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Original Article

Serological, cultural, and molecular evidence of *Brucella* infection in small ruminants in Pakistan

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Abstract

Introduction: The objectives of the present study were to determine the seroprevalence and identify the causative agent of brucellosis in small ruminants in Pakistan.

Methodology: A total of 278 serum and 212 milk samples were collected from sheep and goats that had close contact with seropositive bovine herds. Data related to age, sex, location, and breed were collected on the sampling day. Serum and milk samples were initially screened using two different Rose Bengal plate test (RBPT) antigens and a milk ring test (MRT). Seropositive samples were subjected to bacterial isolation and PCR analysis using *Brucella* genus-specific (bcsp31) and *Brucella* species-specific (IS711 for *Brucella abortus* and *Brucella melitensis*) quantitative real-time polymerase chain reactions (qRT-PCR).

Results: Twenty-four (8.6%) serum samples were positive by RBPT. Twenty (9.4%) animals were positive for *Brucella* antibodies using MRT. No *Brucella* isolates were obtained from the examined blood and milk samples. Of the 24 seropositive serum samples, 18 (75%) were positive in the *Brucella* genus-specific (bcsp31) and *Brucella abortus*-specific (IS711) qRT-PCR, respectively.

Conclusions: *Brucella abortus* was identified as causative agent of ovine and caprine brucellosis in Pakistan. Results of this study can be used for the development of an effective control and eradication strategy for brucellosis in livestock, especially small ruminants.

Key words: brucellosis; sheep; goat; seroprevalence; PCR; Pakistan.

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Introduction

Brucellosis is an important zoonotic disease that affects a wide range of animal species as well as humans [1,2]. The causative agent of brucellosis in goats and sheep is *Brucella melitensis*, while *B. abortus*, *B. ovis*, and *B. suis* may cause infections only under certain condition [3-5]. The major clinical manifestations of brucellosis in sheep and goats are abortion during the last two months of pregnancy, arthritis, fetal membrane retention, weak offspring, orchitis, epididymitis, and sterility [6].

In Pakistan's neighboring countries (Afghanistan, China, India, and Iran), there is evidence of brucellosis in animals and humans [7]. Pakistan is an agriculture-based country, and livestock plays a crucial role in people's livelihood, especially people living in rural areas. Among the different livestock species in Pakistan are 28.1 million sheep and 61.5 million goats [8]. *B. abortus* biotype 1 is known to be the causative agent of brucellosis in cattle and buffaloes [2]. Quantitative real-time polymerase chain reaction (qRT-PCR) assays have confirmed *B. abortus* as the causative agent of human brucellosis in high-risk occupations in Pakistan [1]. However, limited information based only on serological studies is available on brucellosis in small ruminants (*i.e.*, sheep and goats) in Pakistan. In the Punjab region of Pakistan, 31 (1.46%) sheep and 29 (1.93%) goats were positive for *Brucella* antibodies [9].

In order to develop effective control and eradication programs, it is essential to establish the causative agent of ovine and caprine brucellosis in Pakistan. Polymerase chain reaction is an appropriate and rapid method for the correct diagnosis of brucellosis instead of the tedious cultivation of the agent [10,11]. Due to the economic importance of brucellosis, the objectives of the present study were to

determine the seroprevalence and isolate and identify the agent using qRT-PCR assays.

Methodology

Origin of animals and sampling sites

The study area (the Potohar Plateau, including Rawat, Islamabad, and Kherimurat) was chosen because *Brucella abortus* was recently identified there to be the causative agent of bovine and human brucellosis. Sheep and goats that had close contact with seropositive bovine herds (*i.e.*, shared the same housing and grazing area) were selected for sampling. Twelve sheep and goat herds (four from Rawat, two from Islamabad, and six from Kherimurat) were selected. Herds with sheep (n = 2), goats (n = 3), and with both sheep and goats (n = 7) were investigated. Samples were collected from every adult animal in each herd. Herds in Rawat and Kherimurat were large (> 20 animals). A total of 278 (66 male and 212 female) animals were selected for blood collection (118 sheep and 160 goats).

