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PREVALENCE OF PARATUBERCULOSIS IN CATTLE AND BUFFALOES IN FAISALABAD AND ASSOCIATED RISK FACTORS

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ABSTRACT

Bovine Paratuberculosis is a chronic disease primarily of the gastrointestinal tract. The disease is caused by *Mycobacterium avium* subsp. *Paratuberculosis* (MAP). Paratuberculosis is a disease of high economic importance around the globe. The present study was carried out to investigate the prevalence and pathology of paratuberculosis in cattle and buffaloes at two cattle/buffalo colonies of Faisalabad. A total of 133 and 132 adult cattle and buffaloes above two years of age were screened with tuberculin testing for Paratuberculosis. Blood and fecal samples of all the tuberculin positive animals along with the same number of apparently disease-free animals and suspected cases for paratuberculosis (negative by tuberculin test but having chronic diarrhea) were collected and processed for enzyme-linked immunosorbent assay (ELISA) and Ziehl Neelsen (ZN) staining. The study revealed that at two cattle colonies the herd prevalence was 100% and overall animal level prevalence of disease in eight herds was 5.66%. The prevalence rate was non-significantly different in cattle (5.88%) and buffaloes (5.56%). Multivariate logistic regression analysis with the backward elimination procedure in cattle and buffaloes revealed that herd (OR=0.294), age (OR=1.16) and lactation number (OR=1.534) showed significant association with the occurrence of Paratuberculosis. The study revealed that the disease was present at these two cattle/buffalo colonies. The ELISA and ZN showed more positive cases than that of tuberculin testing.

Key word: *Paratuberculosis, Prevalence, ELISA, Cattle, Buffalo*.

INTRODUCTION

The causative agent of bovine Paratuberculosis is *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The disease affects mainly domestic and wild ruminants and results in economic losses, especially in dairy animals. The disease is also of zoonotic importance because the Crohn's disease patients in humans share the same organism (Leonardo *et al.,* 2001). Paratuberculosis is present worldwide and the control programs have started in developed countries to reduce the prevalence of this infection (Nielsen *et al*., 2007). Main reasons to control the infection are zoonosis and economic losses faced by the farmers. The long duration of infection and following deterioration of the health condition of the animals have a considerable impact on productive and reproductive performance (Lombard, 2011). The mode of spread of disease is fecal oral route in dairy herds; while different other routes of transmission, i.e., contaminated feed, water, and in-utero transmission have also been reported (Whittington and Windsor, 2009). Clinically, MAP positive animals shed considerable amounts of organisms in the feces and milk and thus can spread the disease to other animals in the herd (Fletcher *et al.*, 2015). An animal is more susceptible during the first year of life and infection usually occurs within the first month, but it may take 6 months to 5 years to become apparent. Clinical disease may appear after two years or later (Irenge *et al*., 2009). MAP causes chronic diarrhea, muscle wasting and loss of weight (Sivakumar *et al*., 2006). MAP cause reduction in milk yield in dairy cattle and buffaloes. The reduced meat yield was also observed in beef cattle and buffaloes. MAP also causes low reproductive performance in bovines (Kurade *et al*., 2004). It causes the roughening of the hair coat and "Bottle jaw", i.e., intermandibular edema due to the loss of serum proteins. Different diagnostic techniques like tuberculin testing, ZN staining, ELISA, culture isolation and PCR are conventionally used for the detection of *Mycobacterium* subsp. *Paratuberculosis*. Among these tests, tuberculin testing, simply indicates the exposure and the presence of stimulated T-memory cells. The acid fast staining of fecal samples is less sensitive. However, serological tests like enzyme-linked immunosorbent assay (ELISA) was more sensitive test for Paratuberculosis (Hemalatha *et al*., 2013). The combination of ELISA and fecal PCR increases the overall diagnostic sensitivity for the diagnosis of Paratuberculosis. The organism is present worldwide. The prevalence of disease has been reported to be 21.4% in Ireland (Good *et al.,* 2009), 19.3% in Southern Chile (Kruze *et al.,* 2013) and 4% in Italy (Desio *et al.,* 2013). In France, an ELISA based study revealed 2.9% prevalence (Mercier *et al*., 2010). In dairy animals of USA, Paratuberculosis infection has been reported up to 5-10%, and in the herds, it was 33% (Dorshorst *et al*., 2006). In India, its prevalence was 15.14% (Gupta *et al*.,

2012). In Pakistan, few studies have been conducted on abattoirs that reported 11.9% disease prevalence in District Jhang (Sikandar *et al*., 2012). An abattoir based study in Faisalabad showed the prevalence of disease in buffaloes and cattle at the rate of 3.75% and 4.1%, respectively. Because the limited studies on the disease have been carried out, yet no study has been conducted at the herd level, therefore, there was a need to have more studies to know the status of the disease in Pakistan. Therefore, the present study was planned with the objectives to investigate the epidemiology of Paratuberculosis in cattle and buffaloes at selected areas and to compare the suitability of different diagnostic tests, including tuberculin testing, ELISA and ZN staining in the diagnosis of Paratuberculosis.

