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Seroprevalence and Associated Risk Factors of Bovine Brucellosis in Cattle and Livestock Related Persons at District Kurram, Khyber Pakhtunkhwa, Pakistan

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AH conceived the idea and designed the experiment. MA and AA helped in the experimental work. FD and MS supervised, co-supervised, and guided the whole team and provided the research facilities. MS helped in data curation and reviewed the manuscript. All the authors read and approved the final manuscript.

Key words
***Bovine brucellosis, Brucella melitensis,* Public health, *Brucella abortus*, Seroprevalence, i- ELSIA, Rose Bengal plate test, Serum Plate Agglutination Test, AMOS-PCR**

ABSTRACT

Brucellosis is an infectious and highly contagious zoonotic disease caused by the Brucella species and is of great economic and public health importance. This study utilized serological and molecular techniques to investigate the status of brucellosis in cattle and highly occupational risk groups in the district Kurram, Khyber Pakhtunkhwa, Pakistan. For this purpose, three hundred eighty-four (two hundred eighty-four from cattle and one hundred from livestock-related persons) blood samples were screened by rose bengal plate test (RBPT), serum plate agglutination test (SPAT), and indirect enzyme-linked immunosorbent assay (*i*-ELISA). A research proforma was used to evaluate the relationship between infection and different risk factors. Further, all positive samples were investigated for the presence of pathogens by AMOS-polymerase chain reaction (AMOS-PCR). The overall prevalence of brucellosis in cattle was 16.19% by RBPT, 18.30% by SPAT (*Brucella abortus*), 13.38% by SPAT (*B. melitensis*), and 5.9% by *i*-ELISA. In comparison, in livestock-related persons 15% by RBPT, 24% by SPAT, and 6% by *i*-ELISA were recorded. The presence of *B. abortus* DNA was confirmed by AMOS-PCR in 4 cattle and 02 human samples of serological positive samples. Among risk factors, grazing, breeding protocol, animals with repeat breeding and abortion history in animals while exposure to unpasteurized milk, contact with animal aborted material, contact with retained placenta, and related signs and symptoms in humans were significantly associated ($p<0.05$) with the seropositivity of brucellosis. Detection of brucellosis in cattle and livestock-related persons in the targeted district is alarming for the animals and public health. Infected animals can spread bovine brucellosis to other animals and humans. It is essential to implement ongoing surveillance and education programs in Pakistan to create effective control strategies.

INTRODUCTION

Brucellosis is caused by gram-negative, facultative, coco-bacilli, intracellular and non-motile bacterium. Brucellosis is synonymously called Bang's disease, Mediterranean fever, Mediterranean remittent fever, Malta fever, and undulant fever. To date, twelve species belong belong to the genus *Brucella* and are categorized based on the preference of the organism to the host. The only four of them that are discussed in the literature are *B. abortus*, *B. melitensis*, *B. suis* and *B. canis* have been linked to human infection (aside from biovar 2). People who associate with animals or animal products are more likely to get brucellosis than others, particularly in agricultural nations where most of the community is engaged in raising livestock and cultivating land (Yousaf *et al*., 2021). Cattle are the primary hosts of *B. abortus*, while goats and sheep are the primary hosts of *B. melitensis* and *B. ovis*. Although both *B. abortus* and *B. melitensis* infect humans, human cases susceptible to the latter are however more frequently documented (Bonfoh *et al*., 2012). Brucellosis results in significant economic losses in terms of abortions, stillbirths, placenta retention, a decrease in milk output,

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and low fertility rates in both males and females. Animals that are asymptomatic and suffering from the disease typically release infectious materials into the pasture or watering troughs, which are significant sources of infection for healthy animals (Ntivuguruzwa *et al*., 2020).

The mode of transmission is either through contact with placenta, fetus, fluid of fetus, and vaginal secretions or through contaminated animal discharges such as blood, milk, semen, and urine. Furthermore, equipment and clothes also carry the infection (John *et al*., 2010).

The disease commonly emerges in people as a sudden onset of fever characterized by non-specific symptoms (pyrexia, sweating, lack of appetite, lethargy, loss of weight, anxiety, headache, and muscle pain). Human infection may involve multiple organ systems. The illness has the potential to worsen and turn into a persistently disabling condition with serious side effects including endocarditis, neuro-brucellosis, involvement of the bones and joints as well as epididymo-orchitis. Despite this, the reported fatality rate of brucellosis in people is less than 2% (Workalemahu *et al*., 2017).

The detection of brucellosis in animals can be made with the help of disease signs and symptoms, blood tests, or cultures of contaminated materials. Different biochemical tests that are used in the diagnosis of brucellosis include the rose bengal plate test (RBPT), milk ring test (MRT), serum agglutination test (SAT), standard tube agglutination test (STAT) (Godfroid *et al*., 2010). Polymerase chain reaction (PCR) is a confirmatory technique but it is less suitable in endemic areas as it requires specialized equipment and expertise. Enzyme-linked immunosorbent assay (ELISA) tests play a crucial role in diagnosing infectious diseases. So, most clinical settings make it a preferred choice due to its capability to identify IgM and IgG antibodies, thus providing accurate diagnosis and quick results. Asia, the Middle East, Africa, and Latin America still have endemic and uncontrolled brucellosis (Refai, 2003; Shahzad *et al*., 2017).

