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PREVALENCE OF PARATUBERCULOSIS IN CATTLE AND BUFFALOES IN PUNJAB PAKISTAN

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Paratuberculosis is economically and zoonotically an important disease in dairy animals and required continuous surveillance. The study was carried out to investigate the prevalence of Paratuberculosis on four public livestock farms. In overall, a prevalence of 2.4 was recorded at four farms, while it was 1.8% in cattle and 3.6% in buffaloes with 100% herd prevalence. It was noted that Sahiwal cattle had 3.18 time's higher chances of having the disease than Cholistani cattle. Further, it was noted that there were 4.94 time's higher chances of disease in lactating than dry/non-lactating cattle, while there were 2.72 times higher chances of disease in dry/non-lactating than lactating buffaloes. Furthermore, in cattle the chances of disease were 3.18 times higher when small ruminants were also present at the farm. Results revealed that the body weights of animals were significantly higher in positive than disease negative cattle. The results of ELISA+PCR were found positive in 80% cases irrespective of the result of another test, while the ELISA+tuberculin in 65% and ELISA+ZN in 30% cases. The results also indicated that ZN faecal in 3.1% cases were positive without having a positive PCR. Based on diagnostic tests, the overall results for PPD, ZN, and indirect ELISA were 3.06%, 1.96% and 2.45%, respectively. Conclusions: the prevalence of paratuberculosis at animal levels is 2.4%, but 100% at farm level. The tuberculin test can be used as a screening test, but the results are not reliable and the positive/suspected animals must be further confirmed by ELISA in resource poor settings.
Keywords: cattle, buffaloes, farms, Pakistan, paratuberculosis, prevalence

INTRODUCTION

Johne's disease or paratuberculosis (PTB) is a chronic enteric disease of livestock principally of ruminants. *Mycobacterium avium* subsp. *paratuberculosis* (MAP) is an acid fast gram positive slow growing bacterium which is the cause of the paratuberculosis or Johne's disease in different ruminant species (Seyyedin *et al*., 2008). Infection with MAP is slow and leads to chronic enteritis with regional lymphadenitis and lymphangitis (Fernandez-Silva *et al*., 2011). The clinical signs manifested by infected animals include chronic diarrhoea, decreased milk production, emaciation and eventually death (Settles *et al*., 2014). The disease is responsible for economic losses worldwide in terms of medication, premature culling and mortality. Faecal oral transmission is the primary mode of spread of MAP in herds, while contaminated feed, water, soil and in-utero transmission is also possible. MAP is also secreted through milk and colostrum and the calves at younger age got infected by this mean of transmission (Lu *et al*., 2008). The MAP is also of interest due to the possible role in causation of inflammatory bowel disease of humans known as Crohn's disease. Milk and its products are a potential source of infection to humans (Hruska *et al*., 2011). Paratuberculosis still has a notifiable status in different developed countries and they have started various control strategies to restrict the magnitude of disease at herd level (Pozzato *et al*., 2011). In the USA, paratuberculosis is present in 5-10% of dairy animals and in 33% of dairy herds (Dorshorst *et al*., 2006), while in England the prevalence rate is 7.3% in dairy herds (Woodbine *et al*., 2009). In India, there is 15.14% prevalence of the disease (Gupta *et al*., 2012). In Pakistan, the prevalence in breeding bulls at a semen production unit has been reported to be 20% (Abbas *et al*., 2011). Due to lack of quality diagnostic methods it has become difficult to detect the MAP in dairy herds. Paratuberculosis is a challenging and economically important disease not only for the dairy industry, but also from a public health perspective. Thus, it becomes essential to keep on monitoring the status of disease in animals in Pakistan. Therefore, this study was undertaken to investigate the epidemiology of paratuberculosis at four public livestock farms by the use of four diagnostic tests (tuberculin, ZN, ELISA, and PCR) other than culture isolation which was not possible due to regular electricity breakdowns.

MATERIALS AND METHODS

The study was conducted on four public livestock farms. A total of 818 animals were included with 403 Sahiwal cattle (Farm 1), 140 Cholistani cattle (farm 2), 106 Nili-Ravi

buffaloes (farm 3) and 169 Nili-Ravi buffaloes (farm 4). The animals above two years of age were included. These farms have semi-open environment with separate milking parlors and animals are only chained during milking time.

