HISTOPATHOLOGY: AN OLD YET IMPORTANT TECHNIQUE TO DIAGNOSE PARATUBERCULOSIS IN NON-DESCRIPT WATER BUFFALOES

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ABSTRACT

Paratuberculosis (PTB) in buffaloes is a chronic enteric disease triggering health implications and huge economic losses in livestock. This study was designed to explore a simple, cost-effective diagnostic approach for PTB in water buffaloes. Blood (5 ml/animal) and intestinal tissue samples accompanied by lymph nodes associated with mesentery were collected from weak and diarrhoeac animals slaughtered at local abattoirs. Out of total n=771 clinically suspected animals, only n=53 carcasses were sampled based on gross observation. Tissue smears of the gut mucosae were obtained and were made adopting special staining protocol. Tissue samples were processed by paraffin sectioning and stained with Ziehl-Neelsen and Hematoxylin-Eosin staining methods. Acid-fast bacilli were observed only in 11/53 cases on mucosal tissue smears. Pressure atrophy of small intestine villi were evident, and the mucosae were found sloughed off. The submucosae were heavily infiltrated with mononuclear cells and multifocal cellular nodules dominated by epithelioid macrophages. The foamy cytoplasm of the macrophages appeared to be engorged with acid fast bacilli and depicted the positive cases. All tissue sections of the suspected samples showed 100% +ve results while only 20.8% samples were found +ve with smear method. All histo-pathologically positive cases were further confirmed by ELISA based serological analysis. Therefore, it was concluded that histopathology is an economical and yet the most trusted tool for diagnosing bubalian PTB in countries like Pakistan.

Keywords: *Bubalus bubalis*, buffaloes, animals, Johne's disease, histology, gut, serology

INTRODUCTION

Paratuberculosis is a chronic debilitating disease which is caused by *Mycobacterium avium subsp. paratuberculosis* (MAP, Yoshida *et al*., 2018). The isolation of the bacterium from various tissues of cattle, camel, small ruminants and non-ruminants like cat, rat, raccoon and occasionally in horse and birds living in the vicinity of infected farm animals suggests that MAP can infect almost all the animal species (Over *et al*., 2011). In buffaloes, it causes a wasting illness with a prolonged course, during which intractable diarrhea results in dehydration, emaciation and eventually the death of the animal. As a chronic

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wasting disease, paratuberculosis is responsible for huge economic losses to dairy and meat industry (Gupta *et al*., 2018). Moreover, this organism is also suspected to develop a Crohn's disease in humans (Correa-Valencia *et al*., 2018). The infectious agent is shed in large amount in the milk and feces of the infected animals and the final host acquires infection by ingestion of contaminated feed and water (Pavlik *et al*., 2000). The infected animal may also a source of contamination of beef products both pre (via blood stream) and post (via fecal material) slaughter. In the pre-clinical and clinical forms, the organism infects the spleen, lungs, kidneys, uterus and placenta and hence there is a generalized infection in the animal (Jones *et al*., 2006).

For control and eradication of infection, it is fundamental to develop a useable and economical early indicative means to decrease the prevalence in the animals as well as to reduce the possible risk factors for the transmission to humans. A wide array of procedures and laboratory tests are available for the diagnosis of Johne's disease (JD) and the list of these methods is frequently expanded. Many researchers have compared the efficiency, sensitivity and specificity of the existing diagnostic procedures for JD (Pavlik *et al*., 2000; Singh *et al*., 2007). These range from conventional methods including skin sensitivity and histopathological investigation of intestine and associated lymph nodes (Buergelt and Ginn, 2000). Fecal examination and tissue histopathology are standard tools for the diagnosis of MAP (Schaik *et al*., 2007). Parallel to these, serological tests like immunoelectrophoresis (IE), complement fixation test (CFT), haemagglutination inhibition (HI), agar gel immunodiffusion (AGID) (Sherman and Markham, 1984) and new sensitive tests like enzyme-linked immunosorbent assay (ELISA) and DNA amplification-based polymerase chain reaction (PCR) (Singh *et al*., 2007) are also being used. Despite the effectiveness of these tests, a considerable variation in the results and several disadvantages have also been reported like labor-intensive, cost-effective and time involved in these conventional tests. However, histopathology is a useful technique in clinical and sub-clinical infected cases (Sivakumar *et al*., 2006). There are two main histopathological presentations of paratuberculosis in animals; a multi-bacillary or lapromatous wherein macrophages filled with numerous MAP are the major inflammatory cells and a paucibacillary or tuberculoid form in which inflammatory infiltrate consists of lymphocytes and a few macrophages having few MAP cells (Catton, 2002). Acid fast bacterium was found in 60% rectal mucosal scrapings of the cows and hepatic microgranuloma occur in about 25% of the affected animals (McGavin and Zachary, 2007). Hence, there is a demand to improve the existing histopathology-based technique (Sikandar *et al*., 2018) as an inexpensive and efficient tool for the detection of PTB according to the available resources in the developing country like Pakistan. The present study was designed to evaluate the performance efficiency of histopathological technique and to compare its results with those of stained smears and the most trusted ELISA for the diagnosis of bubalian-JD in the tehsil Jhang, Pakistan.