Pakistan is an agricultural country where most people especially from rural areas depend on agriculture and livestock, as a result, this disease has a substantial influence on human and animal health (Shafee *et al*., 2011; Maadi *et al*., 2011). In the subcontinent, particularly in Pakistan, India, and Bangladesh brucellosis rates remained comparatively higher. Across the country, the seropositivity of brucellosis in livestock ranged from 0% to 76% (Gul *et al*., 2014; Ahmad *et al*., 2017; Arif *et al*., 2018). In various parts of Pakistan, the prevalence of *B. abortus* is recorded up to 3.25% (Abubakar *et al*., 2012). These results show that brucellosis is becoming more common across the country. Numerous studies have observed brucellosis frequency among animal handlers ranging from 0.95 to 8.58% (Farooq *et al*., 2011). The disease prevalence in occupational workers was reported viz farmers (2.6–21.6%), milkers (18.6%), butchers (2.5%) and veterinarians (5.3–11.1%). A concerted effort is required to control and eradicate brucellosis from domesticated animals (Islam *et al*., 2013). Studies in Khyber Pakhtunkhwa (Peshawar, Charsadda) and Azad Jammu and Kashmir have been carried out to study the prevalence of brucellosis by utilizing various diagnostic techniques (Niaz *et al*., 2021).

With a population of around a million people, the Kurram tribal zone is situated in the northwest of Pakistan and shares a border with the eastern section of Afghanistan. The primary sources of income and food are agriculture and livestock for the people in District Kurram. Considering the severity of the illness, the present study investigated seroprevalence of brucellosis in cattle and humans (veterinarians, inseminators, livestock farmers, slaughterhouse workers, and milkers) living in remote rural and animal migration areas in district Kurram bordering Afghanistan by using serological approaches to determine *Brucella* seroprevalence in cattle population and livestock-related persons in District Kurram, Khyber Pakhtunkhwa, Pakistan and molecular characterization of brucella pathogens in seropositive samples through AMOS-PCR.

MATERIALS AND METHODS

Study design

Due to unknown status of the disease, a cross-sectional study was performed to find out the seroprevalence of bovine brucellosis in district Kurram which is in the North-west of Pakistan having a land area of around 3,310 Km² and a population of one million people. This was the first ever such study in the district as the seroprevalence of the disease was unknown earlier in the area under study. In this cross-sectional study of brucellosis different small-scale dairy farms and domestic cattle were selected for the serologic survey and PCR-based investigation of brucellosis as well as livestock-related persons from different areas of district Kurram, Khyber Pakhtunkhwa Pakistan. This selection was done on the basis of geographical distribution and the number of animals there.

Sample collection and sample size estimation

Random blood samples were collected from cattle populations of different targeted regions of the district. Additionally, blood samples were also collected from high-risk populations (those individuals who continue to interact closely or physically with livestock) and properly labeled.

The sample size was estimated by considering the unknown status of brucellosis in cattle and highly occupational-risk groups. Sampling was done by maintaining the 50% predicted prevalence with a 95% confidence interval and a 5% required absolute precision and was calculated by using the following equation.

$$n = \frac{1.96^2 P_{exp}(1 - P_{exp})}{d^2}$$

$$n = \frac{1.96^2\ 0.50(1 - 0.50)}{0.50^2}$$

$$n = 384$$

Where n is required sample size, P_{exp} is expected prevalence, and d is desired absolute precision

So, according to the formula, a number of samples were taken by randomly selecting the villages, animals, and livestock-related persons in district Kurram. The total sample size was 384 including 284 from cattle and 100 from livestock-related persons. Animals and humans that were vaccinated against brucellosis as well as those animals that suffer from other chronic or untreatable diseases were excluded from the study. Out of 100 human samples, more than half of the samples were collected from District Headquarters Hospital (DHQ) Parachinar, Kurram. These samples were related to farmers and women who are associated with domestic animals in their daily life.

Data collection

A questionnaire was designed for the recording of animal data like age, sex, breed, purpose, management practices, health status, history of the disease, abortion history, and reproductive disorders (i.e., infertility, retention of fetal membrane, stillbirth, repeat breeding, etc.). Informed consent was also obtained from high-risk group people by explaining the purpose of the investigation. Recording human data including general information, clinical signs and symptoms, spread and key risk factors including exposure to raw unpasteurized or uncooked food, contact and type of contact, hygienic status, and awareness knowledge were obtained.

Blood and serum sample collection and handling

Approximately, 4-5 ml of the blood was collected from each of the animals by puncturing jugular vein. Similarly, 5 ml of blood was taken from selected human population with 5 cc uncontaminated, sterilized, and disposable syringes. After taking a sample, 2.5 ml of the blood sample was added to the EDTA tube and 2.5 ml to the gel clot vacutainer and numerically numbered. The gel clot vacutainers were placed in a slanting position overnight and then centrifuged in Civil Veterinary Hospital Parachinar, Kurram at 3000rpm for 3 min to collect the sera from the blood samples. After collecting serum, it was poured into Eppendorf tubes and stored in a freezer at -20 °C till further serological examination.

Analysis of serum samples

All the collected sera samples of cattle and humans were screened for anti-*Brucella* antibodies by Rose–Bengal agglutination test (RBPT) (RBPT antigen, Veterinary Research Institute, Lahore, Pakistan), Serum plate agglutination test (SPAT) (Laboratory Diagnostics Co., Inc. Morganville, NJ 07751, USA) and *i*-ELISA (ID.vet, Serum Ab Test kit310, rue Louis Pasteur- Grabels- France) for animal samples, whereas *i*- ELISA (vircell, S.L. Parque Technologico de la Salud, Avicena 8,18016 Granada, Spain) for human samples according to the manufacturer's recommendations. The study was conducted in the Center of Microbiology and Biotechnology's Brucellosis Lab at the Veterinary Research Institute in Peshawar, Khyber Pakhtunkhwa.

