Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Histopathological and Serological Studies on Paratuberculosis in Cattle and Buffaloes

Arbab Sikandar*, AH Cheema[1], M Younus[2], A Aslam[1], MA Zaman[2] and T Rehman[3]

Department of Anatomy and Histology, College of Veterinary and Animal Sciences, Jhang; [1]Department of Pathology, University of Veterinary and Animal Sciences (UVAS), Lahore; [2]Department of Pathobiology, College of Veterinary and Animal Sciences, Jhang; [3]Department of Parasitology, University of Agriculture, Faisalabad, Pakistan
*Corresponding Author: drarbab786@gmail.com

ARTICLE HISTORY

Received: November 30, 2011
Revised: March 26, 2012
Accepted: April 06, 2012

Key words:
Buffalo
Cattle
ELISA
Histopathology
Mycobacterium avium subsp. Paratuberculosis
Paratuberculosis

ABSTRACT

Paratuberculosis (Johne's diseases) is responsible for massive economic losses to dairy industry, both in the industrially advanced as well as in the developing countries. To detect its occurrence in cattle and buffaloes locally, blood and tissue samples from clinically weak and grossly suspected slaughtered animals were collected from two abattoirs of Jhang, municipal area, Pakistan. Acid-fast smear staining, gross/histopathology and indirect ELISA were done for the detection of *Mycobacterium avium subsp. paratuberculosis* (MAP). Total 134 samples illustrating gross pathological lesions were collected, only 11.19% (cattle: 6.67%, buffaloes: 12.5%) showed acid fast bacilli through smear staining and were taken as confirmed cases. Thickening of intestines alone was not a reliable indicator of Johne's disease. Tissue sections from intestines and mesenteric lymph nodes from these acid fast positive animals were stained with hematoxylin & eosin (H&E) and Ziehl Neelsen (ZN) methods. Sum of (15/134) impression smear staining as well as (15/15) tissue sections of the intestines were found ZN positive, and only 6.7% of impression smears and 100% of tissue sections of mesenteric lymph nodes showed acid fast bacilli. Through ELISA, two cattle and five buffaloes (07/134) gave positive optical densities, while one cattle and seven buffaloes (08/134) were judged as doubtful. It is concluded that infection of MAP can be identified by histopathology and ELISA. The present study was the first record of paratuberculosis among the dairy animals slaughtered at Jhang abattoirs. The objective was to compare different methods for the diagnosis of Johne's disease.

To Cite This Article: Sikandar A, AH Cheema, M Younus, A Aslam, MA Zaman and T Rehman, 2012. Histopathological and Serological Studies on Paratuberculosis in Cattle and Buffaloes. Pak Vet J, 32(4): 547-551.

INTRODUCTION

Paratuberculosis or Johne's disease (JD) has emerged as one of the most important livestock diseases in recent years. It is a chronic infectious disease of domestic, wild and zoo ruminants and has been recognized throughout the world (Buergelt and Ginn, 2000; Seyyedin *et al*., 2010). The disease is caused by an acid-fast bacillus, *Mycobacterium avium subsp. paratuberculosis* (MAP) which is quite resistant to heat and cold (Whittington and Sergeant, 2001). It is believed that newborn calves become infected through ingestion of organisms with colostrum but they develop the clinical JD in adult life after a prolonged incubation period of many years (Kurade *et al.,* 2004). Susceptibility to infection is greatest in early life although the clinical disease does not usually develop in cattle until 2-5 years of age (Buergelt and Ginn, 2000; Collins, 2003 and Maxie *et al*., 2007). Macrophages phagocytize the MAP bacilli and accumulate in the lamina propria of intestine, causes thickening and corrugation of mucosa (Sivakumar *et al.,* 2006), and animal suffers from malabsorption, diarrhea, decrease in milk yield and loss of body condition (Maxie *et al.,* 2007; Singh *et al.,* 2008 and Delgado *et al*., 2009). MAP has been suspected to be causally associated with regional ileitis or Crohn's disease in man (Erume *et al.,* 2001). Histological examination of tissues obtained at necropsy allows rapid diagnosis. Several classifications based on lesions have been made in sheep (Maxie *et al.,* 2007), goat (AL-Dubaib and Mahmoud, 2008), cattle (Amemori *et al*., 2004; Khan *et al.,* 2010) and buffalo (Sivakumar *et al.,* 2006).