The intradermal tuberculin testing (ITT) was performed as a screening test by administering 0.1 ml avian purified protein derivative (Instituto Zooprofilatico, Perugia, Italy) at cervical region. Skin induration at administration sites were measured by using Vernier calliper by the same operator for all animals. Measurements were recorded prior to and after 72 hours following administration of the antigen. Results were expressed as the difference in skin thickness (mm) between the pre- and post-skin test readings. A positive reaction to the intradermal tuberculin test was shown by an increase in skin thickness at the site of injection of more than 4 mm in diameter. A negative reactor was identified when there was no swelling present at the site of injection (Aagaard *et al*., 2003). All the reactor animals and those suspected for paratuberculosis on the basis of history and clinical signs were further tested by ELISA. Lsivet Ruminant Serum Paratuberculosis "Advanced'' kit was used for this purpose on the serum samples. This kit was based on indirect ELISA (Catalogue No. VETPTRS2, France).

For Ziehl Nielsen (ZN) smear microscopy and also for direct PCR, about 10-15 gm faecal samples were collected from all tuberculin positive and tuberculin negative but suspected animals based on clinical signs. Samples were taken directly from rectum using plastic gloves and were sealed in a plastic container and were numbered corresponding to the identity of the animal before they were transported to the lab. For direct PCR (Paolicchi *et al*., 2003), faecal samples were decontaminated by 0.75 % Hexadecylpyridinium Chloride (HPC) and DNA was extracted by using a phenol-chloroform method. The PCR was performed by using P90 (F 5'GTTCGGGGCCGTCGCTTAGG 3') and P91 (R 5'CCCACGTGACCTCGCCTCCA 3') primers with thermal cycling conditions of Initial denaturation at 94°C for 2 minutes, followed by 35cycles of 30 seconds at 94°C for denaturation, annealing at 65°C for 2 minutes, 3 minutes at 72°C for elongation followed by last cycle of 30 seconds for denaturation, 2 minutes for annealing at 65°C and a final elongation at 72°C for 10 minutes (Stanley *et al*., 2007). The PCR for MAP was carried out in a total volume of 25 µl with 5 µl of the template, 17 µl of PCR-EZ D-PCR master mix (Biobasic, Cat. No. BS294; 10 mM KCl, 10 mM (NH4)2 SO4, 20 mM Tris HCl, 0.1 % Triton X-100, 0.1 mg/ml BSA, 2 mM MgCl2, 200 µM dNTPs), 1 µl Taq DNA polymerase (Biobasic, Cat. No. B0089; 5u/µl) and 1 µl each of the primers (forward+reverse).

The information was collected on sex, age, body weight, breed, milk production, status of the animal (dry, pregnant, lactating), total number of animals at the farm, other animals at the farm and their number, etc. Data thus collected were put into an excel sheet and grouped on various basis including age, body weight etc. Data were then analyzed by frequency analysis using SAS statistical software (SAS 2007). The 95% confidence limits and where appropriate Odds ratio were also worked out. Logistic regression analysis was also applied, but that yielded non-significant result by univariate and multivariate models.

RESULTS

The results of four tests carried out for the diagnosis of paratuberculosis revealed that where ELISA was positive, where at least one of the other tests was also found positive. In total, these were 20 cases where at least two tests gave a positive result (Table 1). The ELISA, ZN faecal and PCR did not give positive results alone, while tuberculin alone was positive in 37.5% cases. The tuberculin test alone was found positive in 40.6% cases along with other tests. The results of ELISA+PCR irrespective of other test were found positive in 16 (80%), ELISA+tuberculin in 13 (65%) and ELISA+ZN in 6 (30%) cases.

Table 1. The results of tuberculin test, ELISA, ZN faecal and PCR alone and in combination in cattle and buffaloes are presented.

