Prevalence of paratuberculosis in water buffaloes on public livestock farms of Punjab, Pakistan

Aziz-ur-Rehman[1], M. Tariq Javed[1*], M. Sohaib Aslam[1], M. Nisar Khan[2], S. Misdaq Hussain[1], Khuram Ashfaque[3] and Asim Rafique[4]

[1] *Department of Pathology, Faculty of Veterinary Science, University of Agriculture Faisalabad, Pakistan.*
[2] *Department of Parasitology, Faculty of Veterinary Science, University of Agriculture Faisalabad, Pakistan.*
[3] *Department of Clinical Medicine and Surgery, University of Agriculture Faisalabad, Pakistan.*
[4] *Department of Poultry Production, Government of Punjab, Faisalabad, Pakistan.*

[*] *Corresponding author at: Department of Pathology, Faculty of Veterinary Science, University of Agriculture Faisalabad-38040, Pakistan. Tel.: +92 41 9200161-70/3120, e-mail: mtjaved@uaf.edu.pk.*

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Summary

The present study had the goal to assess the prevalence of paratuberculosis in 4 public livestock farms of the Punjab (Pakistan). It included 627 total animals of more than 2-year-old tested by Avian, Purified Protein Derivative (PPD). The results of the PPD test were confirmed by indirect ELISA and by Ziehl-Neelsen (ZN) test. An overall prevalence of 1.3% was recorded. The values were between 0.0% and 3.03%. The results of odds ratio suggested that there are 2.18 time's higher chances of infection when body weight of buffaloes is less than 500 kg; 1.65 times in dry animals; 2.58 times when small ruminants are present; and 1.19 times when cattle are absent. The total positive buffaloes observed by Avian PPD were 12, although only 10 were then confirmed by ELISA, and 8 by ZN faecal microscopy. The relative sensitivity and specificity of Avian PPD by considering ELISA as standard test, were 80.0% and 89.47%, respectively. Similarly, the relative sensitivity and specificity of the ZN faecal test were 70.0% and 97.37%, respectively. The relative sensitivity and specificity of Avian PPD by considering ZN faecal test as standard, were 100% and 90%, respectively. Among haematological parameters, platelets significantly decreased and MCH increased in paratuberculosis positive animals.

Prevalenza della paratubercolosi nei bufali indiani negli allevamenti di bestiame statali in Punjab, Pakistan

Parole chiave
Bufali,
ELISA,
Paratubercolosi,
PPD,
Prevalenza,
Punjab,
Tubercolina,
Ziehl-Neelsen.

Riassunto

Scopo di questo studio è stato valutare la diffusione della paratubercolosi in quattro allevamenti statali in Punjab (Pakistan). Con il test per la tubercolina aviare (PPD) sono stati analizzati 627 animali con più di due anni di età. I risultati sono stati poi validati con ELISA indiretta e con il test di Ziehl-Neelsen (ZN). Complessivamente è stata riscontrata una prevalenza dell'1,3% ma il valore subisce variazioni significative a seconda dell'allevamento di provenienza ed è compreso tra 0,0 e 3,03%. I risultati della *odds ratio* hanno suggerito che la probabilità di contrarre l'infezione è di 2,18 volte maggiore quando il peso corporeo dei bufali è inferiore a 500 kg; 1,65 volte in animali all'asciutta, 2,58 volte quando sono presenti piccoli ruminanti, 1,19 volte quando non ci sono bovini. Complessivamente sono risultati positivi 12 bufali, di cui 10 confermati con l'ELISA e 8 con il test di Ziehl-Neelsen (ZN). Considerando l'ELISA come "gold standard", la sensibilità e la specificità relativa della tubercolina aviare PPD sono risultate essere rispettivamente dell'80,0% e dell'89,47%; quelle del test ZN, analogamente del 70,0% e del 97,37%. Considerando invece come standard il test ZN, la sensibilità e specificità relativa della tubercolina aviare PPD sono state rispettivamente del 100% e del 90%. Per quanto riguarda i parametri ematologici, negli animali positivi alla paratubercolosi diminuivano significativamente le piastrine mentre aumentava l'MCH.