Molecular detection and identification

DNA was extracted from all serum samples using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer. The Brucella AMOS PCR assay was used to identify and differentiate 4 species of *Brucella* (*B. abortus*, *B. melitensis*, *B. suis* and *B. ovis*). AMOS-PCR was utilized to evaluate the molecular characterization of pathogens in seropositive samples (Kolo *et al*., 2019).

Statistical analysis

Prevalence (%) and relative 95 % confidence interval were calculated by using SPSS version 25. Chi-square testing was performed to find out significant differences among different risk factors of the disease. Univariate analysis was performed to calculate Odds ratio (OR).

RESULTS

Prevalence of anti-brucella antibodies in bovine and human serum

The prevalence of brucellosis in serum samples of cattle at district Kurram, Khyber Pakhtunkhwa, Pakistan was investigated in which cattle samples have 16.19%, 18.30% 13.38%, and 5.9% prevalence through RBPT, SPAT (*B. abortus*), SPAT (*B. melitensis*) and *i*-ELISA , Perspectively. In comparison, human samples have 15%, 24%, and 6% prevalence through RBPT, both SPAT (*B. abortus* and *B. melitensis*) and *i*-ELISA respectively. In molecular analysis through AMOS PCR, 4 samples of cattle and 2 human samples were found positive for *B. abortus* as shown in Table I.

Table I. Overall seroprevalence of brucellosis in cattle and human.

Serial number	Species	Total number of serum samples	Serological examination				Molecular identification through AMOS-PCR (%)
			RBPT (%)	SPAT		*i*-ELISA (%)	
				B. abortus (%)	*B. melitensis* (%)		
1	Cattle	284	46 (16.19)	52 (18.30)	38 (13.38)	17 (5.9)	4 (1.4)
2	Human	100	15 (15)	24 (24)	24 (24)	6 (6)	2 (2)

RBPT, rose bengal plate test; SPAT, Serum plate agglutination test; i-ELISA, indirect enzyme-linked immunosorbent assay; AMOS (abortus, melitensis, ovis, and suis) PCR

Seroprevalence of bovine brucellosis through RBPT and relationship with disease-related risk factors

In cattle

Breed, age, sex, and purpose of animal keeping showed a non-significant association with the seroprevalence of brucellosis (p>0.05) using RBPT. In breed, 16.2% local, 20.8% cross-bred and 6.1% exotic were positive. While in age, animals less than 5 years were 8.5%, greater than 5 years were 16.7%, and greater than 10 years were 33.3% positive. Through RBPT, males were more positive than females. 20.4% males while 15.2% females were positive. The purpose of animal keeping was categorized into two groups dairy and beef. Beef animals show more positivity than dairy ones through RBPT. Grazing was significantly associated with the disease (p<0.05). Using RBPT, 14.1% animals with open grazing and 35.7% with confined grazing were found positive. In the breeding method, a highly significant association was found between natural mating and brucellosis. Out of 35 positive animals, 40% and 8.3% were found positive for natural mating and AI, respectively with RBPT (p<0.05).

Immunization against brucellosis, other vaccination, health status of animals, pregnancy status, and previous calving history were non-significantly associated with the disease (p>0.05). Retained placenta shows a significant association with the seroprevalence of brucellosis. With RBPT 25.9% with retained placenta history and 11.6% with no RFM were found positive. A highly significant association was found between abortion and disease (p=0.0001). Out of 35 positive animals, 51.6% were with an abortion history. Repeat breeding was also significantly associated with the seroprevalence of the disease (p<0.05). 28.6% of animals which were positive with RBPT had repeat breeding history (Table II).

In livestock-related persons

By using the Chi-square test, it was found that profession, gender, age, and clinical signs were non-significantly associated with brucellosis (p>0.05) using RBPT. Exposure to unpasteurized milk was significantly associated with the disease (p<0.05). Using RBPT, 22.8% people with drinking unpasteurized milk history and 4.7% with no such history were found positive. Exposure to undercooked/Raw meat, contact with animal aborted material, contact with animal retained placenta, contact during slaughtering animals, housing, and residing at the farm were non-significantly associated with the disease (p>0.05). One of the factors is awareness about the disease was significantly associated with the seroprevalence of brucellosis (p<0.05). Out of 15 positive samples, no one knows about the disease and its symptoms as shown in Table III.

Seroprevalence of brucellosis determined through SPAT and relationship with disease-related risk factors

In cattle

Breed, age, sex, and purpose of animal keeping showed non-significant association with the seroprevalence of brucellosis (P>0.05) using SPAT *(B. abortus* and *B. melitensis).* With reference to *B. abortus,* in breed 19% local, 22.2% cross bred while 6.1% exotic were positive. Age was categorized into three groups. 8.5% below 5 years, 18.9% above 5 years while 40% over 10 years cattle were positive. In sex, 22.2% male while 17.4% female were positive. 17.5% dairy animals while 21.8% beef animals were positive through SPAT *(B. abortus).* Grazing was significantly associated with the disease (P<0.05). Using SPAT, 16.4% animals with open grazing and 35.7% with confined grazing were found positive. In the breeding method, a highly significant association was found among natural mating, artificial insemination, and brucellosis. Out of 230 animals, 8.7% tested positive for *B. abortus* (SPAT, p < 0.05) across both in natural mating and AI groups. Immunization against brucellosis, other

Table II. Seroprevalence of brucellosis (*B. abortus*) in cattle through RBPT and SPAT and its relationship with different disease-related risk factors.