MATERIALS AND METHODS

Two cattle/buffalo colonies of Faisalabad, one on Aminpur road (Chakeera) and second on Satyana road (Malkhanwala) were included in the study and a total of 133 and 132 adult cattle and buffaloes, respectively above two years of age were screened for Paratuberculosis on the basis of expected prevalence. For the calculation of the sample size the relevant formula for a 95% confidence interval was used with 10% expected prevalence and 5% desired absolute precision:

$$n = \frac{1.96^2 \, Pexp \, (1-Pexp)}{d^2}$$

The intradermal tuberculin testing (ITT) was performed by administering 0.1 ml avian purified protein derivative (PPD; produced by Instituto Zooprofilattico, Perugia, Italy) at cervical region. Skin induration at administration sites was measured by using Vernier caliper by the same operator for all the animals. The animals were classified (positive and negative) on the basis of skin thickness as described by Aagaard *et al*. (2003).

A specialized proforma was developed and observations were recorded, including sex, age, body weight, breed, milk production, the status of the animal (dry and lactating), stage of lactation, feeding, housing, total number of animals at the farm, other animals at the farm and their number, etc. Animal grouping was done on different basis. Blood samples of all the positive animals were collected without anticoagulant. Five ml of blood was collected without anticoagulant and kept the tubes in a slant position for about half an hour for the separation of serum. Collected serum was stored at -20˚C for determination of total serum proteins and conduction of ELISA for Paratuberculosis. Blood samples from a similar sample size as of positive were collected from apparently disease-free animals along with suspected cases for Paratuberculosis which were PPD negative but had the history of chronic diarrhea. The ELISA was done by using the method described in the commercial kit (LSIVET, Ruminant Serum Paratuberculosis Advanced kit, France; Lot No. 2-Vetptrs-007). Fecal samples were also collected and further Ziehl Neelsen staining was carried out, smears were made from different fecal samples. The slides were stained with ZN method (Murray *et al*., 1999) to detect acid fast bacilli. The data collected was analyzed by using frequency analysis, stratified analysis and logistic analysis procedures, i.e., multivariate logistic regression with backward elimination, bivariate logistic regression and after controlling the age and herd as a constant factor. The 95% confidence limits were also worked out where appropriate (SAS, 2007). The analysis of variance technique was also applied and means were compared by DMR for parameters such as age, lactation length, lactation number, etc.

RESULTS

The results revealed 100% prevalence of Paratuberculosis at two cattle/buffalo colonies at herd level in eight herds. The overall prevalence of 5.66% was observed at animal level in 265 buffaloes and cattle at Proka and Malakhanwala cattle/buffalo colonies, Faisalabad on the basis of combined results of tuberculin and ELISA test. At Proka 9/133 animals were found positive and prevalence rate was 6.76%, while at Malakhanwala colony 6/132 animals were found positive that revealed 4.5% prevalence. The prevalence was non-significantly different in cattle (5.88%) and buffaloes (5.56%). Cattle and buffaloes together were divided into different groups based on age, weight, lactation number, lactation length, status and a total number of animals present in a herd. The statistical analysis revealed non-significant differences between different groups for age, weight, lactation number, lactation length, milk produced and status of the animals (Table 1). However, a significant difference ($P<0.05$) was observed between different groups made for total number of animals present in different herds.

Bivariate and multivariate Logistic Regression in Cattle and Buffaloes at Two Cattle/Buffalo Colonies: The bivariate logistic regression analysis in cattle and buffaloes revealed that herd showed significant association with the occurrence of Paratuberculosis. With the increase in the herd there will be 0.54% chance of occurrence of Paratuberculosis. Multivariate logistic regression analysis with the backward elimination procedure in cattle and buffaloes revealed that herd (OR=0.294), age (OR=1.16) and lactation number (OR=1.534) showed significant association with the occurrence of Paratuberculosis. With the increase in herd there will be 0.71% less chance of occurrence of Paratuberculosis. With the one-year increase in age, there

will be 16% more chance of the occurrence of Paratuberculosis and with the increase in one lactation number, there will be 53% more chance for occurrence of Paratuberculosis. After controlling the age as a constant factor, the bivariate logistic analysis revealed that herd and total animals at farm showed significant association with the occurrence of Paratuberculosis. With the increase in the herd there will be 0.68% chance of occurrence of Paratuberculosis, while the increase in one animal in the herd, there will be 0.042% chance of occurrence of Paratuberculosis (Table 2).