Milk samples (n = 212) were collected only from females that were already selected for blood sampling. On the sampling day, data related to farm location, sex, species, and breed were also collected.

Blood and milk collection and serology

Milk and blood samples were collected, stored, and processed according to standard procedures [12]. Milk samples were collected from both quarters. Serum samples were initially screened with two Rose Bengal plate test (RBPT) antigens: the antigen of the Veterinary Research Institute (VRI), Lahore, Pakistan, and the antigen of IDEXX, Pourquier, France. Briefly, 30 µL of serum was mixed with an equal volume of antigen preparation on a glass plate; the plate was agitated gently for 4 minutes. A serum sample was considered positive if agglutination occurred. The milk ring test (MRT) antigen used was purchased by VRI Lahore, Pakistan. In brief, MRT antigen was brought to room temperature before use. One milliliter of milk was added to a test tube. Then, 30 to 40 µL of antigen was added, and the sample was mixed and incubated at 37°C for 1 hour. A sample that had blue colouration at the surface was considered to be positive. Then, serum samples were shipped on dry ice to the Friedrich-Loeffler-Institute (FLI), Germany, for molecular analysis.

Bacteriology

Culturing of brucellae from seropositive blood (n = 24) and milk samples (n = 20) was done at the bacteriology section, National Veterinary Laboratory, Islamabad, Pakistan, on modified Farrell's serum dextrose agar according to standard procedures [12,13]. Modified Farrell's serum dextrose agar with 5% horse serum, 1% dextrose, and the following antibiotics (added to 1 L medium): cycloheximide (100 mg), bacitracin (25,000 IU), polymyxin B sulfate (5,000 IU), vancomycin (20 mg), nalidixic acid (5 mg), and nystatin (100,000 IU), was used for primary isolation of brucellae. Plates were inoculated with blood and milk and incubated aerobically and in the presence of 5%–10% carbon dioxide at 37°C. The plates were checked for up to 10 days for the presence of bacterial growth.

DNA extraction from serum samples

DNA was extracted from all seropositive serum samples using high Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Purity and concentration of DNA was tested using a Nano-Drop ND-1000 UV-Vis spectrophotometer (Nano-

Table 1. Primers for *Brucella* genus and species-specific qRT-PCR

qRT-PCR	Primer	Sequence (5' to 3')	Target
Brucella genus	Forward	GCTCGGTTGCCAATATCAATGC	bcsp31
	Reverse	GGGTAAAGCGTCGCCAGAAG	
B. abortus	Forward	GCGGCTTTTCTATCACGGTATTC	IS711
	Reverse	CATGCGCTATGATCTGGTTACG	
B. melitensis	Forward	AACAAGCGGCACCCCTAAAA	IS711
	Reverse	CATGCGCTATGATCTGGTTACG	

Table 2. Probes for *Brucella* genus and species-specific qRT-PCR

qRT-PCR Type	Sequence (5' to 3')
Brucella genus	6FAMAAATCTTCCACCTTGCCCTTGCCATCABHQ1
B. abortus	6FAMCGCTCATGCTCGCCAGACTTCAATGBHQ1
B. melitensis	6FAMCAGGAGTGTTTCGGCTCAGAATAATCCACABHQ1

Drop Technologies, Wilmington, DE), and DNA samples were stored at -20°C.

Molecular detection by qRT-PCR

All DNA samples were analyzed by *Brucella* genus-specific (bcsp31) and species-specific real-time PCR assays for *B. abortus* and *B. melitensis* [14]. The detailed procedure used has been described previously [11]. The details of primers and probes are given in Tables 1 and 2 [14]. Amplification was done in 25 µL of total volume. Nuclease-free water, DNA of *E. coli* (DSM 30083, ATCC 11775), and DNA of *Brucella* reference strains (BA 544, BM 16M) were used as negative control (NC), no template positive control (NPC), and positive control, respectively. The amplification reactions were done in duplicate in optical 96-well microplates (Thermo Fisher Scientific Inc., Waltham, USA) using a Mx3000P thermocycler system (Agilent Technologies, Santa Clara, USA). Cycling conditions were as follows: one cycle for decontamination at 50°C for 2 minutes, initial denaturation at 95°C for 10 minutes, followed by 50 cycles at 95°C for denaturation for 25 seconds, and 1 minute for annealing at 57°C. No internal