Introduction

Paratuberculosis is a chronic and progressive infection which leads to debilitating condition and affects a wide range of hosts, mostly ruminants (Hermon-Taylor *et al.* 2004). It is caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which has also been found in Crohn's disease patients in humans (Sechi *et al.* 2001). Although in developed countries control strategies have reduced its prevalence, paratuberculosis is still widely spread (Franken 2005, Kennedy 2007, Kobayashi *et al.* 2007, Kulkas 2007, Nielsen *et al.* 2007). In recent years, dairy farming has gained a lot of attention in Pakistan, which is now number 4th in the world in total milk production. Paratuberculosis can then represent a severe threat to this industry and, considering its zoonotic potential, to the public health.

Economic losses caused by MAP to the farmers (Ott *et al.* 1999), its probable role in Crohn's disease in humans, and the fact that this organism can be present in milk and milk products and can also resist cheese processing and pasteurization, provide compelling reasons to strengthen control measures. Animals mostly get infected before 6 months of age, but clinical manifestations show almost after 2 years of age. The causative agent of the disease is horizontally transmitted in young ruminants through contaminated food and water (Patterson *et al.* 1968). In dairy herds of Spain, MAP prevalence has been reported to be 4.03% (Diéguez *et al.* 2007), while in India its presence was found to be 2.71% (Singh *et al.* 2004). In Lahore Slaughterhouse, Pakistan, reports on paratuberculosis indicate a prevalence of 14% and 12% in goats and sheep, respectively (Chaudhary *et al.* 2009). A study on breeding bulls at the semen production unit reported a prevalence ranging from 16.6% to 24.6% (Abbas *et al.* 2011). Another study from Pakistan reported a prevalence of 10.63% in sheep (Sikandar *et al.* 2013). These data suggest that MAP infection is present in different animal species in the country. The present study aims to investigate the prevalence of paratuberculosis in buffaloes at 4 government livestock experimental stations by using Avian purified protein derivative (PPD) and the most suitable methods to assess MAP prevalence in buffaloes.

Materials and methods

The study included 627 buffaloes distributed in 4 government livestock experimental stations, 174 at farm 1, 201 at farm 2, 87 at farm 3, and 165 at farm 4. At the time of selection no animal showed clinical signs of the disease. All animals having more than 2 years of age were screened by delayed hypersensitivity test using an intradermal injection of Avian PPD on the left side of the neck. The reactions were read 72 hours after injection. The interpretation was made according to the diameter of swelling at the injection site and increase in thickness of skin folds. Animals were considered as positive, doubtful or negative according to the method described by Aagaard and colleagues (Aagaard *et al.* 2003). For the test comparison, about 10-15 g of the faecal sample were collected from all the tuberculin positive animals (n = 12) and 36 randomly selected tuberculin negative animals. The samples were taken directly from rectum using plastic gloves. They were sealed in a plastic container and were numbered with the animal ID number. Samples were then transported to the lab and used for Ziehl-Neelsen (ZN) microscopy.