There is a need to develop ELISA based method as an inexpensive, efficient, single step and sensitive to use for

the detection of antibodies in cattle and buffaloes in developing countries like Pakistan for herd screening. The present study was planned and executed with the aims to compare the results of ZN stained smears, histopathology and ELISA technique for the diagnosis of JD in cattle and buffaloes in the Jhang district; no such study has been done in this region previously and prevalence of JD in local animals is unknown.

MATERIALS AND METHODS

Histopathological Studies

Sampling procedure: Intestines along with mesenteric lymph nodes were randomly collected from the carcasses. For this purpose two abattoirs in the Jhang Municipal Area were visited three to four times a week during October, 2007 to June, 2011. Majority of the animals brought for slaughter were weak and emaciated. Soon after the removal of skin, the intestines were carefully taken out from each carcass in one lot and spread on the floor. Those showing thickened wall, especially around ileocecal junction along with the neighboring mesenteric lymph nodes were taken to the lab for further investigation.

Gross lesions: The following gross pathological changes were looked for in the thickest part of the intestines after opening; thickening of the intestinal wall (especially its mucosal lining); presence of elevated corrugations (which did not disappear on stretching) and enlargement of mesenteric lymph node (MLN) with or without edema and calcification.

Confirmation of Johne's disease: A few hard pressed mucosal impression smears were prepared from where the corrugations were most prominent. The intestinal impression smears (ISs) were stained with ZN method of staining (Sivakumar *et al.,* 2006). Under oil immersion lens the acid fast bacilli were bright/ rose red rods with a blue background. Samples, in which the impression smears of the intestines were ZN stain negative, were all discarded. In case the intestinal ISs showed acid fast bacilli the samples were processed further as under:

Histopathology: Representative specimen from intestines and MLNs were fixed in 10% neutral buffered formalin and further processed including dehydration, clearing, embedding, sectioning (0.5-0.7 µm thickness) and staining was routinely done following the method of Bancroft and Cook (2007). Huntley *et al*. (2005) were followed for ZN staining except the heating step of carbol fuchsin. This heating was done with the help of a spirit lamp flame for 07-10 seconds (until bubbles appeared).

Data analysis: Data thus collected for Ziehl Neelsen acid fast staining of smears and tissue sections were analyzed by applying descriptive statistical techniques like frequencies, percentage and proportion (Zar, 2003).

ELISA Test

Collection of blood: The blood samples for the ELISA test were collected from animals suspected for Johne's disease. Blood samples (5-8 ml) were taken from jugular veins in 10cc disposable syringes, sera were isolated and ELISA test was performed with a kit for the detection of anti-*Mycobacterium avium paratuberculosis* antibodies. As per manufacturers instructions (Serelisa^{TM} 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 extra conjugate was removed through washing step. Link of the enzyme to the complex was exposed by the addition of a substrate which is transformed into a colored product. After end of the reaction, the ODs were measured. The presence or absence of antibodies was determined using threshold values obtained from the positive control) and by using indirect immunoenzymatic technique by adapting short protocol. The presence or absence of antibodies against MAP was determined by comparing their optical densities (ODs) with the threshold values obtained from the positive control.

RESULTS

Histopathological studies: Of the 134 samples so tested only 11.19% (15) were positive for Paratuberculosis. Among those, 6.7% (two) Cattle and 12.5% (13) buffaloes showed the acid fast bacilli. Taking them as the confirmed naturally occurring JD cases, further investigations were restricted only to the examination of impression smears and tissues sections from these animals.

Gross lesions: The intestinal wall was found variably thickened in its different regions but it was more so around the ileo-cecal junction. Although all the 134 samples showed some thickening and variably corrugated mucosa yet the lesions were most prominent in 11.19% (15) specimens which as stated above were confirmed JD cases. Their thickest parts when opened showed corrugations of different sizes and elevations which did not disappear on stretching (Fig. 1). However, mesenteric lymph nodes of only nine animals were found enlarged and edematous.