Table 3. Comparative results of serological tests for samples of sheep and goat sera and milk

Variables	Factors	Samples examined	Positive for		
			RBPT (IDEXX-Pourquier) Positive (%)	RBPT (VRI) Positive (%)	MRT (VRI) Positive (%)
Sex (n = 278)					
	Male	66	2 (3.03)	1 (1.5)	0 (0.0)
	Female	212	22 (10.4)	22 (10.4)	20 (9.4)
Region					
	Rawat	122	12 (9.8)	12 (9.8)	10 (8.2)
	Islamabad	50	2 (4)	1 (2)	0 (0.0)
	Kherimurat	106	10 (9.4)	10 (9.4)	10 (9.4)
Species					
	Sheep	118	3 (2.5)	3 (2.5)	1 (0.85)
	Goat	160	21 (13.1)	20 (12.5)	19 (11.9)
Breed					
	Salt Range	84	3 (3.6)	3 (3.6)	1 (1.2)
	Afghani	34	0 (0.0)	0 (0.0)	0 (0.0)
	Beetal	55	10 (18.2)	9 (16.4)	9 (16.4)
	Local hairy	105	11 (10.5)	11 (10.5)	10 (9.5)

RBPT: rose Bengal plate test; MRT: milk ring test; VRI: veterinary research institute, Pakistan

Table 4. Comparative results of bacteriology and molecular methods for samples of sheep and goats

		Blood culture (n = 24)	Milk culture (n = 20)	qRT-PCR (n = 24)		
				Bcsp31	*B. abortus*	*B. melitensis*
Variables	Factors	Positive (%)	Positive (%)	Positive (%)	Positive (%)	Positive (%)
Sex						
	Male	0 (0.0)	0 (0.0)	2 (100)	2 (100)	0 (0.0)
	Female	0 (0.0)	0 (0.0)	16 (72.7)	16 (72.7)	0 (0.0)
Region						
	Rawat	0 (0.0)	0 (0.0)	11 (91.7)	11 (91.7)	0 (0.0)
	Islamabad	0 (0.0)	0 (0.0)	1 (50)	1 (50)	0 (0.0)
	Kherimurat	0 (0.0)	0 (0.0)	6 (60)	6 (60)	0 (0.0)
Species						
	Sheep	0 (0.0)	0 (0.0)	1 (33.3)	1 (33.3)	0 (0.0)
	Goat	0 (0.0)	0 (0.0)	17 (81)	17 (81)	0 (0.0)
Breed						
	Salt Range	0 (0.0)	0 (0.0)	1 (33.3)	1 (33.3)	0 (0.0)
	Afghani	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Beetal	0 (0.0)	0 (0.0)	8 (80)	8 (80)	0 (0.0)
	Local hairy	0 (0.0)	0 (0.0)	9 (81.8)	9 (81.8)	0 (0.0)

%: percentage of positive samples

amplification control (IAC) was used in the procedure to ensure a high sensitivity. For the same reason, the originally described multiplex qRT-PCR [14] was performed in three separate reactions. Samples with cycle threshold (Ct) values of ≤ 40 were considered positive for *Brucella* genus-specific qRT-PCR and *Brucella* species-specific qRT-PCR. Visual confirmation of positive samples was recorded from graphical representation of cycle numbers versus fluorescence values.

Results

A total of 24 (8.6%) and 21 (7.6%) serum samples were found to be positive for *Brucella* antibodies using IDEXX and VRI RBPT antigens, respectively. Twenty (9.4%) animals were positive for *Brucella* antibodies using MRT (Table 3). Based on parallel interpretation of RBPT antigens, female animals (10.4%) were found to be more often seropositive than were males (3.03%). Moreover, 7 (58.3%) of 12 herds were positive for *Brucella* infection.