From these animals blood samples were also taken. Five ml of blood were collected in a glass test tube having 0.5 ml of 1% EDTA from the jugular vein for haematology. Similarly, 5 ml of blood were collected in another test tube without anticoagulant for serology. Collected sera were stored at - 40°C before being tested for the presence of MAP antibodies by using a commercial indirect ELISA (Lsivet Ruminant Serum Paratuberculosis Advanced Catalogue No. VETPTRS2). Information on sex, age, body weight, breed, milk production, status of the animal (dry, pregnant, lactating), the stage of lactation, recent culling or purchase, management of calves, feeding, housing, total number of animals at the farm, other animals at the farm, and their number, was recorded. The data were analysed by using Winpepi software version 11.43. For each datum the 95% confidence interval was calculated. The Fisher chi-square test was also applied to the epidemiological data. The odds ratio was defined for comparing 2 groups. The data on diagnostic tests, including ELISA, tuberculin, and ZN microscopy were analysed using screening and diagnostic test command in Winpepi software. When the performance of ELISA, tuberculin and ZN microscopy tests were compared, ELISA was considered as standard test. When the performance of the ZN faecal and tuberculin tests were compared, the ZN faecal test was used as standard. The diagnostic techniques mentioned in the OIE Manual in chapter 2.1.11 on paratuberculosis were used as a guide to compare these tests (OIE 2014). Data on haematological parameters were analysed by analysis of variance; while means were compared with a Bonferroni-T test by using SAS statistical software (SAS 2007).

Results

An overall prevalence of 1.3% (8/627) was found in buffaloes at 4 public livestock farms of Punjab. The highest prevalence (3.03%) was found in farm 1, while the lowest (0.0%) was in farm 3 (Table 1).

Table I. *Prevalence (%) of paratuberculosis in buffaloes at four Public Livestock Farms of Punjab on the basis of positive results both by Avian PPD (Purified Protein Derivative) test + ELISA.*

Parameter	Negative	Positive (%)	95% CI	Odds Ratio
Farms				
1	160	5 (3.03)	1.12-6.59	NS
2	173	1 (0.57)	0.03-2.8	
3	201	0 (0)	0.00-1.48	
4	85	2 (2.29)	0.39-7.39	
Body weight				
300-500	268	5 (1.83)	0.67-4.01	OR = 2.18
> 500	351	3 (0.85)	0.22-2.29	
Age				
< 4	2	0 (0)	0.00-0.77	NS
4-8	370	4 (1.1)	0.34-2.56	
> 8	247	4 (1.59)	0.51-3.80	
Lactation length				
0	5	0 (0)	0.00-0.45	NS
1-200	86	1 (1.14)	0.06-5.54	
201-300	513	7 (1.34)	0.59-2.64	
> 300	15	0 (0)	0.00-0.18	
Milk yield				
0	5	0 (0)	0.00-0.45	NS
1-4.9	194	4 (2.02)	0.65-4.8	
5-9.9	417	4 (0.95)	0.30-2.28	
10-15	3	0 (0)	0.00-0.63	
Lactation number				
< 5	346	6 (1.70)	0.69-3.51	NS
5-10	229	2 (0.86)	0.15-2.83	
> 10	44	0 (0)	0.00-0.60	
Status				
Dry	311	5 (1.58)	0.58-3.47	OR = 1.65
Lactating	308	3 (0.96)	0.25-2.60	
Total buffalo				
< 100	85	2 (2.29)	0.39-7.39	NS
100-200	333	6 (1.8)	0.72-3.64	
> 200	201	0 (0)	0.00-1.48	
Total sheep				
0	286	2 (0.70)	0.12-2.28	NS
1-500	160	5 (3.03)	1.12-6.59	
> 500	173	1 (0.6)	0.03-2.80	
Total cattle				
0	361	5 (1.36)	0.50-3.00	NS
1-100	85	2 (2.35)	0.39-7.39	
> 100	173	1 (0.6)	0.03-2.80	
Total animals				
< 500	286	2 (0.70)	0.12-2.28	NS
500-1,000	160	5 (3.03)	1.12-6.59	
> 1,000	173	1 (0.6)	0.03-2.80	
Small ruminants				
Absent	286	2 (0.70)	0.12-2.28	OR = 2.58
Present	333	6 (1.8)	0.72-3.64	
Other animals				
Absent	201	0 (0.5)	0.00-1.48	
Present	418	8 (1.87)	0.88-3.53	
Cattle				
Absent	361	5 (1.36)	0.50-3.00	OR = 1.19
Present	258	3 (1.14)	0.29-3.10	

NS = non-significant; OR = odds ratio.