Preferred organ and preparation for ZN Stain: As detailed above, the sample tested in the present study although small, comprised of confirmed cases of naturally occurring JD.

Intestine vs MLNs: Fifteen of the 134 impressions (Fig. 2) and all the 15 sections of intestines were found ZN stain positive, total 20.13 % (30/149), whereas nine of the 134 impressions and nine of the nine tissue sections of mesenteric lymph nodes, total 12.58% (18/143) showed acid fast bacilli (Table 1). This gave a slight edge to intestines over mesenteric lymph nodes for the detection of MAP by ZN stain.

Impression smear vs Tissue section: But when the impression smears of the two organs under investigation were compared with their tissue sections, the former showed much lower positives 8.95% (24/268) as compared to the latter 100% (24/24). Irrespective of the organs, therefore, tissue section is a relatively better preparation for diagnosis of JD with ZN staining.

Table reviled considerably, higher proportion (100%) of acid fast bacilli in tissue sections of intestines as compared to intestinal impression smears (11.19%).

Table 1: Comparison of acid-fast ZN staining of impression smears and tissue sections from intestines and MLNs

Organs	Intestine		MLN	
Preparation/Animal Tested in two abattoirs	Impression Smears	Tissue Sections	Impression Smears	Tissue Sections
Cattle: 30+00= 30	02	02	02	02
Buffaloes: 30+74= 104	13	13	07	07
Total +ve ISs	15/134		09/134	
Total +ve TSs		15/15		09/09
Percentage (%age)	11.19	100	6.71	100
Total ISs		24/268	8.95%	
Total TSs		24/24	100%	

MLN =Mesenteric lymph node, -ve = negative, +ve = Positive

Fig. 1: Large intestine of cattle (upper) and buffalo (lower). Mucosa of distal colon is quite thickened forming prominent longitudinal folds (cattle) and cecum of buffalo showing transverse ridges which did not disappear on stretching. The corrugations are like convolutions of the cerebrum.

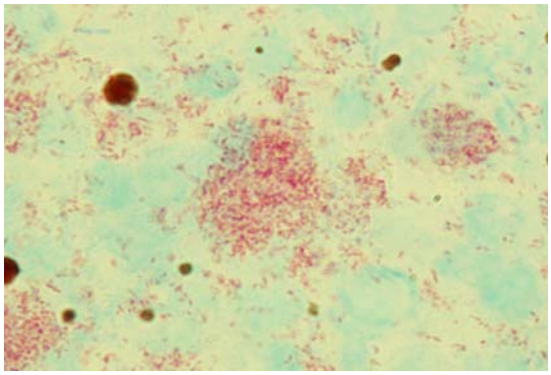

Fig. 2: Hard pressed Impression smear prepared from the mucosa of ileo-cecal valve. A multibacillary form in which the ruptured cells are stuffed with acid fast bacilli of *Mycobacterium avium subsp. paratuberculosis*. The bacilli appear as rose red rods. ZN (1000X).

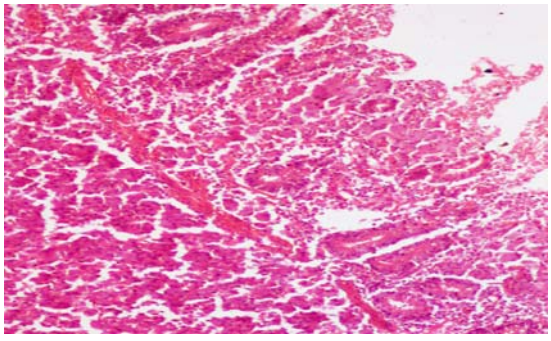

Fig. 3: Small intestine of Buffalo. Villi and crypts of Lieberkuhn are atrophied and widely separated. There is heavy infiltration by mononuclear cells in lamina propria and submucosa and disruption of muscularis mucosae. H & E (100X).