Animals kept in Rawat region were more often seropositive than animals kept in Islamabad and Kherimurat. The seroprevalence of *Brucella* antibodies was higher in goats than in sheep. Of the two breeds of sheep investigated, only 3 (3.6%) Salt Range were found to be seropositive. In goats, the prevalence was slightly higher in Beetal and local hairy goats. No isolate of *Brucella* was recovered from blood and milk samples of sheep and goats. Of the 24 seropositive samples, 18 (75%) were positive in the *Brucella* genus-specific (bcsp31) qRT-PCR. The serum samples positive with the *Brucella* genus-specific (bcsp31) qRT-PCR were also positive with the *Brucella abortus*-specific (IS711) qRT-PCR (Table 4). None of the serum samples was positive for *Brucella melitensis* DNA. Both male and female animals were found positive for brucellosis in qRT-PCR.

Discussion

Brucellosis is an infectious disease found in a wide range of animal species; is also transmitted to humans via secretions and excretions of infected animals. For the development and onset of control and eradication programs, knowledge of the prevalent species and biotypes is important in order to understand the chains of infection. This study provides first evidence that *Brucella abortus* is the causative agent of brucellosis in small ruminants in Pakistan.

A total of 24 (8.6%) serum samples were found to be positive for *Brucella* antibodies in the present study. These data are in contrast to a previous study conducted in the Punjab region of Pakistan, when a seroprevalence of only 1.6% was reported in small ruminants [9]. A comparable high prevalence (11.2%) was found in livestock research stations of the Punjab region of Pakistan [15].

A reason for the higher seropositivity of animals in this study is that these small ruminants had close contact with seropositive herds of cattle and buffalo, a possible source of *Brucella* spp. It is also common practice that livestock farmers do not cull and dispose of brucellosis-positive animals, but rather sell them to other farmers, thereby enabling infected animals to enter *Brucella*-free herds and transmit infection to other animals. Thus, prevalence of brucellosis is increasing day by day in Pakistan.

In this study, the seroprevalence was higher in goats than in sheep. These results are in line with previous studies in Pakistan [9,15]. In previous studies conducted in various countries (Bangladesh, Kosovo, Sudan, Nigeria), the same trend was noticed [16-19]. Higher seroprevalence in sheep was only reported from Egypt and Tajikistan [20,21]. This variation might be due to the differences in countries' local herd management systems.

The seroprevalence was higher in female animals than in male animals. A higher seroprevalence in female sheep and goats has been reported from the district of Peshawar, Pakistan [22], whereas higher seropositivity in male sheep and goats has been reported in goats in Bangladesh and Mexico [23,24]. In the present study, the number of male animals was lower than the number of female animals because female animals were served by positive rams of the same herd or were of older age, increasing the risk of getting infected.

The seroprevalence in sheep and goats of the three regions investigated varied greatly. The highest seroprevalence was reported from Rawat (9.8%), followed by Kherimurat (9.4%) and Islamabad (4%). One of the possible reasons for the high prevalence in Rawat and Kherimurat is that serum samples were collected from large herds. It is a well-known fact that larger herds have a higher probability of contacting infected animals. Herd prevalence is associated considerably with herd size [25]. Variation in seropositivity was also seen in goats in Mexico [23].

In the present study, two breeds of goats (Beetal and local hairy) were investigated; the prevalence was different in both breeds. Differences in goat breeds were also reported from Mexico [23]. However, it was surprising to find that only one breed of sheep was infected (Salt Range). Brucellosis-negative sheep of

the Afghani breed also shared the same grazing area and housing with seropositive Salt Range sheep. The most likely reason for this finding is that all of these animals were males and were reared to be sold to farmers for breeding.

No isolates of *Brucella* were recovered from milk and blood samples of sheep and goats in the present study using Farrell's modified serum dextrose agar. This agar has been used successfully to isolate *Brucella abortus* biotype 1 from milk samples of sheep from Nigeria [26,27]. The possible reason for failure of culture is that the animals were infected chronically and so no living bacteria were circulating any longer.

Molecular detection of *Brucella* genus DNA based on qRT-PCR (bcsp31) from serum samples confirmed the presence of *Brucella* in these sheep and goat serum samples. Bcsp31 qRT-PCR and other PCR assays have been used by various authors in different countries to amplify *Brucella* DNA from blood, milk, and serum [28-30], and PCR is a validated technique in the diagnosis of brucellosis.