MATERIALS AND METHODS

Blood and tissue sampling and preparation of impression smears

The samples were collected from the buffaloes (n=771) suspected of Paratuberculosis

brought for slaughter at local abattoirs of tehsil Jhang. Out of total clinically suspected animals, only n=53 carcasses were sampled (intestinal tissues and mucosal smears) based on gross observation (Sikandar *et al*., 2012) along with weak and emaciated health status. Blood samples (5 ml/ animal) were collected from weak, debilitated and diarrhoeac animals. The blood was centrifuged at 2000 rpm for 10 minutes, separated serum was collected and preserved at -20°C until further use. Anti-MAP antibodies detection kit (Serelisa™, France) was used in ELISA as per manufacturer's instructions.

The tissue samples were collected immediately after the slaughtering of the animals. Small (chiefly from the terminal portion of ileum) and large intestines along with the neighboring mesenteric lymph nodes were isolated based on the gross thickened/corrugated gut mucosal walls. Impression smears were prepared from the gut mucosal sites (n=3/per animal) where the corrugations were well noticeable and stained with ZN for confirmation of PTB. The carbol fuchsin and methylene blue staining methods of the smears were adopted as previously mentioned (Sikandar *et al*., 2013).

Histopathological processing of tissue samples

Intestinal tissue specimens were cut into transverse pieces of 1cm and fixed at 4°C in freshly prepared 4% v/v formaldehyde solution for 48 h and instantly handled for histological study as previously described (Ali *et al*., 2017). The tissues sections of 4 to 5µm thickness were prepared by microtome (AMOS Scientific AEM-450, St. Veit/ Glan, Austria), processed by paraffin sectioning and stained with Hematoxylin-Eosin (H&E) and Ziehl-Neelsen (ZN) stains.

Microarchitectural analysis of the tissues

A total of three sections (one after every 10 sections) were collected from each tissue sample and the structural changes in the mucosae were examined under light microscope (Olympus CX31, New York, USA) maintained with live image digitalized program (Olympus DP20, Olympus, USA). Descriptive statistical techniques were applied to analyze the data collected for grossly affected histopathological tissues, impression smears and ELISA methods.

RESULTS AND DISCUSSION

Gross pathology

Gross examination revealed that intestinal wall was unsteadily thickened at various portions. After opening of the gut, the thickened sites were found to have prominent elevated portions and variable sized corrugations. The elevations were not vanished on gentle thumb pressure. A thick mucous layer in the lumen and the hyperemia were evident on the gut mucosal surface. The associated mesenteric lymph nodes were edematously swollen and the lymphangiectasia was apparent.

Sample confirmation

The samples were confirmed positive for PTB by staining the impression smears obtained from the corrugated mucosal sites of the gut. Acid-fast bacilli were detected simply in 11 cases on smear examination and the bacteria appeared bright rose--red rods in a blue background under 100X. Though all the 53 samples exhibited certain corrugations in the mucosae and had thickening in the walls, however these gross lesions were recorded more obvious in the smeared positive cases. These samples were further processed by

histopathology and ELISA for additional studies.

Tissue microarchitecture and serology

In the histopathological observations of the gut sections, the lining epithelium of the intestinal mucosa was found sloughed-off (Figure 1). Villi were not very prominent while the crypts were very deep in the small intestine. In the lamina propria, pressure atrophy and displacement of the crypts of Lieberkuhn were evident (Figure 2).