Result	Positive cases (%)	95% Confidence limits
Tuberculin + ELISA + ZN faecal + PCR	10 (31.2)	17.09 - 48.67
Tuberculin + ELISA + ZN faecal	0 (0.0)	0.00 - 8.94
Tuberculin + ELISA + PCR	2 (6.3)	1.06 - 19.15
Tuberculin + ZN faecal + PCR	0 (0.0)	0.00 - 8.94
ELISA + ZN faecal + PCR	3 (9.4)	2.44 - 23.43
ELISA + ZN faecal	3 (9.4)	2.44 - 23.43
ELISA + PCR	1 (3.1)	0.16 - 14.46
Tuberculin + ELISA	1 (3.1)	0.16 - 14.46
Tuberculin + ZN faecal	0 (0.0)	0.00 - 8.94
Tuberculin + PCR	0 (0.0)	0.00 - 8.94
Zn faecal + PCR	0 (0.0)	0.00 - 8.94
Tuberculin	12 (37.5)	22.15 - 55.03
ELISA	0 (0.0)	0.00 - 8.94
ZN faecal	0 (0.0)	0.00 - 8.94
PCR	0 (0.0)	0.00 - 8.94
Total	32	

The results of various parameters recorded in cattle with relation to the animal or its environment revealed that there was no statistical difference between positive animals in various groups made as shown in Table 2. The odds ratio indicated that there were 3.18 time's higher chances of occurrence of paratuberculosis in cattle at farm 2 where the Sahiwal breed was kept than farm 1 where Cholistani breed was kept.

Table 2. Results of frequency analysis and 95% confidence limits in paratuberculosis positive animals.

Parameters	Negative	Positive (%)	95% CI
Cattle			
Farms			
1	139	1 (0.71)	0.04 - 3.47
2	394	9 (2.23)	1.09 - 4.06
Age (years)			
< 5	40	0 (0.00)	0.00 - 7.22
5 - 10	335	7 (2.05)	0.90 - 4.01
> 10	158	3 (1.86)	0.48 - 4.99
Weight (kg)			
< 300	57	0 (0.00)	0.00 - 5.12
> 300	476	10 (2.06)	1.05 - 3.64
Breed			
Cholistani	139	1 (0.71)	0.04 - 3.47
Sahiwal	394	9 (2.23)	1.09 - 4.06
Lactation Number			
< 5	319	5 (1.54)	0.57 - 3.39
5 - 10	206	5 (2.37)	0.87 - 5.17
> 10	8	0 (0.00)	0.00 - 31.23
Lactation Length			
0	74	0 (0.00)	0.00 - 3.97
1 - 200	112	1 (0.88)	0.04 - 4.29
201 - 300	303	9 (2.88)	1.33 - 5.41
> 300	44	0 (0.00)	0.00 - 6.58
Milk Production			
0	74	0 (0.00)	0.00 - 3.97
1 - 4.9	108	3 (2.70)	0.69 - 7.18
5 - 10	333	7 (2.06)	0.90 - 4.03
> 10	18	0 (0.00)	0.00 - 15.33
Status			
Non-lactating/dry	189	1 (0.53)	0.03 - 2.57
Lactating	344	9 (2.55)	1.25 - 4.63
Small Ruminants			
No	139	1 (0.71)	0.04 - 3.47
Yes	394	9 (2.23)	1.09 - 4.06
Buffalo			
Farms			
1	169	0 (0.00)	0.00 - 1.76
2	96	10 (9.43)	4.89 - 16.17
Age (years)			
< 5	12	0 (0.00)	0.00 - 22.09
5 - 10	155	4 (2.52)	0.80 - 5.96
> 10	98	6 (5.77)	2.37 - 11.61
Weight (kg)			
< 400	21	2 (8.7)	1.48 - 25.87
400 - 550	171	6 (3.39)	1.39 - 6.92
> 550	73	2 (2.67)	0.45 - 8.53
Lactation Number			
< 5	188	7 (3.59)	1.58 - 6.97
5 - 10	77	3 (3.75)	0.96 - 9.86
Lactation Length			
0	63	1 (1.56)	0.08 - 7.46
1 - 200	5	1 (16.67)	0.83 - 59.09
201 - 300	89	6 (6.32)	2.60 - 12.67
> 300	108	2 (1.82)	0.31 - 5.88
Milk Production			
0	63	1 (1.56)	0.08 - 7.46
1 - 4.9	12	0 (0.00)	0.00 - 22.09
5 - 10	189	9 (4.55)	2.24 - 8.18
> 10	1	0 (0.00)	
Status			
Non-lactating/dry	156	8 (4.88)	2.29 - 9.05
Lactating	109	2 (1.80)	0.30 - 5.82

The results also revealed that there were 4.94 time's higher chances of disease in lactating than non-lactating/dry cattle. The results also revealed that there were 3.18 time's higher chances of disease in cattle when small ruminant were present at the farm than when these were absent. The results in buffaloes showed a significant difference in prevalence of paratuberculosis between two farms studied. There were 2.72 time's higher chances of paratuberculosis in non-lactating/dry animals than lactating animals.

