FOODBORNE PATHOGENS AND DISEASE
Volume 10, Number 6, 2013

DOI: 10.1089/fpd.2012.1360

Seroprevalence and Risk Factors Associated with Brucellosis as a Professional Hazard in Pakistan

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Abstract

The present study was conducted to determine the seroprevalence and identify risk factors associated with brucellosis in humans at high risk in the Potohar plateau of northeastern Pakistan. A total of 262 serum samples were collected from persons of different occupational groups: veterinary personnel, milkers, abattoir workers, livestock farmers, and others (drivers, security guards, housewives). Data related to gender, age, occupation, contact with animals, brucellosis-related symptoms, consumption of raw milk, and geographical region were collected. The Rose Bengal plate test and the serum agglutination test were performed to determine the seroprevalence of brucellosis. The overall seroprevalence was found to be 6.9% (95% confidence interval [CI]: 4.1, 10.6). Real-time polymerase chain reaction assay showed that all cases were affected by *Brucella abortus*. Individuals who consumed raw milk had higher odds of brucellosis seropositivity. This is the first report of human brucellosis related to *B. abortus* in high-risk professionals from Pakistan by the combined use of serological and molecular methods.

Introduction

Brucellosis is an important zoonotic disease of animals and humans. It causes high economic losses worldwide, particularly in developing countries of the Middle East, of the Mediterranean basin, India, Iran, and countries of central and South America (Vrioni *et al*., 2004; Queipo-Ortuno *et al*., 2005; Nikokar *et al*., 2011).

Humans most often get infected by *Brucella* (*B.*) *abortus*, *B. melitensis* or *B. suis*. Being a zoonosis, brucellosis is transmitted from infected animals to humans who are in close contact with infected vaginal secretions, urine, feces, blood, aborted fetus, or who consume unpasteurized milk or other raw milk products. Shepherds, milkmen, butchers, knackers, veterinary assistants, and abattoir workers are at high risk (Agasthya *et al*., 2007). The symptoms of human brucellosis are undulant fever, headache, weakness, body pain, and sometimes endocarditis, orchitis, or arthritis may develop (Zaks *et al*., 1995; Swai and Schoonman, 2009).

Pakistan is an agricultural country, where mostly underprivileged people, particularly from rural areas, depend on livestock for their livelihood. Only snippet seroprevalence studies have been previously conducted in Pakistan. Hussain *et al*. (2008) reported 14% and 11% seroprevalence using the Rose Bengal plate test (RBPT) and enzyme-linked immunosorbent assay, respectively, in humans having direct contact with animals. It was also found that 6.79% of individuals tested positive to the serum agglutination test (SAT) (Rashid *et al*., 1999). Likewise, a 21.7% seroprevalence was found in slaughterhouse workers of Lahore district of Pakistan (Mukhtar, 2008). Similar findings related to human brucellosis have also been reported from neighboring countries (i.e., Bangladesh [4.4%], India [15.6%], and Iran [7.1%]) (Agasthya *et al*., 2007; Nikokar *et al*., 2011; Rahman *et al*., 2012).

Various serological methods including RBPT, SAT, and enzyme-linked immunosorbent assay have been used for the diagnosis of brucellosis. Currently, polymerase chain reaction assay (PCR) and quantitative real-time PCR (qRT-PCR) assays are applied and have several advantages when compared to traditional serological techniques: they are robust, rapid, reliable, sensitive, specific, and need less turnaround time for final results. In a recent study, a combination of serological and state-of-the-art molecular techniques (qRT-PCR) showed that the detection rate of brucellosis can be increased (Gwida *et al*., 2011).

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This study was designed to evaluate the seroprevalence and the risk factors associated with brucellosis among high-risk professionals in Potohar, Pakistan. To the best of our knowledge, this is the first study where genus and species-specific qRT-PCR were used for the identification of *Brucella* species prevalent in the human population of Pakistan.

Materials and Methods

Origin of humans and sampling sites

The study was conducted on the Potohar Plateau including Islamabad Capital Territory (ICT), Rawalpindi, and Attock districts of Pakistan. This plateau is located between Punjab and Azad Kashmir (Chaudary *et al*., 2007). Blood samples ($N=262$) were randomly collected from high-risk professionals including veterinary personnel, milkers, abattoir workers, livestock farmers, and others (drivers, security guards, housewives) (Table 1). Approximately 30–35 million persons of the rural population of Pakistan are engaged in livestock production and 30–40% of their income comes from livestock (Government of Pakistan, 2005–2006). The group "veterinary personnel" included veterinary doctors, assistants, and students. More than 100 people of this group are working for the government and in nongovernmental sectors in three districts. The group "livestock farmers" included farm owners, farm workers and shepherds. They were recruited from 157 cattle farms of the three study districts. The actual number of milkers and abattoir workers working in the study districts is unknown. The group "milkers" included persons who collected milk from small and large animal farms and sold it either by themselves or supplied this milk to different shops of the study area. In the group "abattoir workers," slaughterhouse workers (butchers/throat cutters, blood collectors, digesta collectors, leather cleaners/helpers) and meat sellers were included.

Basic data regarding descriptive epidemiology were gathered using a questionnaire.

Ethical approval

This study was approved by the ethical committee of Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan. Oral and written consent was taken from each participant prior to blood sample collection.

Data collection and handling

Data collection started in July 2011 and ended in December 2011. Information regarding age, sex, contact with animals, presence of symptoms (fever, sweating, joint pain, back pain, body pain, abdominal pain, joint swelling, fatigue, lack of appetite, chills) related to brucellosis (Mantur *et al*., 2007), profession (occupation), consumption of raw milk and geographical region was collected.