McNemar Test to Compare the Sensitivity of Diagnostic Tests: The McNemar's test revealed that the test sensitivity of ELISA and PPD, ELISA and ZN, and PPD and ZN were not different (Table 3). However, the results revealed that ELISA test showed more positive results than that of tuberculin and ZN staining.

Table 1. Different parameter wise percentage of Paratuberculosis in cattle and buffaloes at two cattle/buffalo colonies, Faisalabad, on the basis of tuberculin and ELISA together.

Parameters	Positive / Negative	Positive %	Mantel Haenszel Chi Square Test
Herd			
1	2/10	16.66	P = 0.38
2	2/54	3.57	
3	2/16	11.11	
4	3/85	3.14	
5	1/8	11.11	
6	1/13	7.14	
7	3/49	5.7	
8	1/15	6.25	
Age (Years)			
<5	13/60	7.51	0.07
>5	2/90	2.17	
Weight (Kilograms)			
<350	11/132	7.69	0.121
>350	4/118	3.28	
Lactation Number			
<5	14/212	6.19	0.36
>5	1/38	2.56	
Lactation Length (Months)			
<3	5/76	6.17	
3-6	9/160	5.33	0.89
>6	1/114	6.67	
Milk Yield (Liters)			
0	2/37	5.13	0.63
1-4.9	1/16	5.88	
5-9.9	6/121	4.72	
10-15	6/76	7.22	
Status			
Dry	2/37	5.13	0.87
Lactating	13/213	5.75	
Total Number of Animals			
<50	6/190	3.06	0.002
>50	9/60	13.04	

Table 2. Parameters showed significant association with Paratuberculosis in cattle and buffaloes in logistic regression analysis procedure.

Parameters	Odd Ratio	95% Confidence Limit	P-Value
Bivariate Logistic Regression			
Herd	0.454	0.291-0.710	0.0005
Multivariate Logistic Regression			

Herd	0.294	0.165-0.525	<0.001
Age	1.341	1.173-1.673	0.0019
Lactation Number	1.534	1.068-2.202	0.0204
After Controlling Age as constant Bivariate Logistic Regression			
Herd	0.318	0.185-0.544	0.03
Total Animals	1.042	1.004-1.083	0.0016

Table 3. Comparison of Sensitivity between Diagnostic Tests.

Parameters	**ELISA**		
PPD	Negative	Positive	95% CI
Negative	250	2 (0.79)	0.13-2.60
Positive	0	13 (100)	79.42-100
McNemar's Test Statistic (S) = 2.00, P= 0.157			
Kappa = 0.9246			
ZN			
Negative	250	1 (0.39)	0.02-1.94
Positive	0	14 (100)	80.74-100
McNemar's Test Statistic (S) = 1.00, P= 0.317			
Kappa = 0.9635			
ZN		PPD	
Negative	250	1 (0.39)	0.02-1.94
Positive	2	12 (85.71)	60.26-97.53
McNemar's Test Statistic (S) = 0.33, P= 0.563			
Kappa = 0.8829			

DISCUSSION

The present study was conducted on eight herds of cattle and buffaloes at Proka and Malkhanwala, two cattle/buffalo colonies of Faisalabad. Tuberculin testing was carried out and blood samples were collected. Further processing was carried out by ZN staining of fecal samples, ELISA of blood samples. The main focus of study was to observe the prevalence of Paratuberculosis at these eight herds at these two cattle/buffalo colonies. The present study revealed overall prevalence of 5.66% in the total population of 265 of cattle and buffaloes in eight herds at two cattle/buffalo colonies, Faisalabad on the basis of tuberculin and ELISA together. The highest prevalence of Paratuberculosis was found to be at herd1. The chi-square test and 95% confidence interval revealed non-significant difference in prevalence between eight herds. The prevalence, however, ranged from 3.4 to16.66%.

