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PREVALENCE AND PATHOLOGY OF PARATUBERCULOSIS IN CATTLE AND BUFFALOES AT FAISALABAD ABATTOIR

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Bovine Paratuberculosis is a chronic, debilitating disease resulting in huge economic losses to the dairy industry. The present study was conducted to determine the frequency of Paratuberculosis infection in cattle and buffaloes slaughtered at Faisalabad abattoir, Pakistan. The study carried out on a total of 400 animals including cattle (200) and buffaloes (200). On the basis of PCR, 0.5% buffaloes and 1.5% cattle were found positive for Paratuberculosis. From 25 cattle and 20 buffaloes the suspected morbid samples of intestine and mesenteric lymph nodes were collected and were further processed for histopathology analysis and molecular characterization using polymerase chain reaction techniques (PCR). A total of 18 cases of intestine showed haemorrahages, out of these 2 cases showed thickened mucosa and corrugation. These samples were further processed for histopathology and PCR. The results revealed that out of 45 morbid samples (25 cattle + 20 buffaloes), 4 were confirmed by PCR for *Mycobacterium Paratuberculosis*. The histopathology examination of intestine tissues revealed diffuse inflammatory reaction, mononuclear cell infiltration along with degenerative and necrotic changes in sub- mucosal glands of the intestine. The immature granuloma formation was also observed in both intestine and lymph nodes.The study concluded that the disease was prevalent (1%) in animals coming for slaughtering at Faisalabad abattoir.
Keywords: Prevalence, pathology, cattle buffaloes, paratuberculosis, abattoir.

INTRODUCTION

Paratuberculosis in cattle is a chronic, intestinal granulomatous infection caused by *Mycobacterium avium* subsp. *Paratuberculosis* (Gulliver *et al*., 2015). Clinically Paratuberculosis in cattle can be identified by chronic diarrhea, weight loss and drastic emaciation leading to death (Koets and Grohn, 2015). The disease mainly affects the cattle, sheep, goats and wild ruminants. Animals are usually infected in their early age (<6 months), but clinical signs develop later in life (Awadin *et al*., 2016). The MAP is intracellular, acid-fast and slow growing pathogen that requires mycobactin in its media for growth (Munir *et al*., 2014). The disease adversely effects the productive and reproductive performance of the animals i.e., decrease milk production, increase cull rate, decrease in animal fertility and constant spread of organism. Many developed countries have started efficient control and eradication program to decrease the disease prevalence rate (Geraghty *et al*., 2014). As the organism has prolong incubationperiod, it disturbs the productive efficiency and the animals remain infected sub clinically (Gregor *et al*., 2015).

The organism can also be present in milk and colostrum, and can pass to young ones through colostrum. Persistent diarrhoea, weight loss and decrease in milk yield leads to huge economic losses all over the world. In advanced phase of disease, there is a severe decrease in milk yield and severe diarrhoea, can be observed due to intestinal inflammation and malabsorption of the nutrients. Mostly MAP positive animals shed considerable amounts of organisms in the faeces and milk and thus can be cause of spread of the disease to their herd mates (Fletcher *et al.*, 2015). The pathogenesis of the disease is poorly known. The organism is usually assumed to pass through the lumen of the small intestine and move towards the Peyer's patches via Microfoldcells (M cells) of the follicle associated epithelium, soon this is phagocytized by macrophages (Koets *et al.*, 2015). MAP has ability to survive and replicate within macrophages in the intestine and in the lymph nodes. This association of M cell and peyer's patches indicate that the follicle associated epithelium has concern with the uptake of the organism (Koets *et al.*, 2015). The macrophages engulf these bacteria and gradually lead to severe granulomatous inflammation in small and large intestine and in associated mesenteric lymph nodes. Acid fast bacilli (AFB) can be observed in the lesions; however, the number of bacilli increases with the passage of time (Kurade *et al.*, 2004).