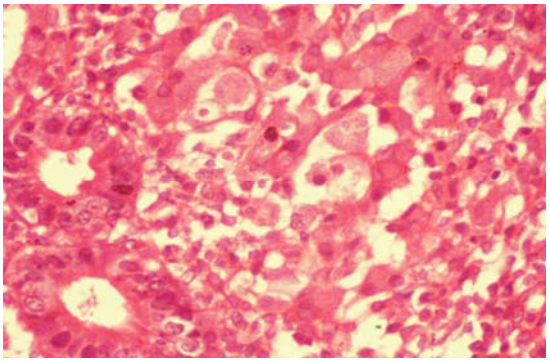

Fig. 4: Large intestine of Cattle. Epithelioid Macrophages have foamy cytoplasm and eccentrically placed nucleus in the lamina propria of the mucosa of cecum; Crypts also pushed wide apart from each other. H&E (400X).

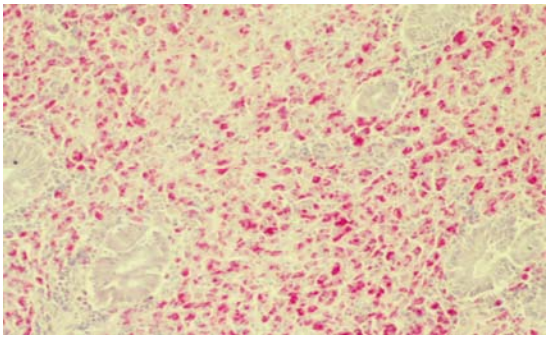

Fig. 5: Large intestine of Cattle. In the mucosa of cecum, macrophages laden with rose red AFB. ZN (100X).

Similarly, the number of positive cases was also high (100%) through tissue section of mesenteric lymph nodes as compared to impression smears (6.71%).

Histopathology

Intestine of cattle and buffaloes (H&E): The walls of intestine were much thickened due to increase in the size of mucosa and mostly of the submucosa. There was extensive infiltration by epithelioid macrophages, lymphocytes and plasma cells in mucosa and upper part of submucosa. Infiltration by a few eosinophils and some neutrophils had also occurred. Crypts of Lieberkuhn were reduced in numbers and atrophied (Fig. 3) and widely separated from each other (Fig. 5). Pressure atrophy of the crypts was due to mononuclear cells (MNCs) infiltration. The epithelial cells lining the villi of small intestine had sloughed off in initial infection, and the lamina propria was fully engorged with MNCs. Collection of macrophages (Microgranuloma) were seen in the mucosa (Fig. 3, 4). Disruption of the muscularis mucosae was also observed (Fig.3). Submucosa was edematous with deposition of proteinaceous material, and infiltration of mononuclear cells and proliferation of fibrous connective tissue (FCT). A small number of multinucleated giant cells were also present in the upper part of submucosa in buffaloes. Diffused granulomatous lesions were seen in the submucosa (Fig.3). Fatty change and few infiltrations by mononuclear cells were seen in the tunica muscularis. Epithelioid macrophages had large foamy cytoplasm and eccentric, round to oval, darker nuclei (Fig. 4). Macrophages in the lamina propria were mostly fused to

each other. Small collections of mononuclear cells were also seen in the serosa.

Cattle and buffalo intestines (ZN): Large collections of macrophages containing acid fast bacilli in the cytoplasm were present in the mucosa and mostly in the submucosa (Fig. 5). The organisms were pink to rose-red in colour and the approximate size of the individual microorganism was about 3 µm in length (Fig.2).

Lymph nodes (H&E): Mesenteric lymph nodes were surrounded by thick capsules of fibrous connective tissue (FCT). There were large, round or irregular variable sized areas containing amorphous/acellular, brown to pink caseous materials with small amount of dark blue calcification. These necrotic areas had thin fibrous capsules and were located in the cortical and paracortical areas of the lymph node. Same macrophages like intestinal sections were present in the subcapsular sinuses and paracortical areas of the lymph nodes. Most of the subcapsular and cortical areas were replaced by these epithelioid macrophages. Macrophages contained dark brown granular material in their cytoplasm. A few Langhan's giant cells were also present in the sections.