Although species-specific PCR assays have a lower analytical sensitivity than do genus-specific PCRs, *B. abortus* was identified as the causative agent of brucellosis in sheep and goats. Interestingly, no evidence of *B. melitensis* infection was found. *B. abortus* was confirmed as the causative agent of ovine and caprine brucellosis in previous studies using PCR assays [31] and of sheep brucellosis using bacteriology [32-34].

Conclusions

B. abortus has recently been identified as the causative agent of bovine and human brucellosis in the Potohar Plateau of Pakistan. No evidence for *B. melitensis* was found in this study. A possible reason for this is that the present study focused on small ruminants that had close contact with cattle and buffalo herds infected with *B. abortus*. Moreover, only a small number of samples was collected for this study. In future studies, after involving large numbers of samples and new study areas, *B. melitensis* may also be added to the list of prevalent *Brucella* species in small ruminants of Pakistan. We have demonstrated that there is an immense need to develop a control and eradication program that includes vaccination, screening, and culling of brucellosis-positive cattle, sheep, and goats. Further studies will also have to include camels.

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References

1. Ali S, Ali Q, Neubauer H, Melzer F, Elschner M, Khan I, Abatih E N, Ullah N, Irfan M, Akhter S (2013) Seroprevalence and Risk Factors Associated with Brucellosis as a Professional Hazard in Pakistan. Foodborne Pathog Dis 10: 500-505.
2. Ali S, Ali Q, Melzer F, Khan I, Akhter S, Neubauer H, Jamal S M (2014) Isolation and identification of bovine *Brucella* isolates from Pakistan by biochemical tests and PCR. Trop Anim Health Prod 46: 73-78.
3. Paolicchi FA, Terzolo HR, Campero CM (1993) Isolation of *Brucella suis* from the semen of a ram. Vet Rec 132: 67-67.
4. Radostits O M, CC Gay, DC Blood, Hinchcliff KW (2000) Veterinary Medicine, 9th edition. London: ELBS Bailliere Tindall. p. 2162.
5. Moshkelani S, Javaheri-Koupaei M, Fathpour H, Alirezaei M (2011) Detection of *Brucella melitensis* from aborted Caprine fetuses in Iran. Global Vet 6: 495-497.
6. European Commission (2001) Brucellosis in sheep and goats (*Brucella melitensis*). Report of the Scientific Committee on Animal Health and Animal Welfare. SANCO.C.2/AH/R23/2001. 20 p.
7. Ali S, Ali Q, Nji Iabatih E, Ullah N, Muhammad A, Khan I, Akhter S (2013) Sero-prevalence of *Brucella abortus* Among Dairy Cattle and Buffaloes in Pothohar Plateau, Pakistan. Pak J Zool 45: 1041-1046.
8. Government of Pakistan, Ministry of Finance (2012) Pakistan Economic Survey 2011–12. Available: http://www.finance.gov.pk/survey_1112.html. Accessed 12 Junuary 2015.
9. Nasir AA, Shah MA, Rashid M (2000) Prevalence of antibodies of *Brucella* in sheep and goats of Punjab region. Pak J Biol Sci 3: 1943.
10. Baily G, Krahn J, Drasar B, Stoker N (1992) Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. J Trop Med Hyg 95: 271-275.
11. Gwida MM, El-Gohary AH, Melzer F, Tomaso H, Rosler U, Wernery U, Wernery R, Elschner MC, Khan I, Eickhoff M (2011) Comparison of diagnostic tests for the detection of *Brucella* spp. in camel sera. BMC Res Notes 4: 525.
12. Alton GG, Jones LM, Angus RD, Verger JM (1988) Techniques for the brucellosis laboratory. Paris: INRA. p. 191.
13. Farrell I, Robertson L (1972) A comparison of various selective media, including a new selective medium for the isolation of brucellae from milk. J Appl Bacteriol 35: 625-630.
14. Probert WS, Schrader KN, Khuong NY, Bystrom SL, Graves MH (2004) Real-time multiplex PCR assay for detection of *Brucella* spp., *B. abortus*, and *B. melitensis*. J Clin Microbiol 42: 1290-1293.
15. Qureshi M, Masood S (1988) Latest trend of brucellosis in livestock at Livestock Experiment Stations in the Punjab. Pak J Vet Res 1: 39-44.
16. Brisibe F, Nawathe D, Bot C (1996) Sheep and goat brucellosis in Borno and Yobe States of arid northeastern Nigeria. Small Ruminant Res 20: 83-88.