There is an association between MAP and the Crohn's disease in human because of its similarity in clinical signs and lesions. For the diagnosis of disease, several methods have been used, but most of the tests have low sensitivity and specificity (Timms *et al*., 2016). Due to slow progress of disease and low immune response, it is difficult to diagnose the magnitude of disease efficiently with single diagnostic tool. Different

surveys showed that paratuberculosis is present worldwide, causing huge economic losses in the different countries. In china, seroprevalence study on commercial dairy and beef cattle was carried out that showed that 11.79% animals were affected with Paratuberculosis (Sun *et al.*, 2015). The study revealed that the estimated herd prevalence of Paratuberculosis was 7% in Austria (Europe), while in Denmark it was 55% (Kennedy *et al.*, 2001). The disease prevalence was 7% in Egypt cattle (Awadin *et al*., 2016). The disease prevalence rate in the south Brazil was 97.2% (Gomes *et al.*, 2005). Only few countries including Sweden have very low disease prevalence. In Pakistan, an abattoir based study has been conducted that reported 11.9% disease prevalence in district Jhang (Sikandar *et al*., 2012). The prevalence in breeding bulls at a semen production unit has been reported to be 20%, while in case of teaser bulls it was 33% (Abbas *et al*., 2011). Because the limited studies on the disease have been carried out, there is need to have more studies in different areas to know the status of the disease. Thus, it is essential to know the present status of the disease in Faisalabad, Pakistan. Therefore, the present study was carried out to investigate the epidemiology of Paratuberculosis in cattle and buffaloes at Faisalabad slaughterhouse to understand the pathology of Paratuberculosis in cattle and buffaloes.

MATERIALS AND METHODS

The study was conducted at Faisalabad slaughterhouse. A total of 400 consecutive animals, including 200 cattle and 200 buffaloes, were included in the study. Morbid tissue samples of 25 suspected cattle and 20 suspected buffaloes were collected. Morbid organs, including intestinal pieces, exhibiting thickening (corrugation) of mucosa, along with mesenteric lymph nodes were collected. The data about each animal, including its tentative age, weight, sex and parity were also recorded. The age of the animal was assessed by eruption of permanent teeth. To assess the weight of the animal, the girth was measured and then their tentative weights were estimated. The judgement of butchers about the weight was also recorded. The weight of the animals judged by butchers was correlated well with the results of the girth of the animal. The tissues showing lesions suspected for paratuberculosis were collected. These tissues samples were stored in 10% buffered formalin for histopathology. Tissue sections of 5 micron meters thick were cut and stained with haematoxylin and eosin staining procedure and observed under microscope. The tissue slides containing morbid tissues along with normal tissues portions were processed for histopathology using hematoxylin and eosin staining protocol (Bancroft and Gamble, 2007). A part of sample was not fixed in formalin and was used for PCR amplification. For DNA extraction Phenol – Chloroform extraction method was used as described by Okwumabua *et al*. (2010). Direct PCR was performed on these tissue samples for the rapid diagnosis of Paratuberculosis using specific primers sequences, P90 (GTTCGGGGCCGTCGCTTAGG) and P91 (GAGGTCGATCGCCCACGTGA) (Bartos *et al*., 2006). Conditions were as follows: Initial denaturation at 94°C for 2 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 65°C for 2 minutes, elongation 72°C for 3 minutes (35 cycles) and a final elongation at 72°C for 10 minutes (Stanley *et al*., 2007). The amplified PCR product was analyzed by using 1% agarose gel electrophoresis. The gel was placed into an electrophoresis gel documentation system (Syngene, USA) for visualizing DNA bands in a dark room through ultraviolet light trans illuminator. The data collected was analyzed by using frequency analysis (SAS, 2007). The 95% confidence limits were also worked out.

RESULTS

The study revealed that the disease was prevalent in animals at slaughterhouse with 1% prevalence.On the basis of PCR, 0.5% buffaloes and 1.5% cattle were found positive for Paratuberculosis (Fig. 1).

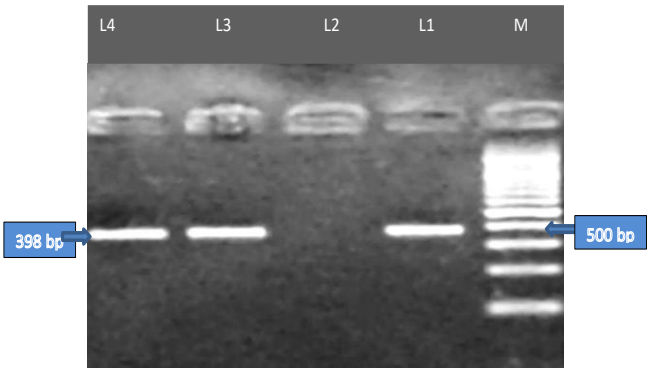

Figure 1. L1 and L2 showing PCR product of 398 bp with Primer 90/91 M: represent the DNA ladder.