The results on mean age, body weight, lactation number, lactation length and milk production showed non-significant difference between positive and negative animals in both cattle and buffaloes, except for body weight in cattle which was significantly ($P<0.05$) higher in positive than negative cattle (Table 3).

Table 3. The results of mean ± SD of different parameters in negative and positive cattle and buffaloes.

Parameters	Negative	Positive
Cattle		
Age (years)	8.5	9
Weight (kg)	379.3 B	431.1 A
Lactation Number (n)	4.14	4.4
Lactation Length (days)	200	231
Milk Production (litres)	5.6	5.65
Buffalo		
Age (years)	9.3	10.4
Weight (kg)	502.8	481
Lactation Number (n)	3.2	3.8
Lactation Length (days)	233	255
Milk Production (litres)	4.9	6.2

Note: values in a row with capital letters are significantly different at $P<0.05$

DISCUSSION

In overall, 20/818 animals were found positive including 10/543 cattle and 10/275 buffaloes with a prevalence of 2.4, 1.8 and 3.6%, respectively, while the herd prevalence was 100%. Previous studies carried out in Pakistan reported a varied prevalence. An abattoir-based study conducted in Jhang City that included both cattle and buffaloes showed 11.19% prevalence on the basis of an ELISA (Sikandar *et al*., 2012). Another abattoir-based study in Lahore reported a prevalence of 12.4% in buffaloes and 14.2% in cattle by ELISA, respectively (Khan *et al*., 2010). It clearly indicates that the prevalence at an abattoir is higher than at the farm. It is quite understandable that mostly the low producer or untreatable animals are sold out by the farmers and those come to slaughter at the abattoirs and thus the prevalence at the abattoir is higher. The disease prevalence in India was reported to be 15.14 to 18.33% based on sero-prevalence study (Gupta *et al*., 2012). Another study from India reported prevalence of 13.4% in Gujarat and 16.3% in Andhra Pradesh

based on serology (Trangadia *et al*., 2012). Another study carried out by Singh *et al*. (2008) reported 28.0% and 29.8% sero-prevalence of Johne's disease in buffalo and cattle, respectively in Northern India. The prevalence in Punjab and Uttar Pradesh was 23.3% and 21.9%, respectively (Singh *et al*., 2008). In buffaloes, southern and west UP had the prevalence of 40.3% and 25.5%, respectively. South and west UP showed the prevalence of 42.6% and 30.0%, respectively in cattle (Singh *et al*., 2008). The reports from Pakistan and India suggest that the disease is more prevalent in India than in Pakistan. Similarly, the results from Iran also showed 12% prevalence in dairy cattle (Hanifian *et al*., 2013) which is lower than what has been reported from India and close to reports from Pakistan. Most of the results in India showed an almost similar prevalence in cattle and buffaloes, but the results of the present study showed that the prevalence in buffaloes is 2.01 times higher than in cattle. These results have to be further clarified in future studies, but it may be possible that Nili-Ravi buffaloes are more susceptible to paratuberculosis than Sahiwal and Cholistani Cattle. However, among cattle breeds, the results of the present study suggested that Sahiwal cattle have 3.18 time's higher chances of having the disease than Cholistani cattle. These results are of just two farms and thus have to be looked with caution as maybe there were some management differences including the culling of diseased animals.

The results of logistic regression analysis carried out by univariate, bivariate and multivariate model did not reveal a significant association of epidemiological factors, including age, weight, breed, lactation number, lactation length, milk production, the status of animals (lactating or dry) and presence of small ruminants or other animals at the farm. However, the results of odds ratio suggested that there were 4.94 time's higher chances of disease in lactating than dry/non-lactating animals. However, the results in buffalo were otherwise and there were 2.72 times higher chances of disease in dry/non-lactating than lactating buffaloes. Furthermore, in cattle the chances of disease were 3.18 times higher when small ruminants were also present at the farm. The later findings were otherwise as were noted in Tuberculosis in large ruminants and the prevalence was lower where small ruminants were also present (Javed *et al*., 2011). As it was noted that the prevalence of tuberculosis was low in small ruminant which may have contributed to low prevalence in large ruminant. However, there is no such data available on paratuberculosis in small ruminants and large ruminants at the same farm.