Variables/ Category	N	RBPT				SPAT			
		Positive (%)	P value	OR	Confidence interval L.L -U. L	Positive (%)	P value	OR	Confidence interval L.L - U. L
Breed									
Local	179	29 (16.2%)	0.162			34 (19%)	0.129		
Cross bred	72	15 (20.8%)				16 (22.2%)			
Exotic	33	02 (6.1%)				02 (6.1%)			
Age									
<5years	47	4 (8.5%)	0.070			4 (8.5%)	0.020*		
> 5 years	222	37(16.7%)				42(18.9%)			
>10 years	15	05(33.3%)				06(40%)			
Sex									
Male	54	11 (20.4%)	0.355	1.425	0.671- 3.028	12 (22.2%)	0.409	1.357	0.656- 2.806
Female	230	35 (15.2%)				40 (17.4%)			
Purpose									
Dairy	229	35 (15.3%)	0.394	0.722	0.340- 1.531	40 (17.5%)	0.454	0.758	0.367- 1.566
Beef	55	11 (20%)				12 (21.8%)			
Grazing									
Open grazing	256	36 (14.1%)	0.003*	0.295	0.126- 0.689	42 (16.4%)	0.012*	0.353	0.152- 0.819
Confined grazing	28	10 (35.7%)				10 (35.7%)			
Breeding protocol									
A.I	180	15 (8.3%)	0.0001*	0.136	0.063- 0.296	20 (8.7%)	0.0001*	0.188	0.090- 0.390
Natural mating	50	20 (40%)				20 (8.7%)			
Immunized against Brucellosis									
Yes	0	0				0			
No	284	46 (16.2%)				52 (18.3%)			
Other vaccination									
Yes	257	42 (16.3%)	0.838	1.123	0.369- 3.415	48 (19.7%)	0.976	0.985	0.355- 2.734
No	27	4 (14.8%)				4 (10%)			
Health status									
Healthy	244	42 (17.2%)	0.251	1.871	0.632- 5.539	42 (17.2%)	0.143	2.204	0.748- 6.491
Emaciated	40	04 (10%)				04 (10%)			
Retained placenta									
Yes	58	15 (25.9%)	0.009*	2.651	1.252- 5.614	16 (27.6%)	0.018*	2.349	1.144- 4.824
No	172	20 (11.6%)				24 (14%)			
Abortion history									
Yes	31	16 (51.6%)	0.0001*	10.10	4.326- 23.604	16 (51.6%)	0.0001*	7.778	3.414- 17.722
No	199	19 (9.5%)				24 (12.1%)			
Pregnancy status									
Pregnant	78	7 (9%)	0.059	0.437	0.181- 1.051	10 (12.8%)	0.190	0.598	0.276- 1.298
Non-pregnant	152	28 (18.4%)				30 (19.7%)			
Repeat breeding									
Yes	70	20 (28.6%)	0.0001*	3.867	1.840- 8.126	21 (30%)	0.001*	3.180	1.579- 6.408
No	160	15 (9.4%)				19 (11.9%)			
Previous calving									
Normal	204	33 (16.2%)	0.257	2.316	0.522- 10.274	38 (18.6%)	0.166	2.747	0.622- 12.127
Dystocia	26	2 (7.7%)				2 (7.7%)			

OR, odds ratio; For abbreviations, see Table I.

Table III. Seroprevalence of brucellosis in livestock-related persons through RBPT, SPAT and *i*-ELISA and its relationship with disease-related risk factors.