At many locations, the demarcation between mucosa and submucosa were not so prominent. Both mucosa and submucosa were found to be distended with focal and multifocal cellular nodules dominated by epithelioid macrophages. A heavy infiltration of mononuclear cells including lymphocytes, plasma cells and macrophages were seen both in the mucosa and submucosa (Figure 1). Microgranulomas composed of Langhan's type multinucleated giant cells and the epithelioid macrophages were evident in the mucosal lining. Blood vessels were dilated, and fibrous connected tissue was dominant in the submucosa. After special staining with ZN, the epithelioid macrophages were appeared to be engorged with acid fast bacilli (Figure 2) depicting clear positive cases. The connective tissue capsules of the lymph nodes were quite thickened with fibrous capsules and the CT septae were also thickened. The subcapsular, paracortical and medullary sinuses were widened and enlarged as well as filled with proteinaceous fluid. In few cases (n=5), there was evidence of caseous necrosis in the area of cortico-medullary junctions. Necrosis and hypoplasia were also seen in the lymphatic nodules. All the histo-pathologically positive cases were further confirmed serologically by indirect ELISA test.

DISCUSSION

Buffaloes are principal domestic animals in Pakistan contributing considerably to the meat, milk, leather and other similar industries of the country. Paratuberculosis caused by MAP is chronic enteric disease in buffaloes triggering health implications and drastic economic losses in the form of decreased milk production and low post-slaughter carcass value. Gastrointestinal tract is the major target site for the bacterium (Amemori *et al*., 2004). After getting entrance into the body, MAP bacilli are phagocytosed by macrophages but instead of being destroyed by intra-cellular machinery, rather the bacterial cells continue to multiply within the macrophage. Hence, they become a persistent intracellular infection in the intestinal mucosa (Maxie *et al*., 2007) and thereafter spread to the regional associated lymph nodes (Pavlik *et al*., 2000). Accumulation and multiplication of laden macrophages in the lamina propria of intestine particularly in the ileum and cecum (Jones *et al*., 2006) triggered the characteristic patho-morphological lesions of paratuberculosis in buffaloes. Comparable gross lesions of the disease in the gut were observed during the current study including thickening and corrugations of intestinal mucosa especially in the lower ileum and edematous swelling of the mesenteric lymph nodes as reported in an earlier study (Amemori *et al*., 2004). These mucosal thickening and congestion were due to mass infiltration of mononuclear cells (Sivakumar *et al*., 2006) and lead to sloughing of the lining epithelial layer and pressure atrophy of the intestinal crypts. The disruption of mucosal lining severely affects the absorption of nutrients and water from the gut and the affected animal suffers from malabsorption, malnutrition, diarrhea, loss of body condition and

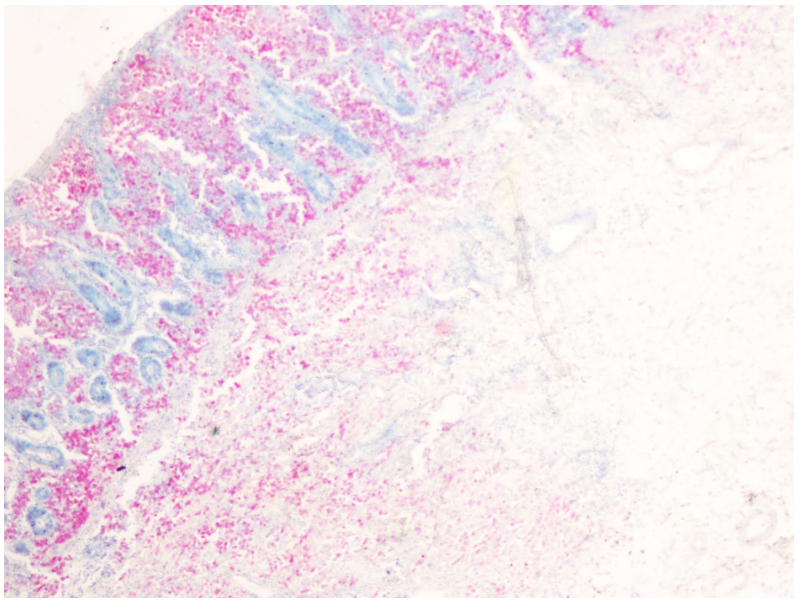

Figure 1. Semi thin slide of the small intestine showing mass infiltration of the MAP-laden macrophages in the mucosa and submucosa. The lining epithelium is sloughed-off and disruption of the muscularis mucosae is evident. Ziehl Neelsen (40X).