No significant differences were found between the prevalence rates observed among farms and similarly none of the variables considered in this study significantly influences the MAP prevalence.

The total positive buffaloes observed by Avian PPD were 12, by ELISA were 10, and by ZN faecal microscopy were 8. The results of Avian PPD, while considering ELISA as standard test revealed a relative sensitivity of 80.0% and a relative specificity of 89.47% (Table 2). Similarly, the relative sensitivity and specificity of the ZN faecal test were 70.0% and

Table II. *Sensitivity of Avian PPD (Purified Protein Derivative) test, Ziehl-Neelsen (ZN) faecal test based on ELISA as standard test, and of Avian PPD based on ZN faecal test as standard test in buffaloes at the Four Public Livestock Farms of Punjab.*

Paratuberculosis	ELISA		95% CI	Statistics
	Negative	Positive (%)		
Avian PPD				
Negative	34	2 (5.56)	0.94-17.16	Sensitivity = 80.0%
Positive	4	8 (66.67)	37.69-88.39	Specificity = 89.47%
ZN faecal				
Negative	37	3 (7.5)	1.94-19.07	Sensitivity = 70.0%
Positive	1	7 (87.50)	51.97-99.37	Specificity = 97.37%
Avian PPD	**ZN faecal**			
Negative	36	0 (0.00)	0.00-7.98	Sensitivity = 100%
Positive	4	8 (66.67)	18.41-81.59	Specificity = 90%

Table III. *Comparison of haematological parameters in Avian PPD (Protein Purified Deriviative) positive and negative reactor buffaloes at four Livestock Experiment Station of Punjab Pakistan.*

Parameter	PPD positive animals Mean ± SD	PPD negative animals Mean ± SD
RBC 10^{12}/l	7.976 ± 2.766	6.376 ± 0.285
PCV%	46.500 ± 18.185	24.733 ± 3.271
Haemoglobin g/dl	18.050 ± 5.8585	13.336 ± 0.317
WBC 10^9/l	9.600 ± 2.657	7.366 ± 1.171
MCH pg	20.366 ± 0.592 A	18.033 ± 2.003 B
Platelet 10^9/l	144.500 ± 47.094 B	356.666 ± 70.945 A
Differential Leukocyte Count		
Lymphocyte 10^9/l	6.666 ± 2.344	4.633 ± 1.401
Lymphocyte %	59.350 ± 21.756	56.800 ± 1.473
Granulocyte 10^9/l	2.083 ± 2.435	1.333 ± 0.251
Granulocyte %	19.266 ± 15.450	15.200 ± 0.264

97.37%, respectively, considering ELISA as standard test. When the performances of ZN faecal and avian PPD tests were compared, the relative sensitivity and specificity of Avian PPD were 100% and 90%, respectively.

Apart from platelet and mean corpuscular haemoglobin, non-significant differences were observed in the the haematological parameters (Table 3). In paratuberculosis positive animals, the platelet significantly decreased while MCH increased.

Discussion

The present study revealed a prevalence of 1.3% on 4 public livestock farms on the basis of tuberculin testing. Studies on MPA are only sporadic in Pakistan, a previous study focusing on slaughterhouse reported a prevalence of 6.67% in cattle and 12.5% in buffaloes (Sikandar *et al.* 2012). The prevalence rate differences found at farm level and in slaughtered animals were expected, as, most of the time, non-productive or low productive animals are taken to a slaughterhouse and culled. The infection may cause chronic diarrhoea which is difficult to treat, and affects the production of animals, thus the animals are culled (Ott *et al.* 1999). A study from Iran reported a prevalence between 4.2% and 7.7% (Razieh *et al.* 2012) while another study from India reported a prevalence of 15.14% in cattle (Gupta *et al.* 2012). To the best of our knowledge, this is the first report on paratuberculosis in buffaloes on public livestock farms in Punjab. It indicates the presence of paratuberculosis in the area.