The prevalence; however, was relatively higher in cattle. The results of prevalence of Paratuberculosis with respect to sex in both cattle and buffaloes on the basis of PCR revealed that 0.56% male and 1.36% females were positive for Paratuberculosis. However, higher prevalence was found in females. The results of prevalence of Paratuberculosis in different weight groups of both cattle and buffaloes on the basis of PCR showed that out of two weight groups, i.e., <350 and >350 kg body weight, the chi-square analysis and 95% confidence interval revealed significant difference ($P<0.05$) in prevalence between two weight groups. Higher prevalence was found in group having weight <35 0kg. The results on prevalence in different age groups in both cattle and buffaloes on the basis of PCR showed that 0.59% animals were positive for Paratuberculosis between 1-5 years of age, while 0.72% animals were between 5.1-10 years of age and 2.12% were in

age group >10 years. The results on prevalence in different parity groups revealed non-significant difference in prevalence of Paratuberculosis in cattle and buffaloes between four parity groups. However, relatively higher prevalence was found in nulliparous animals (Table 1).

Table 1. Distribution of Paratuberculosis in cattle and buffaloes with respect to specie, sex, parity and lesions.

Parameters	PCR		95% CI
Specie	Negative	Positive	
Cattle	197	3(1.5)	0.38-4.03
Buffalo	199	1(0.5)	0.03-2.44
	OR = 0.33		
Sex			
Male	178	1(0.56)	0.03-2.72
Female	218	3(1.36)	0.35-3.65
	OR = 2.45		
Weight (Kg)			
<350	157	4(2.48)	0.79-5.88
>350	222	0(0.00)	0.01-1.34
	OR = 2.45		
Age (Years)			
1-5	166	1(0.59)	0.03-2.81
5.1-10	138	1(0.72)	0.04-3.50
>10	92	2(2.12)	0.36-6.85
Parity			
0	237	3(1.25)	0.32-3.36
1	83	1(1.19)	0.06-5.73
2	44	0(0.00)	0.11-10.47
3	32	0(0.00)	0.08-8.94
Lymph Node			
Normal	384	0(0.00)	0.00-0.78
Swollen	12	4(25.00)	8.49-49.89
Intestine			
Normal	380	0(0.00)	0.00-0.79
Hemorrhagic	16	2(11.11)	1.91-32.17
Thickened	0	2(100)	2.36-100

The results on prevalence of Paratuberculosis with respect to histopathological lesions in lymph nodes on the basis of PCR revealed that out of 16 swollen lymph node cases, four yielded positive PCR for *Mycobacterium avium* subsp. *paratuberculosis.* The results on prevalence of Paratuberculosis in intestine with respect to histopathological lesions on the basis of PCR showed that out of 18 cases that showed haemorrahage, only 2 of these cases were found positive by PCR for *Mycobacterium avium* subsp. *paratuberculosis*, while in 2 cases, intestine appeared thickened and all these cases yielded positive PCR for MAP (Fig. 2). Out of total 45 morbid samples collected from slaughterhouse (25 cattle + 20 buffaloes), 4 were confirmed by PCR. The histopathological examination of intestine samples revealed diffuse inflammatory reaction, mononuclear cell infiltration along with degenerative and necrotic changes in sub- mucosal glands of the intestine (Fig. 3).

Figure 2. Gross pictures of corrugated, thickened intestine.

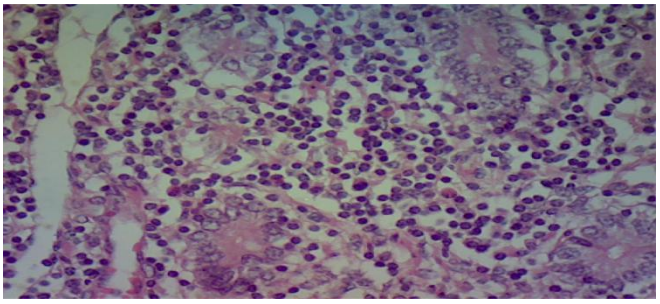

Figure 3. Photomicrograph of intestine showing diffuse inflammatory reaction with mononuclear cell infiltration and degeneration and necrotic changes in sub-mucosal glands (400x).