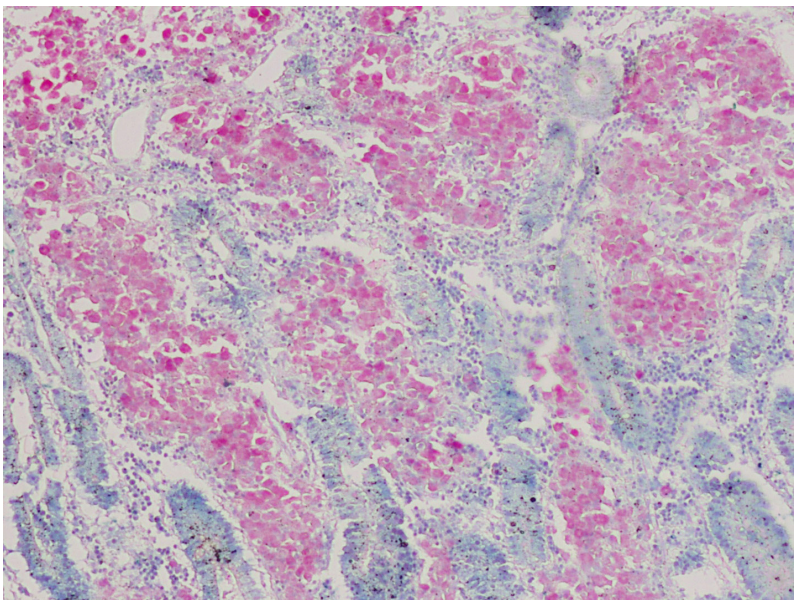

Figure 2. Semi thin slide of the small intestinal mucosa. The MAP-laden macrophages and other MNCs infiltrated in the mucosa causing atrophy of the crypts. Ziehl Neelsen (100X).

thus decrease in milk yield (Maxie *et al*., 2007). Like our observations, transverse corrugations, lymphadenopathy and granulomatous lesions were also reported in the intestinal mucosa consistent with paratuberculosis in buffaloes (Sivakumar *et al*., 2006). Moreover, these morphological changes in subclinical JD has been characterized by microgranuloma in the MLN in North American bison (Buergelt and Ginn, 2000).

Mycobacterium is responsible for causing Crohn's disease in humans, but the cost-effective diagnosis of the disease is required for an effective control measure to overcome the drawback of the available vaccines being unable to prevent the bacterial shedding during the infection (Antognoli *et al*., 2007). The prevention and eradication of JD is significantly hindered due to dearth of simple and sensitive tools for detection of infection at an early stage (Muhammed *et al*., 1978). Usually more than one detection methods are combined for complete diagnosis of the disease (Singh *et al*., 2007). For this purpose, the diagnostic tests are divided into antigen detection and specific antibody detection in serum (Buergelt and Ginn, 2000). Similar to serological tests, careful histological examination of the intestine especially jejunum to ileocecal valve and mesenteric lymph nodes is another effective method for the detection of mycobacterium (Amemori *et al*., 2004). Apart from these tests, DNA based detection of the infection was found to be more sensitive than bacterial culture and staining techniques (Buergelt and Ginn, 2000). Similarly, nested PCR was also found to be a rapid and useful tool for the diagnosis of later stage paratuberculosis in animals (Uy *et al*., 2018). Compared to tissue culture, ELISA was stated as less sensitive, but others claimed it as quickest and least expensive screening tool (Schaik *et al*., 2007).

Sub-clinically infected animals are an important source of transmission of infection and appropriate diagnosis of these animals is crucial for controlling bovine paratuberculosis. Although, serological diagnosis includes ELISA, CFT and agar gel immunodiffusion but these tests have low sensitivity as antibodies may not be detected until late infection. Bacteriological examination of the infection demonstrates the detection of acid-fast bacilli in impression smears of mucosa and gut tissues stained with ZN for presumptive confirmation by microscopic examination (Jones *et al*., 2006; Sivakumar, 2006). In the present study, the observed thickening and corrugation of the mucosa, hypoplasia and atrophy of the intestinal crypts was due to MNCs in the mucosa and submucosa as previously described elsewhere (Maxie *et al*., 2007). In the current study, it was explored that the lesions also extend anteriorly as far as duodenum and posteriorly into the rectum. Histo-pathology of tissue sections detected 100% of the suspected carcasses while the smear method declared only 20.8%. Hence, both histopathology of the infected gut with the MLN tissues are the best combination for confirmation of the positive samples. It was thus concluded that the technique has better specificity compared to other similar conventional tools and can be considered as a useful tool for the diagnosis of bubalian PTB in Pakistan.

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