ELISA test: Blood samples were collected from these animals before slaughtering at abattoirs and sera were subjected to ELISA. Two cattle and five buffaloes 05.02% (07/134) were clear positive whereas one cattle and seven buffalo 05.97% (8/134) species gave doubtful ODs.

DISCUSSION

The present study was designed to detect paratuberculosis in cattle and buffaloes by conventional methods, i.e., ZN smear staining and histopathology as well as detection of its antibodies by using indirect ELISA. Although sporadic cases of this chronic disease of domestic ruminants are not uncommon, yet there is no available published report on JD in Jhang area of Pakistan.

In small ruminants, e.g., sheep, goats and deer, the intestinal gross lesions were often mild, therefore, easily overlooked at necropsy (Maxie *et al.,* 2007). In the present study thickened and corrugated intestines were obvious in cattle and buffaloes. Lesions in the intestine may possibly expand from duodenum up to the rectum. In highly developed cases, there is diffuse thickening along with transverse and longitudinal corrugations of the intestinal wall making irregular folds (Buergelt *et al.,* 2000; Sivakumar *et al.,* 2006 and Khan *et al.,* 2010). The surfaces of the folds are red but not ulcerated (Maxie *et al*., 2007). Similar observations were made in this study (Fig.1). The present study showed caseous necrosis and calcification in the mesenteric lymph nodes which is similar to the findings reported by Tafti and Rashidi, (2000). Thickening and corrugations of mucosa was due to mononuclear cells (epithelioid macrophages) infiltration in mucosa and in submucosa (Maxie *et al*., 2007) as seen in (Fig. 3). Diffuse lesions were found mostly in the submucosa in advanced cases. Multinucleated giant cells were present in the submucosa of buffaloes which were absent in the intestine of cattle. Giant cells in the MLNs of only two buffalo sections were observed as reported by Tafti and Rashidi, (2000). Prominent morphological alterations in paratuberculosis were microgranulomas composed of epithelioid macrophages (Fig. 3) and multinucleated giant cells of Langhan's type. Similar changes have been reported in North American bison (Buergelt and Ginn, 2000) and water buffalo (Sivakumar *et al*., 2006).

It may, however, be mentioned that intestines of only 15 cases of confirmed Johne's disease are reported in the present study: which include two cattle and 13 buffaloes. Therefore, the conclusions made are no more than trends only. A single test cannot recognize all the infected animals in a herd at a given time. Therefore, use of more than one test was a superior choice in chronic infections like paratuberculosis. The ELISA is both rapid and efficient and a large number of samples can be processed each day. In the present study only 05.02% animals were clear cut positive and 05.97% animals were adjudged as doubtful through ELISA, this may be because of species differences (still not reported). Further studies of more animals of known age etc. are required to know the actual position. The ELISA negativity recorded in the present study might be due to insufficient amount of targeted antibodies in their sera.

- This study has emphasized the importance of a careful histopathological examination of the intestine and mesenteric lymph nodes for diagnosis of paratuberculosis in domestic ruminants.
- Smear method alone cannot be trusted to diagnose paratuberculosis until and unless confirmed by histopathology and ELISA. Although histopathology is time consuming but it is most reliable technique and has higher specificity as compared to other conventional methods. It can be claimed as a routine diagnostic tool for JD.
- The evidence in the present study for histopathology suggests that intestines were the better site than mesenteric lymph nodes, and tissue section was a preferred preparation than hard pressed mucosal impression smear.

Information of Johne's disease in domestic animals is limited in Pakistan (Abbas *et al.,* 2011); this is why paratuberculosis is not a priority disease for control in this country. It can be concluded from this research that paratuberculosis is prevalent in Pakistan and reliable, rapid and specific diagnosis is possible by ELISA test and histopathological techniques.

Acknowledgement: The authors would like to thank Prof. Dr. MA Majeed for endless cooperation and guidance. We would like to acknowledge Pathology lab and University diagnostic lab, UVAS; Histology and Pathology lab, UAF, for providing chemicals, reagents and help in this study.

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