The immature granuloma formation was also observed (Fig. 4). Out of these 4 confirmed positive samples, the histopathology examination of 3 lymph node samples revealed immature granulomatous reaction with inflammatory cells (Fig. 5). Diffuse cellularity with prominence of macrophages along with presence of lymphocytes and mixture of chronic inflammatory cells were also observed (Fig. 6).

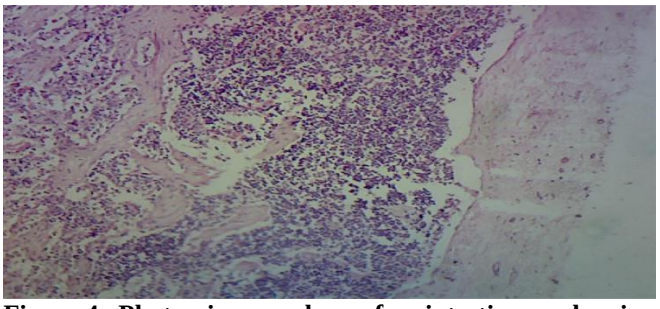

Figure 4. Photomicrograph of intestine showing immature granuloma formation (100x).

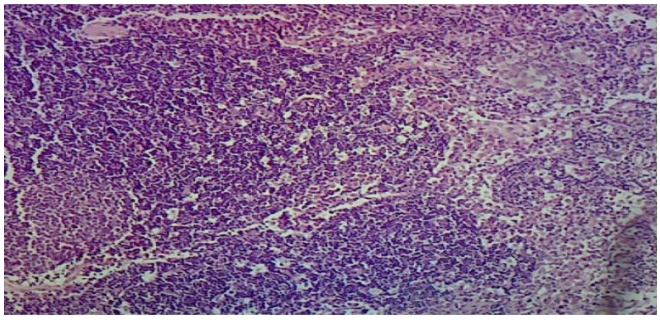

Figure 5. Photomicrograph of lymph node showing immature granulomatous reaction with inflammatory cells (100x).

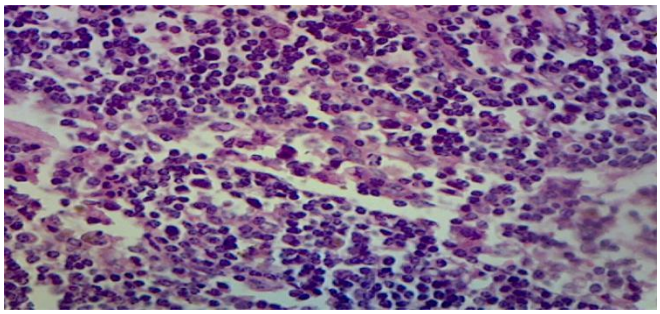

Figure 6. Photomicrograph of lymph node diffuse cellularity with prominence of macrophages along with presence of lymphocytes and mixture of chronic inflammatory cells (200x).