Variables/ Category	N	RBPT				SPAT				*i*-ELISA			
		Positive (%)	P value	OR	Confidence interval L.L - U. L	Positive (%)	P value	OR	Confidence interval L.L - U. L	Positive (%)	P value	OR	Confidence interval L.L - U. L
Profession													
Farmer	23	6 (26.1%)				9 (39.1%)				2 (8.7%)			
Butcher	5	2 (40%)				2 (40%)				1 (20%)			
Milker	11	1 (9.1%)	0.154			2 (18.2%)	0.358	_		0	0.567	_	
Vet.Dr/ Assistant	14	2 (14.3%)				3 (21.4%)				1 (7.1%)			
Livestock Owner	17	0				2 (11.8%)				0			
Housewife	30	4 (13.3%)				6 (20%)				2 (6.7%)			
Age													
<30 Years	17	02(11.8%)				03 (17.6%)				0			
31-40 Y	35	08(22.9%)				12 (34.3%)				03 (8.6%)			
41-50 Y	36	05(13.9%)	0.263			08 (22.2%)				03 (8.3%)	0.456		
> 50 Y	12	0				01 (8.3%)	0.253	_		0		_	
Gender													
Male	69	10(14.5%)	0.832	0.881	0.274- 2.835	17 (24.6%)	0.824	1.121	0.411- 3.060	04 (5.8%)	0.899	0.892	0.155-5.150
Female	31	05(16.1%)				07 (22.6%)				02 (6.5%)			
Clinical signs (C/S)													
With C/S	62	12(19.4%)	0.119	2.800	0.735- 10.660	19 (30.6%)	0.047*	2.916	0.986- 8.627	06 (9.7%)	0.048*	1.679	1.421-1.983
No signs	38	03 (7.9 %)				05 (13.2 %)				0			
Exposure to unpasteurized milk													
Yes	57	13(22.8%)	0.012*	6.057	1.288- 28.488	18 (31.6%)	0.041*	2.846	1.019- 7.953	06 (10.5%)	0.028*	1.843	1.531-2.219
No	43	02 (4.7%)				06 (14%)				0			
Exposure to raw/undercooked meat													
Yes	05	02 (40%)	0.108	4.205	0.640- 27.626	03 (60%)	0.053	5.286	0.828- 33.74	0	0.562	1.056	1.007-1.108
No	95	13 (13.7%)				21 (22.1%)				06 (6.3%)			
Contact with animal aborted material													
Yes	16	04 (25%)	0.222	2.212	0.605- 8.093	04 (25%)	0.919	1.067	0.309- 3.679	04 (25%)	0.0001*	13.667	2.254-82.86
No	84	11 (13.1%)				20 (23.8%)				2 (2.4%)			
Contact with animal retained placenta													
Yes	61	12 (19.7%)	0.102	2.939	0.772- 11.182	18 (29.5%)	0.107	2.302	0.823- 6.444	06 (9.8%)	0.043*	1.709	1.442-2.026
No	39	03 (7.7%)				06 (15.4%)				0			
Contact during slaughtering													
Yes	15	03 (20 %)	0.556	1.521	0.373- 6.197	03 (20 %)	0.694	0.762	0.196- 2.962	02(13.3 %)	0.195	3.115	0.517-18.763
No	85	12 (14.1%)				21 (24.7%)				04 (4.7%)			
Work/live at farm													
Yes	12	1 (8.3%)	0.491	0.481	0.057- 4.025	3 (25%)	0.931	1.063	0.263- 4.294	1 (8.3%)	0.717	1.509	0.161-14.136
No	88	14(15.9%)				21 (23.9%)				05(5.7%)			
Awareness about brucellosis													
Yes	18	0	0.049*	1.269	1.136- 1.416	3 (16.7%)	0.421	0.581	0.153- 2.208	0	0.237	1.237	1.121-1.365
No	82	15 (18.3%)				21 (25.6%)				06 (7.3%)			

For abbreviations, see Table I and Table II.

vaccination, health status of animals, pregnancy status and previous calving history were non-significantly associated with the disease (p>0.05). Retained placenta showed a significant association with the seroprevalence of brucellosis. With SPAT *(B. abortus)* 27.6% of animals with retained placenta and 14% with no RFM history were found positive. A highly significant association was found between abortion and the disease (p=0.0001). Out of 40 positive female animals, 51.6% were positive with an abortion history. Repeat breeding was also significantly associated with seroprevalence of the disease (p<0.05). 30% of animals that were positive for SPAT *(B. abortus)* had a repeat breeding history (Table II).

With reference to *B. melitensis* age was significantly associated with the disease and animals above 10 years of age showed a highly significant association of 33.3% with the disease through SPAT *(B. melitensis)*. Grazing was also significantly associated with the disease (P<0.05). Using SPAT *(B. melitensis)*, 10.9% animals with open grazing and 35.7% with confined grazing were found positive. In the breeding method, a highly significant association was found among natural mating, artificial insemination, and brucellosis. In 230 animals, 7.8% showed statistically significant *B. melitensis* positivity (SPAT, p < 0.05), found in both natural mating and artificial insemination groups. Immunization against brucellosis, other vaccinations, health status of animals, pregnancy status, and previous calving history were non-significantly associated with the disease (p>0.05). Retained placenta showed a significant association with the seroprevalence of brucellosis. With SPAT *(B. melitensis)* 20.7% with retained placenta and 9.3% with no RFM history were found positive. A highly significant association was found between abortion and the disease (p=0.0001). Out of 28 positive female animals, 45.2% had with abortion history. Repeat breeding was also significantly associated with seroprevalence of the disease (p<0.05). 27.1% animals which were positive for SPAT *(B. melitensis)* had repeat breeding history (Table IV).

In livestock-related persons

By using chi-square test, it was found that profession, gender, and age were non-significant associated with the seroprevalence of brucellosis (P>0.05) using SPAT. In profession, 39.1% farmers, 40% butchers, 18.2% milkers, 21.4% veterinary assistants, 11.8% livestock owners and 20% housewives were positive. Age was divided into 4 groups. Below 30 years 17.6%, between 31 to 40 years 34.3%, between 41 to 50 years 22.2% and above 50 years 8.3% were positive. In Sex, male 24.6% and female 22.6% were positive through SPAT. Clinical signs showed a significant association with the disease with p=0.047. Out of 100 people, 30.6% had related clinical signs and 13.2% with no signs showed positivity through SPAT. Exposure to unpasteurized milk was significantly associated with the disease (P<0.05). Using SPAT, 31.6% people with drinking unpasteurized milk history and 14% with no such history were found positive. Exposure to undercooked/ Raw meat, contact with animal aborted material, contact with animal retained placenta, contact during slaughtering animals, housing and residing at a farm and awareness about brucellosis were non-significantly associated with the disease (*p*>0.05) (Table III).