The results of the present study suggests that the MPA prevalence might depend on the number of animals present at the farm. In this study, it was higher in farms with more than 500 animals. Another study reported a herd prevalence of 28% of small herds, 53% of medium herds, and 100% of larger herds (Kruze *et al.* 2013). Similarly, lower prevalence in smaller herds (< 100 animals) and higher in larger herds (> 100 animals) has been reported (Woodbine *et al.* 2009). During the present study, the prevalence of disease increased from 0% in animals of less than 4 years to 2.69% in > 4 years of age. These results showed an increase in prevalence with age. A study from India reported an overall prevalence of 29% in bovines, with 13.6% in young, and 29.8% in adults (Singh *et al.* 2008). Another study reported an increase in sero-prevalence of paratuberculosis with the increase in age and reported a higher prevalence at the age between 2 and 3 years (Woodbine *et al.* 2009). However, the prevalence found in bovines in India was much higher than that observed in the present study.

This study also revealed a possible association between infection and presence of sheep or small ruminants at the farm: MPA prevalence was lower when small ruminant or sheep were absent and was instead higher when they were present, suggesting some role of small ruminants in maintenance and/or spread of infection in large ruminants or buffaloes. Similarly, another study reported a positive association of the prevalence of paratuberculosis with the co-grazing of sheep and cattle (Woodbine *et al.* 2009). Nonetheless, a study on the same farms revealed a protective effect of the presence of sheep on the occurrence of bovine tuberculosis (Javed *et al.* 2011, Javed *et al.* 2012). Further studies are required to clarify the role of small ruminants in spreading MPA infection. However this study seems to confirm that keeping multiple species of animals on the farm is a risk factor for both, tuberculosis and paratuberculosis. According to these results, it seems to have more chance of finding MPA infection in animals with lower body weight (OR = 2.18). It is known that the disease causes a decrease in the body weight due to consistent chronic diarrhoea (Kudahl and Nielsen 2009).

When the three tests were compared, results were discordant. If, on the one hand, all animals positive to PPD were not detected either by ELISA or ZN test, on the other hand, some of the animals which were negative to PPD resulted positive to ELISA. These data suggest that tuberculin test may be considered as a screening test along with ZN faecal test in resource-poor countries. ELISA, instead, can be used as a routine test for screening animals for prevalence studies on paratuberculosis (Singh *et al.* 2014). Discordant results were also observed in a study by Singh and colleagues where PPD positive animals were not confirmed by ELISA and similarly, in a Weber and colleagues survey, results obtained by ELISA and ZN were not fully concordant (Weber *et al.* 2009).

The results of haematological parameters revealed no effect of disease on almost all the parameters, with the only exception of the platelet counts and mean corpuscular haemoglobin. These results indicate a decrease in the platelet count, and an increase in MCH. Another study reported a significant decrease in the haematological parameters including RBC count, WBC count, PCV, MCHC, Hb, and an increase in monocyte count and platelet in sub-clinically infected camel (Salem *et al.* 2012). However, another study performed on cattle reported that haematocrit, haemoglobin, and erythrocyte values were within normal ranges in paratuberculosis positive cows, but lower than in control cows (Senturk *et al.* 2009). They also found lower platelet count in infected animals. These changes were linked to decreased serum iron and iron binding capacity. They further reported that as the disease progresses the values of the haematological parameters may fall below the reference range. Thus, it can be concluded that the haematological changes are likely species-dependent and can vary at different stage of disease. However, more studies are required in buffaloes to have a better idea of the effects of paratuberculosis on these parameters.

It can be concluded from the present study that tuberculin testing can be used in conjunction ZN faecal test for screening of animals for paratuberculosis in resource-poor countries. The prevalence of paratuberculosis was not high in the farms included in this study, as it was less than 1.3%.

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