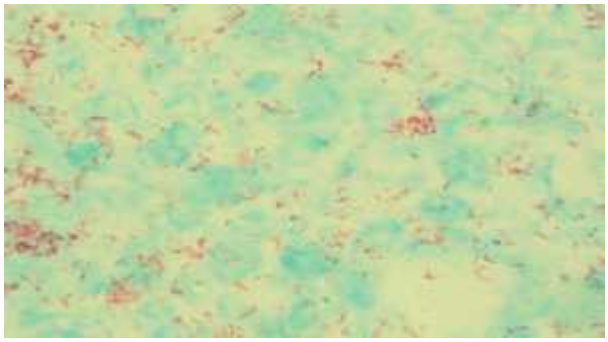

Fig. 1: Mucosal impression smear (hard pressed) prepared from ileum. The acid fast bacilli appear as rose-red to pink rods. ZN (1000X).

Favorite organ and methods for ZN Stain:

Intestine vs Mesenteric lymphnodes: Six impressions out of grossly suspected forty seven (Fig. 1) and all the six intestinal tissue sections were found ZN stain positive 12/53(22.64 %), whereas five of the forty seven impressions and five of the five tissue sections of mesenteric lymph nodes 10/52(19.23%) showed acid fast bacilli (Table-1). This indicated the preeminence of intestinal samples over mesenteric lymph nodes for the detection of MAP using ZN staining.

Impression smears vs Tissue sections: The comparison of tissue sections to impression smears yielded unrivaled results. The percentage of positive tissue sections was 100% (11/11) while that for impression smears was only 11.70% (11/94). Tissue section is, therefore, comparatively a superior preparation for the analysis of JD with ZN staining technique.

Statistical analysis signified considerably higher number of AFB in tissue sections of intestines (100%) as compared to intestinal impression smears (12.76%) as shown in Table-1. Likewise, the number of positive cases was also greater (100%) using tissue section of MLNs as compared to its impression smears (10.63%).

B. Histopathology: The intestinal walls were extremely thickened. Extensive infiltrations by mononuclear cells (epithelioid-macrophages, plasma cells and lymphocytes) were observed especially in the mucosa and upper portions of submucosa of intestinal sections. Cytoplasm of the epithelioid macrophages was foamy in appearance and darker round to oval nuclei were eccentric in position (Fig. 2). Few eosinophils and neutrophils had also infiltrated. The mucosal lining epithelial cells of small intestine were mostly sloughed off. Crypts of Lieberkuhn (intestinal mucosal glands) were atrophied, became slit-like and wide apart from each other. Atrophy of the crypts of lieberkuhn was due to pressure of the infiltrated mononuclear cells (MNCs). Lumens of some of the crypts were filled with exfoliated cells mixed with inflammatory cells. Invasion by macrophages was also observed in the wall of crypts. The lamina propria of the mucosa was abundantly puffed-up with MNCs. Edematous swelling occurred in submucosa consisting of proteinaceous substance and fibrous connective tissue (FCT) proliferation. Fatty change was observed in tunica muscularis. A mild mononuclear cells infiltration was also observed in serosa.

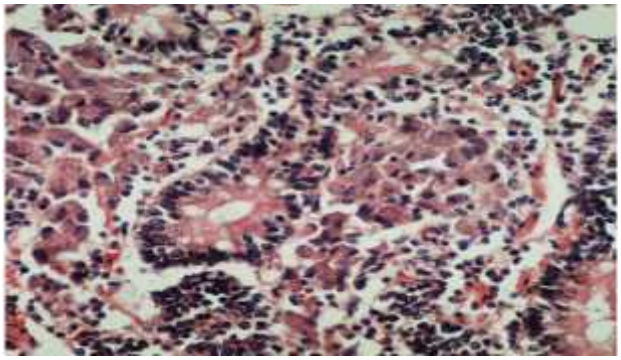

Fig. 2: Small Intestine, epithelioid macrophages having foamy cytoplasm and eccentric nuclei are seen in the mucosa. H & E (400X).
Intestine (ZN):

The cytoplasm of epithelioid macrophages was stuffed with microorganisms. The appearance of microorganisms in macrophages was red to pink. Laden macrophages were mostly present in the mucosa; however, small aggregates were also existed in the upper submucosa. Epithelioid macrophages in the outer serosa contained only a few AFB. The acid fast microorganisms were rose-red to pink in color (Fig.3).

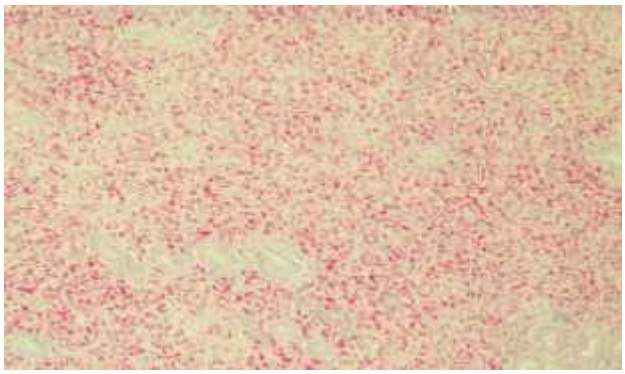

Fig. 3: In the cecum mucosa of large intestine, the infiltrated epithelioid macrophages laden with rose-red to pink AFB. Crypts are present wide apart from each other. ZN (100X).