The analysis of variance techniques revealed that the body weight of animals was significantly higher in positive than disease negative cattle. So, the results of the odds ratio and analysis of variance have indicated an association of breed, status of the animal (dry/non-lactating or lactating), presence of small ruminants and live body weight with the disease in cattle. The same analysis revealed an association of the status of the animal with the disease in buffaloes. Similar to our findings, a study from England reported a relatively higher prevalence of disease in older age animals than that of young ones (Woodbine *et al*., 2009), the same case was in the present study also where we found 0% prevalence in animals between 2-5 years of age, while 1.9% in cattle of more than 5 years of age, almost similar results were in buffaloes with relatively lower prevalence in younger than old buffaloes. In Irish dairy herds the sero-prevalence study showed that the disease was more prevalent in high producing animals (Hoogendam *et al*., 2009) which according to present study sound similar for cattle but not for buffaloes. The stress of high milk production might make the animal more vulnerable to the MAP or there may be some genetic association in high producing animals.

The tuberculin base study was also carried by Lilenbaum in 2010, which concluded that though the tuberculin test is a diagnostic tool (Lilenbaum, 2000) but it interferes with the reactions of other Mycobacterium so the problem of cross reactivity may be present (Olsen *et al*., 2001; Marassi *et al*., 2005). The cross reactivity might be the reason that 12 (37.5%) cases which were found positive by PPD were not found positive by ELISA or any other test carried out. This suggests that PPD can only be considered as a screening test, but the positive cases have to be confirmed by the use of another test, especially ELISA. The results of ELISA+PCR were found positive in 80% cases irrespective of the result of another test, while the ELISA+tuberculin in 65% and ELISA+ZN in 30% cases. These Results suggest that ELISA is a more suitable test for diagnosis, while PCR might have some limitations. There may be some hindering factors present in faecal matter for PCR and in cases of very low number of Mycobacterial in the faecal matter that may not be picked by pipetting during PCR. which may include skipping of Mycobacterium during. The results also indicated that ZN faecal in 3.1% cases were positive without having a positive PCR, which can be linked to hindering factors in faecal matter for the PCR or that the DNA purification needs to be improved. Kaur *et al*. (2011) reported that 55% of faecal samples collected from 153 paratuberculosis suspected animals in Indian Punjab based on clinical signs were found positive by PCR. ELISA as a diagnostic tool was used in most of the other studies on paratuberculosis (Singh *et al*., 2014). In addition to this, Ziehl Nielsen (ZN) staining of the faecal samples was also performed that is another important and confirmatory diagnostic tool for the identification of Mycobacterium. Interestingly, during present study all the ZN positive cases were also found positive by PCR or ELISA or both. Kaur *et al*. (2011) tested faecal specimens by ZN-staining and DNA was extracted by freeze and thaw method. The Ziehl-Neelsen (ZN) staining showed 71% positive results for acid fast bacilli, while 55% samples were detected by polymerase chain reaction. These findings confirm our results of PCR positive cases among ZN positive cases and that not all ZN positive cases can be found positive by PCR. It may be

due to the presence of Mycobacterium, other than Mycobacterium avium paratuberculosis or may be due to hindering factors present in the faecal samples or chance missing of Mycobacterium in the 4-6 micro-liter of the sample collected from faecal maters for PCR while ZN staining results are confirmed in larger sample spread over the slide or there could be some more unknown factors involved. Based on diagnostic tests, the overall results of the present study for PPD, ZN, and indirect ELISA were 25/818 (3.06%), 16/818 (1.96%), and 20/818 (2.45%), respectively. Also the culturing could not be done here. Variation on diagnostic results would be further interpreted if the culture results were available. There was no strong association of clinical signs with MAP as most animals those were found positive did not had consistent but had intermittent diarrhea.

Conclusions: the prevalence of paratuberculosis at animal level is 2.4% but 100% at farm/herd level. The tuberculin test can be used as a screening test, but the results are not reliable due to false positives results and the positive/suspected animals must further be confirmed by ELISA in resource poor settings.

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