Seroprevalence of brucellosis determined through i-ELISA and relationship with disease-related risk factors

In cattle

Breed, sex, age, and purpose of animal keeping showed non-significant association with the seroprevalence of brucellosis (*p*>0.05) using *i*-ELISA. Local breeds 6.1%, cross-bred 8.3% were positive. In age groups, animals less than 5 years 6.4%, above 5 years 5.4% and above 10 years 13.3% were positive. In sex, 9.3% male while 5.2% female animals were positive through *i*-ELISA (indirect ELISA).

In grazing, confined grazing showed a highly significant association with the disease. 25% of animals with confined grazing and 3.9% with open grazing were positive through *i*-ELISA. In the breeding method, a highly significant association was found between natural mating and brucellosis. Out of 230 animals, 16% were found positive for natural mating and 2.2% were positive for artificial insemination with *i*-ELISA (p<0.05). Immunization against brucellosis, other vaccinations, health status of animals, pregnancy status, retained placenta history and previous calving history were non-significantly associated with the disease (p>0.05). Abortion showed significant association with the seroprevalence of brucellosis (p<0.05). With *i*-ELISA 29% with abortion history and 1.5% with no such history were found positive. Repeat breeding was also significantly associated with seroprevalence of the disease (p<0.05). 12.9% of animals that were positive with *i*-ELISA had repeat breeding history while only 1.9% were positive with no such history through *i*-ELISA as shown in Table IV.

In livestock-related persons

By using the chi-square test, it was found that profession, gender, and age were non-significantly associated with the seroprevalence of brucellosis (P>0.05) using SPAT i-ELISA. In the profession, 8.7% farmers, 20% butchers, 7.1% veterinary assistants, and 6.7% housewives were positive. Age was divided into 4 groups. Between 31 to 40 years 8.6%, and between 41 to 50 years 8.3% were positive

Table IV. Seroprevalence of brucellosis in cattle through SPAT (*B. melitensis*) and *i*-ELISA and relationship with disease related risk factors.

Variables/ Category	N	SPAT				*i*-ELISA			
		Positive (%)	P value	OR	Confidence interval L.L - U. L	Positive (%)	P value	OR	Confidence interval L.L - U. L
Breed									
Local	179	25 (14%)	0.152			11 (6.1%)	0.245		
Cross bred	72	12 (16.7%)				06 (8.3%)			
Exotic	33	01 (03%)				0			
Age									
< 5years	47	4 (8.5%)	0.047*			3 (6.4%)	0.453		
> 5 years	222	29(13.1%)				12(5.4%)			
> 10 years	15	05(33.3%)				02(13.3%)			
Sex									
Male	54	10 (18.5%)	0.218	1.640	0.742-3.621	05 (09.3%)	0.260	1.854	0.624-5.504
Female	230	28 (12.2%)				12 (5.2%)			
Purpose									
Dairy	229	28 (12.2%)	0.244	0.627	0.284-1.383	12 (5.2%)	0.280	0.553	0.186-1.641
Beef	55	10 (18.2%)				05 (9.1%)			
Grazing									
Open grazing	256	28 (10.9%)	0.0001*	0.221	0.093-0.526	10 (3.9%)	0.001*	0.122	0.042-0.353
Confined grazing	28	10 (35.7%)				07 (25%)			
Breeding protocol									
AI	180	14 (7.8%)	0.0001*	0.217	0.095-0.494	04 (2.2%)	0.0001*	0.119	0.034-0.415
Natural mating	50	14 (7.8%)				08 (16%)			
Immunized against brucellosis									
Yes	0	0				0			
No	284	38 (13.4%)				17 (6%)			
Other vaccination									
Yes	257	34 (13.2%)	0.818	0.877	0.286-2.691	14 (05.4%)	0.238	0.461	0.124-1.718
No	27	4 (14.8%)				03 (11.1%)			
Health status									
Healthy	244	35 (14.3%)	0.239	2.065	0.604-7.065	17 (07%)	0.085	1.176	1.118-1.237
Emaciated	40	03 (7.5%)				0			
Retained placenta									
Yes	58	12 (20.7%)	0.022*	2.543	1.123-5.761	03 (5.2%)	0.986	0.988	0.258 -3.780
No	172	16 (09.3%)				09 (5.2%)			
Abortion									
Yes	31	14 (45.2%)	0.0001*	10.882	4.460-26.55	09 (29%)	0.0001*	26.727	6.731-106.128
No	199	14 (07%)				03 (1.5%)			
Pregnancy status									
Pregnant	78	06 (7.7%)	0.136	0.492	0.191-1.270	03 (3.8%)	0.503	0.636	0.167-2.418
Non-pregnant	152	22(14.5%)				09 (5.9%)			
Repeat breeding									
Yes	70	19 (27.1%)	0.0001*	6.251	2.660-14.687	09 (12.9%)	0.001*	7.721	2.022-29.479
No	160	09 (05.6%)				03 (1.9%)			
Previous calving									
Normal	204	26 (12.7%)	0.458	1.753	0.391-7.856	12 (5.9%)	0.204	1.135	1.081-1.192
Dystocia	26	2 (7.7%)				0			

For abbreviations, see Table I and Table II.

while below 30 years and above 50 years have no positivity through i-ELISA. In Sex, male 5.8% and female 6.5% were positive through i-ELISA. Clinical signs showed a significant association with the disease with p=0.048. Out of 100 people, 9.7% had related clinical signs. Exposure to unpasteurized milk was significantly associated with the disease (P<0.05). Using i-ELISA, 10.5% people with drinking unpasteurized milk history were found positive. Exposure to undercooked/raw meat, contact during slaughtering animals, housing and residing at a farm and awareness about brucellosis were non-significantly associated with the disease (p>0.05). Contact with animal aborted material was highly significantly associated with the disease (p<0.05). 25% cases were positive with such history while 2.4% were positive without a history of contact with aborted material. Contact with animal-retained placenta was also significantly associated with seroprevalence of brucellosis. 9.8% were people who had contact with the animal-retained placenta in their lives and were positive through i-ELISA (Table III).