17. Jackson R, Pite L, Kennard R, Ward D, Stack J, Domi X, Rami A, Dedushaj I (2004) Survey of the seroprevalence of brucellosis in ruminants in Kosovo. Vet Rec 154: 747-751.
18. Omer MM, Abdelaziz AA, Abusalab SM, Ahmed AM (2007) Survey of brucellosis among sheep, goats, camels and cattle in Kassala area, Eastern Sudan. J Anim Vet Adv 6: 635-637.
19. Rahman M, Faruk M, Her M, Kim J, Kang S, Jung S (2011) Prevalence of brucellosis in ruminants in Bangladesh. Vet Med-Czech 56: 379-385.
20. Hamdy M, Amin A (2002) Detection of *Brucella* species in the milk of infected cattle, sheep, goats and camels by PCR. The Vet J 163: 299-305.
21. Jackson R, Ward D, Kennard R, Amirbekov M, Stack J, Amanfu W, El-Idrissi A, Otto H (2007) Survey of the seroprevalence of brucellosis in ruminants in Tajikistan. Vet Rec 161: 476-82.
22. Ghani M, Siraj M, Zeb A, Naeem M (1995) Sero-Epidemiological study of Brucellosis among Goats and Sheep at Peshawar district. Asian-Aust J Anim Sci 8: 489-494.
23. Solorio-Rivera J, Segura-Correa J, Sanchez-Gil L (2007) Seroprevalence of and risk factors for brucellosis of goats in herds of Michoacan, Mexico. Prev Vet Med 82: 282-290.
24. Islam M, Samad M, Rahman A (2012) Risk factors associated with prevalence of brucellosis in black Bengal goats in Bangladesh. Bangladesh J Vet Med 8: 141-147.
25. Akakpo A, Bornarel P (1987) Epidemiology of animal brucellosis in tropical Africa: clinical, serological and bacteriological surveys. Res Sci Tech 6: 981-1027.
26. Ocholi R, Kwaga J, Ajogi I, Bale J (2004) Phenotypic characterization of *Brucella* strains isolated from livestock in Nigeria. Vet Microbiol 103: 47-53.
27. Ocholi RA, Kwaga JKP, Ajogi I, Bale JOO (2005) Abortion due to *Brucella abortus* in sheep in Nigeria. Rev Sci Tech Off Int Epiz 24: 973-979.
28. Al-Bayatti S, Al-Thwani A (2009) Evaluation of PCR, ELISA, and culture methods for the diagnosis of animal brucellosis. Diyala Agri Sci J 1: 1-17.
29. Gupta VK, Shivasharanappa N, Kumar V, Kumar A (2014) Diagnostic evaluation of serological assays and different gene based PCR for detection of *Brucella melitensis* in goat. Small Ruminant Res 117: 94-102.
30. Izadi A, Moslemi E, Tabatabaei Panah A, Kheiri Manjili H (2014) *Brucella* spp. detection in dairy products using Nested and Hemi Nested PCR techniques. Ann Biol Res 5: 124-131.
31. Dehkordi F S, Saberian S, Momtaz H (2013) Detection and Segregation of *Brucella abortus* and *Brucella melitensis* in Aborted Bovine, Ovine, Caprine, Buffaloes and Camelid Fetuses by Application of Conventional and Real-time Polymerase Chain Reaction. Thai J Vet Med 42: 13-20.
32. Bannatyne C (1960) *Brucella abortus* infection in a black face ewe. Vet Rec 72: 660-661.
33. Allsup TN (1969) Abortion in sheep associated with *Brucella abortus* infection. Vet Rec 84: 104-108.
34. Zowghi E, Ebadi A (1988) Abortion due to *Brucella abortus* in sheep in Iran. Rev Sci Tech Off Int Epiz 7: 379-382.

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