DISCUSSION

The results showed that on the basis of PCR out of total 400 animals slaughtered at slaughterhouse, 3 cattle and 1 buffalo were found positive for Paratuberculosis. The overall prevalence of the disease was 1%. Similarly, 2% prevalence of the disease was reported by Hajikolaei *et al.* (2006) in Iran. The observed prevalence is less as compared to other studies carried out in Pakistan as a study conducted by Sikandar *et al*. (2012) at two abattoirs of Jhang revealed 11.19% prevalence in cattle and buffaloes, similarly, another histopathological study in Nili-Ravi buffaloes of Jhang showed 22.64% prevalence. In Faisalabad, an overall prevalence of Paratuberculosis was 4.5% and 2.5% in buffaloes and cattle, respectively reported in another study (Ashraf *et al*., 2015). The disease is also present all over the world with different prevalence rate. An abattoir based study in Uganda showed 8.8% prevalence of MAP (Okuni *et al*., 2011). Similarly, a study in northern India revealed prevalence of disease as 28.6 and 29.8% in buffaloes and cattle, respectively (Singh *et al*., 2008). In Italy, the prevalence of Paratuberculosis in dairy animals was 2.8-5.5% (Marchetti *et al*., 2013). Another prevalence study of Johne's disease in Iran reported upto12% (Hanifian *et al*., 2013). The disease prevalence in India was reported to be 15.14 to 18.33% (Gupta *et al*., 2012). Sixty percent disease prevalence was found in Jordan (Hailat *et al*., 2012). Nielsen *et al*. (2000) described 70% herd-level prevalence in Danish dairy cattle. The present study showed that more cattle were found positive for Paratuberculosis as compared to buffaloes. Khan *et al.* (2010) conducted a study at Lahore slaughterhouse that revealed higher prevalence of disease in cattle i.e., 14.2% than of buffaloes 12.4%. Regarding the sex of the animal, 0.56% of the positive animals were male and 1.36% were females. Salgado *et al.* (2009) conducted a study that also showed higher prevalence in females than males. A prevalence based in southern Iran was carried out by Hajikolaei *et al*. (2006) that revealed that out of total 250 samples, 2 (0.8%) males and 3 (1.2%) females were found positive for Paratuberculosis. It has reported that poor hygienic conditions at traditional farms enhance the chance of occurrence of disease. In addition to this, higher number of animals in a limited confined area, constant shedding of Mycobacterium by diseased animals and ability of the bacteria to persist for a long time in the surrounding environment are the major predisposing factors of Paratuberculosis. According to weight groups, the animals found positive had less body weight, i.e., < 350 kg. This is because most of the animals are brought to the slaughterhouse in debilitating condition and when don't recover from disease and become weak they are sold for slaughter. The results of the present study revealed that the prevalence was higher in older age groups, i.e., >10 years old as compared with 1-5 years age group. A prevalence based study in England was conducted by Woodbine *et al.* (2009) which revealed that old animals were more prone to disease than the young ones. The gross findings showed that 1.25% nulliparous females were suspected for Paratuberculosis. The results with respect to histopathological lesions revealed swollen lymph nodes and intestine, while intestine also showed heamorrahages. A study conducted by Tiwari *et al*. (2006) also revealed that in cattle the main lesions of Johne's disease were usually confined to the intestine and associated lymph nodes. The thickening of the intestinal wall up to three or four times than normal with corrugation of the mucosa was characteristic for Paratuberculosis. A histopathological study in Indian buffalo revealed inflammation of lymph node and intestine, thickening and corrugation of small intestine and granulomatous inflammation (Sivakumar *et al.,* 2006). A study conducted by Koets *et al.* (2015) revealed that thickening and corrugation of intestine along with enlargement of mesenteric and lymph nodes were prominent in diseased animals. This corrugation and thickening of mucosa may be due to infiltration of mononuclear cells, i.e., epithelioid macrophages infiltration in mucosa and in submucosa (Maxie *et al*., 2007). The main microscopic lesions observed in intestine were the diffuse inflammatory reaction with mononuclear cell infiltration along with degenerative and necrotic changes. The disintegration of villi structure with macrophages and lymphocytes were observed.

A histopathological study revealed that the epithelial cells lining the intestinal villi had mostly sloughed-off, especially in the distal part of the intestine and granulomatous infiltrations were shown in lamina propria (Sikandar *et al*., 2013). The epithelioid macrophages were the predominant infiltrating cells in the intestine and lymph nodes. Similarly, histopathological studies further showed the sloughed-off epithelium and infiltration of inflammatory cells in the mucosa and the granuloma formation. This infiltration was mainly by the macrophages that were predominantly present (Sikandar *et al*., 2013). In case of mesenteric lymph nodes, prominence of macrophages along with presence of lymphocytes and immature granulomatous reaction with inflammatory cells were observed microscopically.

***Conclusion*:** The study concluded that paratuberculosis is prevalent at slaughterhouse of Faisalabad, Punjab, Pakistan and histopathology along with PCR are reliable and specific diagnostic technique for the diagnosis of Paratuberculosis.

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