Detection of Brucella *spp. DNA in bovine sera*

Brucella DNA was checked in serum samples positive by *i*-ELISA. *B. abortus* DNA was identified in 4 samples of cattle and 2 samples of humans through AMOS-PCR.

DISCUSSION

Brucella is a member of the order Rhizobiales and class a-Proteobacteria. It is a gram-negative, facultative intracellular, anaerobic, non-motile coccobacilli (Gupta *et al*., 2006). The present investigation was devised for brucellosis detection in the district of Kurram, Khyber Pakhtunkhwa, Pakistan through a combination of serological techniques, namely RBPT, SPAT, and *i*-ELISA, along with molecular testing using the AMOS PCR for differentiation of species prevailing in cattle and livestock-related person. The overall prevalence of brucellosis in cattle was recorded to be 16.19% by RBPT, 18.30% by SPAT (*B. abortus*), 13.38% by SPAT (*B. melitensis*), 5.9% by *i*-ELISA, while in livestock-related persons 15% by RBPT, 24% by SPAT, and 6% by *i*-ELISA was recorded. *B. abortus* DNA was detected in sera from 4 cattle and 2 human samples through AMOS PCR.

In the current study, the overall incidence of brucellosis in cattle was 16.19% and 15% in humans by RBPT. Our results agree with a previous study in which *Brucella* seroprevalence was 15% in animals, but his human results were only 6% (Muhammad *et al*., 2017). Our finding showed a higher pervasiveness in crossbred cattle of more than 5 years of age. Similarly, a cross-sectional epidemiological survey indicated a higher seroprevalence of brucellosis among older animals compared to younger ones (Gumi *et al*., 2013). This aligns with reported data, which proposed that the animals aged 6 years and above exhibited higher seropositivity when contrasted with those below the age of 6 (Swai and Schoonman, 2010). In pregnant animals, the production of erythritol in the placenta creates a favorable environment for the rapid multiplication of bacteria, resulting in conditions like endometritis, infections of cotyledons, and placentitis. This phenomenon results from several factors, including an impaired immune system brought on by disease, aging, and pregnant animals (Gul and Khan, 2007). Our findings align with Magersa *et al*. (2011) wherein brucellosis was found to be responsible for 13.8% of bovine abortions. However, one study demonstrated a significantly greater percentage of cattle having a history of abortion positive for *Brucella* 32.92% and 34.64% through RBPT and SPAT, respectively. This alteration in the seroprevalence is thought due to the variations in the immune status of animals under investigation (Otlu *et al*., 2008). Contrary to our findings, one study reported an animal-related prevalence of 5% using RBPT and SPAT (Magona *et al*., 2009).

Our results are in agreement with a former report about the overall prevalence of brucellosis in cattle of 15%, and only 6% in humans (Khan *et al*., 2017). Conversely, a study about human brucellosis showed a 3.6% prevalence which does not support our findings (Ahmed *et al*., 2017). Previous findings supported our study as they determined the prevalence of brucellosis in working humans. According to them, the general value of positive tests of brucellosis was about 15.7%. They considered professionals to be at higher risk of brucellosis than others (Franco *et al*., 2017).

Statistical association of risk factors of the study showed that animals with confined grazing showed a higher prevalence of brucellosis than open grazing. Similarly, animals with abortion, repeat breeding, and retained placenta history have more prevalence of the disease. Mai *et al*. (2012) conducted an epidemiological study to estimate the seropositivity of brucellosis in cows. They subjected all samples to RBPT and then confirmed them through *c*-ELISA. Their findings support our study as animals aged above seven years showed higher seropositivity of 35%. Similarly, animals under zero grazing systems showed higher seropositivity of 23.8% (Mai *et al*., 2012). It means a significant correlation has been found between different management systems and seroprevalence of brucellosis in animals. So, management systems are the major factors responsible for brucellosis infection and stressing the need for awareness campaigns about the disease, and its risk factors along with the adoption of vaccination programs,

proper preventive, and control measures. The introduction of infected animals to farms without going through the proper brucellosis screening tests, the failure to separate and eliminate animals with a high abortion rate, frequent mixing with infected flocks, insufficient disposal of aborted fetuses and placental membranes, and the consumption of contaminated water and feed by healthy animals all contribute to the observed seroprevalence (Sadhu *et al*., 2015).

In the current investigation, the seroprevalence rate exhibited no significant variation between male and female animals. Interestingly, the absence of erythritol in the reproductive organs of males renders them less susceptible to infection (Jain *et al*., 2013). The higher occurrence of brucellosis in males can be due to the majority of infected animals had reached sexual maturity, and male animals were frequently employed for breeding purposes. Additionally, it is noteworthy that a smaller number of samples were collected from male animals in the research area, which could potentially influence the frequency of recorded Brucella cases in males. As indicated livestock owners often refrain from culling or removing brucellosis-infected animals from their herds. Instead, these are utilized for breeding purposes or sold to other ranchers. This practice provides a plausible explanation for the high prevalence of brucellosis in males (Saeed *et al*., 2019).