Lymph Nodes (H&E): Mesenteric lymph nodes had thick capsules of fibrous connective tissue (FCT). Variable sized necrotic and calcified areas surrounded by thin layer of FCT were located in the cortical and paracortical regions of the lymph nodes (Fig. 4). Epithelioid macrophages seen in the intestinal sections had also occupied most of the cortical areas of MLNs. Micro-granulomas were present in parenchyma of lymph nodes as well. Fibrous connective tissue followed by a zone of mononuclear cells was noticeable around the granulomas. Central areas of most of the granulomas were necrotic and calcified. Giant cells of Langhan's type were detected in the medullary area.

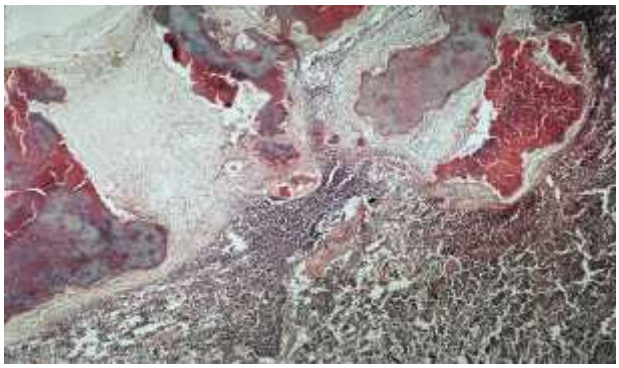

Fig. 4: Ileal mesenteric lymph node, representing several large irregular areas of necrosis, caseation and calcification. H&E (40X)

C. Serology: For ELISA test, similar lot was randomly selected, from the same two abattoirs of Jhang, during the

same period and from the same animals (47 in number). The optical densities of only 05/47 (10.63%) were declared as positive.

DISCUSSION

This present project was designed to evaluate the effectiveness of conventional diagnostic tools *i.e.* histopathological examination, ZN staining and ELISA for the prompt diagnosis of ovine paratuberculosis and to ascertain the qualitative prevalence of this disease in Jhang (Pakistan).

Previously it has been postulated that in case of sheep, goats and deer, the intestinal thickening and associated transverse ridges appear to be less conspicuous and are therefore easily overlooked at necropsy. However, Catton (2002) had noticed the intestinal corrugations in goat. During recent study, we also found definite evidences of classical intestinal lesions in sheep. Our results are in accordance with those of (Maxie *et al.* 2007 and Alharbi *et al.* 2012) who observed that advanced cases of JD were typified by diffused intestinal thickening coupled with longitudinal and transverse corrugations that gave rise to asymmetrical folds having red surfaces but no ulcerations. We also noticed caseous necrosis and calcification of mesenteric lymph nodes that reinforce the findings documented by (Kheirandish *et al.* 2009 and Sikandar *et al.* 2012). Previously, the histopathological examination has been reported as a better indicator for the diagnosis of ovine Paratuberculosis (Kurade *et al.* 2004 and Hailat *et al.* 2010) and current study also concurs on this verity. Mucosal thickening and corrugations occurring due to mononuclear cells penetration and edema in the mucosa and submucosa (Maxie *et al.* 2007), have been illustrated in (Fig. 2). Our results are in conformity with the findings of former studies that reported the occurrence of epithelioid cells along with MNCs (Al-Dubaib and Mahmoud, 2008) and giant cells in MLNs of goat (Tafti and Rashidi, 2000). Discrete infection among human population proposes that this bacterium has the potential to get incorporated in grounded meat intended for human consumption and thus acts as a source of human revelation to MAP (Collins 2003 and Antognoli *et al.* 2007). Therefore, appropriate hygienic measures are critical to safeguard human population against meat-borne Mycobacterium paratuberculosis infection. The existing means are insufficient to avert MAP infected tissues from contaminating the human food (Antognoli *et al.* 2007).

The reliability of a single test to identify all the infected animals in a herd at a given time is considered as doubtful. Therefore, the application of more than a single diagnostic tool provides a better option for the precise diagnosis of chronic infections like Johne's disease (Singh *et al.* 2007). Provision of adequate information concerning the epidemiological and pathological trends of JD in domestic animals in Pakistan will facilitate the consideration of this disease on priority basis and it will significantly contribute towards the acquisition of optimal control measures.

Conclusions: This study has accentuated the significance of a careful histopathological appraisal using intestinal samples and mesenteric lymph nodes for the diagnosis of paratuberculosis in sheep. Despite being a time consuming process, the histopathological examination is preferred over other conventional and serological protocols by virtue of its low cost and higher specificity. It is considered as an efficient diagnostic tool that can be routinely performed for the diagnosis of JD. Furthermore it is proposed that intestinal samples and tissue sections must be preferred over mesenteric lymph nodes and mucosal impression smears respectively for the diagnosis of ovine paratuberculosis.

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