Between two species, prevalence was higher in cattle (5.88%) than buffaloes (5.56%). In Pakistan, various studies on the prevalence of Paratuberculosis in different species have been carried out that revealed a different prevalence. A study was carried out in the abattoirs of Jhang in cattle and buffaloes. This ELISA based study revealed 11.9% prevalence of disease (Sikandar *et al*., 2012). In Spain, MAP prevalence in dairy herds was 4.03% (Dieguez *et al.*, 2007). In Southern Iran, PCR confirmed 8.6 to 23% prevalence of MAP in dairy herds (Haghkhah *et al*., 2008). While the prevalence of MAP in Northern Iran in dairy cattle ranged from to 4.2-7.7% by PCR (Sadati *et al*., 2012). The statistical analysis showed a non-significant difference in prevalence between two age groups. However, relatively higher prevalence was found in animals of >5 years of age. In England, the study on Paratuberculosis was carried out by Woodbine *et al*. (2009) which revealed a relatively higher prevalence in adult animals than that of young ones. Similarly, relatively higher prevalence was found in animals having less body weight, i.e., <350 kg and in animals having lactation length of >6months. A study conducted by Nielsen *et al*. (2002) also revealed that the prevalence of Paratuberculosis was relatively higher in those animals that were in higher lactation length stage. The statistical analysis revealed non-significant difference in prevalence between milk yield groups. However, relatively higher prevalence was found in milk yield group of 10-15 liters. The high milk yield can act as a stress factor in high producing animals and make animals susceptible for Paratuberculosis. The sero-prevalence study in Irish dairy herds showed that the disease was more prevalent in high producing animals (Hoogendam *et al*., 2009). The statistical analysis showed non-significant difference in prevalence between lactation number groups. However, the prevalence was relatively higher in animals of <5

lactation number. Similarly, animals were also divided into two groups on the basis of status, i.e., dry or lactating. The results revealed that the disease was more prevalent in the lactating animals. On the basis of a total number of animals present in herds, animals were divided into two groups, i.e., <50 and >50. The statistical analysis showed significant difference ($P<0.05$) in prevalence between two groups. The study also showed that the prevalence was higher where total animals were >50. Similarly, prevalence-based study was also conducted by Pillars *et al*. (2009) which revealed higher prevalence in those herds where stock density was more than 200. Similarly, lower prevalence in smaller herds (<100 animals) and higher in larger herds (>100 animals) had been reported (Woodbine *et al*., 2009). Multivariate logistic regression analysis revealed that herd, age and lactation number showed significant association with the occurrence of Paratuberculosis. After controlling the age as a constant factor, the bivariate logistic analysis revealed that herd and total animals at farm showed significant association with the occurrence of Paratuberculosis while, after controlling the herd as a constant factor, the bivariate logistic analysis revealed that specie showed significant association with occurrence of Paratuberculosis.

ELISA was included in the study for further diagnosis and confirmation after tuberculin testing. ELISA showed more positive results as compared to tuberculin testing. In addition, ZN staining was also performed as diagnostic tool for the detection of Mycobacterium. To compare the sensitivity of diagnostic tests McNemar test was applied to test the sensitivity of ELISA and PPD, and ELISA and ZN and it was found that the sensitivity was not different between these tests. The study conducted by Hemalatha *et al*. (2013) showed that ZN staining of fecal samples was less sensitive than ELISA, which was more sensitive test for Paratuberculosis. Another study conducted in Pakistan concluded that ELISA was rapid, reliable and specific diagnostic test for the diagnosis of Paratuberculosis (Sikandar *et al*., 2012). The study revealed that the ELISA test was more sensitive and specific than ZN and PPD. Similarly, ZN revealed more sensitive and specific results than PPD. In dairy cattle population of Latium region (Italy), a study conducted by Lillini *et al*. (2005) revealed that the ELISA was the most reliable and useful diagnostic tool for the screening of dairy herds. Another study also suggested that ELISA was rapid, reliable and specific diagnostic test for the diagnosis of Paratuberculosis (Sikandar *et al*., 2012). Tripathi *et al*. (2006) conducted a study to compare the different diagnostic tests, sensitivities of the tests were calculated. The study revealed that among all the tests, overall highest sensitivities were showed by ELISA. In Netherlands, a study was conducted by Weber *et al*. (2009) that concluded that the sensitivity and specificity of ELISA test in suspected and clinical positive cattle were higher than ZN-test. Therefore, for the confirmation of disease ELISA was preferred over the ZN-test.

Conclusions: The study concluded that Paratuberculosis is present in cattle and buffalo population at cattle/buffalo colonies, of Faisalabad, Pakistan. At two cattle/buffalo colonies prevalence rate varied significantly ($P<0.05$) based on total number of animals. The study also revealed that tuberculin testing, ELISA and ZN staining are efficient diagnostic tools for the diagnosis of Paratuberculosis. Tuberculin testing and ELISA together can help to diagnose the disease in animals and ELISA is a better rapid test for the diagnosis of Paratuberculosis.

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