In the present study, managemental practice grazing shows a highly significant association with the prevalence of the disease. Detection of brucella species in the research area signifies the potential occurrence of interspecies transmission. Notably, factors such as shared watering spots, Pasture lands, and the mixing of sheep and goats with cattle can serve as sources for the transmission of brucellosis among different animal species. Moreover, the presence of brucellosis positivity in cattle is believed to be a result of natural infection since vaccination practices are not implemented in the research area of Pakistan.

In our research, seroprevalence exhibited a significant difference between animals with a history of abortion and those without. The prevalence of brucellosis among animals with a history of abortion was recorded at 29%, whereas in non-aborted animals, the prevalence stood at 1.5%, as determined through the *i*-ELISA. Abortion at the late stages of pregnancy emerged as the most common clinical mark of brucellosis in breeding animals. In our study, we observed that animals that had previously experienced an abortion displayed a higher likelihood of infection compared to those that had never encountered such complication. These findings align closely with the research that reported a significant correlation between brucellosis and abortions occurring during the final trimester of pregnancy in cattle (Ali *et al*., 2017).

The overall prevalence of brucellosis in cattle and livestock-related people through *i*-ELISA is 5.9% and 6% respectively. In managemental practices confined grazing, in breeding protocol natural mating, animals with abortion, and repeat breeding history show highly significant association with seroprevalence of the disease in our study. Animals above the age of 10 years show more positiveness at 13.3%. Similarly, male animals have more prevalence (9.3%) than female animals. In livestock-related persons prevalence of brucellosis is much higher in those who have relative signs and symptoms of the disease, those who have been exposed to unpasteurized milk, and those who have contact with animal-retained placenta and animal aborted material. Our study links with previous reports about the brucellosis prevalence in occupationally exposed individuals. They also determined a 6% prevalence in humans by *i*-ELISA (Pathak *et al*., 2014). *i*-ELISA exhibited the lowest rate of brucellosis positivity, whereas the RBPT demonstrated a higher positivity rate. This could be due to *i*-ELISA being a quantitative test that specifically detects IgG, while RBPT and SPAT measure both IgG and IgM qualitatively (Valarmathy *et al*., 2007).

AMOS-PCR is utilized to evaluate the molecular characterization of pathogens in seropositive samples (Kolo *et al*., 2019). AMOS PCR detected only one *Brucella* species and that was *B. abortus* in 4 samples of cattle and 2 samples of human. Pathak *et al*. (2014) supported our study as they determined brucellosis prevalence in occupationally exposed individuals, and they also identified only one species of *Brucella* which was *B. abortus*. One study documented seroprevalence and predisposing factors for caprine, ovine, and bovine brucellosis. They identified *B. melitensis* in positive DNA samples by performing qRT-PCR (Saeed *et al*., 2019). Other authors are strongly in line with us as they conducted an experiment to establish the seroprevalence of human brucellosis and identified risk factors. According to them, overall seropositivity is 6.9%, and PCR techniques determined that all patients were infected by *B. abortus*. Those who had high rates of consumption of unpasteurized raw milk had higher prevalence (Ali *et al*., 2013). Our study also revealed the same factors and identified those who have contact with animal aborted material are at greater risk and were positive through serological tests. PCR detects DNA even in minimal quantities within blood samples, regardless of antibody titer. Due to its capacity for antigenic detection rather than antibody detection, PCR is more dependable and sensitive (Akhter *et al*., 2010).

It is imperative to introduce an awareness campaign targeting high-risk populations and the public to educate them about the dangers of brucellosis. Emphasis should be positioned on discouraging the consumption of raw,

unpasteurized milk, which poses a significant risk for transmission. It is crucial to conduct thorough screening for brucellosis in newly introduced animals before integrating them into existing herds, thereby preventing the introduction, and spread of the disease. To tackle the challenges posed by brucellosis, it is imperious to approach it within the comprehensive framework of the "One Health" initiative. This approach brings together multiple disciplines to ensure efficient control and eradication of the disease. As part of this strategy, it is recommended to isolate the prevalent Brucella species for further in-depth investigations. Whole genome sequencing molecular epidemiology will help in the detection of new species and in the development of new vaccines which will play a vital role in controlling the disease.

CONCLUSION

The presence of *Brucella* species in the research area provides compelling evidence of interspecies transmission, emphasizing the potential for brucellosis to spread among different animal species. Detection of *B. abortus* in cattle and humans focuses on the significant threat to community health. Although the SPAT and RBPT are first-line screening tests for brucellosis in livestock in Pakistan, their lack of specificity is of concern. To effectively control and eradicate brucellosis from Pakistan requires the elimination of positive shedders, an interactive extension program, and more specific and confirmatory tests should be considered, so that the risk to humans can be minimized.

DECLARATIONS

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Ethical statement and IRB approval

The study was approved by the Institutional Review Board and the ethical clearance Committee of the University of Agriculture, Faisalabad (vide letter No.B.E-1177/ORIC dated 15-09-2025. Informed consent was taken from all participants for which a specially designed proforma was used containing information regarding the name of the patient, father's name, gender, age, nationality, locality, family history, diagnosis, and treatment, etc.

Statement of conflicts of interest

The authors have declared no conflict of interests.

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