Blood collection and serology

Five milliliters of blood was collected aseptically. These samples were then immediately transferred to an ice box and transported to the National Veterinary Laboratory Islamabad, Pakistan for further processing. Blood samples were kept upright at 4°C for a maximum of 24 h. Blood samples were centrifuged at 5000 rpm for 10 min. After centrifugation, supernatants were collected in 1.5 mL sterile Eppendorf tubes which were stored at −20°C. Samples were shipped on dry ice to Germany for further analysis.

Serological evaluation of serum samples

Rose Bengal plate test (RBPT). The RBPT was performed according to standard procedures (Alton *et al*., 1988). Briefly, 30 μL of serum was mixed with an equal volume of antigen preparation (Veterinary Research Institute, Lahore, Pakistan) on a glass plate; the plate was agitated gently for 4 min. A serum sample was considered positive if agglutination occurred.

Serum agglutination test (SAT). The SAT test was performed according to standard procedures (Alton *et al*., 1988).

Table 1. Potential Risk Factors for Human Brucellosis Seropositivity in the Regions of Islamabad Capital Territory (ICT), Rawalpindi, and Attock of Pakistan Based on a Parallel Interpretation of the Serum Agglutination Test and Rose Bengal Plate Test

Variable	*Category*	*Sera tested*	*Positive (%)*	*CI*	p-*Value*
Locality	ICT	82	10 (12.2)	(6.0, 21.3)	0.073
	Rawalpindi	110	4 (3.6)	(1.0, 9.0)	
	Attock	70	4 (5.7)	(1.6, 14.0)	
Contact with animals	No	17	0 (0.0)	(0.0, 19.5)	0.615
	Yes	245	18 (7.3)	(4.4, 11.4)	
Occupation	Veterinary personnel	31	0 (0.0)	(0.0, 11.2)	0.005
	Milker	53	1 (1.9)	(0.0, 10.1)	
	Abattoir worker	54	10 (18.5)	(9.3, 31.4)	
	Livestock farmer	107	7 (6.5)	(2.7, 13.0)	
	Others	17	0 (0.0)	(0.0, 19.5)	
Gender	Female	42	2 (4.8)	(0.6, 16.2)	0.746
	Male	220	16 (7.3)	(4.2, 11.5)	
Age	≤30	110	9 (8.2)	(3.8, 15.0)	0.373
	31–50	97	6 (6.2)	(2.3, 13.0)	
	≥51	55	3 (5.5)	(1.1, 15.1)	
Consumption of raw milk	No	250	6 (2.4)	(0.9, 5.2)	<0.0001
	Yes	12	12 (100)	(73.5,100)	

CI, confidence interval.

All serum samples were tested for up to five dilutions, from 1:20 to 1:320. Moreover, *Brucella* agglutination antigen was used according to instructions of the manufacturer (Veterinary Research Institute, Lahore, Pakistan). Results were interpreted according to manufacturer's instructions. A serum sample was considered positive with an agglutination titre of 1:160 or higher.

DNA extraction. DNA was extracted from all seropositive serum samples ($n = 18$) and randomly selected negative samples ($n = 20$). Extraction was done by High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. DNA purity and concentration was checked using a Nano-Drop ND-1000 UV-Vis spectrophotometer (Nano-Drop Technologies, Wilmington, DE), and DNA samples were stored at −20°C for subsequent analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR). All DNA samples were examined by a *Brucella* genus–specific and two species-specific (*B. abortus* and *B. melitensis*) qRT-PCR assays (Probert *et al*., 2004) as described previously. The detailed procedure has been previously described (Gwida *et al*., 2011). In short, the qRT-PCR assays were prepared using the TaqMan™ Universal Master Mix (Applied Biosystems, NJ) containing the following components per reaction: 12.5 μL Taq-Man™ Universal Master Mix, 0.75 μL of each primer (0.3 μM) and 0.25-μL probe (0.1 μM) and 2 μL of template and nuclease-free water sum up to a total reaction volume of 25 μL. No Template Controls that contained 2 μL of water instead of template and positive controls that contained DNA of the relevant *Brucella* spp. were included in each run to detect any amplicon contamination or amplification failure. The qRT-PCR reaction was performed in duplicate in optical 96-well microtiter plates (qPCR 96-well plates, Micro Amp™, Applied Biosystems) using a Mx3000P thermocycler system (Stratagene, La Jolla, Canada) with the following run condition: one cycle of 50°C for 2 min, one cycle of 95°C for 10 min, followed by 50 cycles of 95°C for 25 s and 57°C for 1 min.

Primers and probes were supplied by TIB MOLBIOL (Berlin, Germany) (Tables 2 and 3). A cut-off value of ≤40 was used based on analytical sensitivity and linear measuring range of the used qRT-PCR (Gwida *et al*., 2011). The threshold value was automatically generated by the instrument. Visual confirmation of positive samples was recorded from graphical representation of cycle numbers versus fluorescence values. No internal amplification control was used in the procedure to ensure a high sensitivity. For the same reason, the originally described multiplex qRT-PCR (Probert *et al*., 2004) was performed in three separate reactions.

Statistical analysis. For the statistical analysis, individuals were considered positive based on a parallel interpretation of the results of the two serological tests in which an individual was positive if he/she tested positive to either RBPT or SAT. To determine the potential risk factors associated with human brucellosis seropositivity, a two-stage modeling approach was used. First, a univariate analysis was performed to determine the strength of the association between seropositivity status and each of the risk/indicator factors in turn using Fisher's exact test. Secondly, variables with a p-value ≤ 0.10 in the univariate analysis were further analyzed using a multivariable Firth's logistic regression analysis. Firth's logistic regression analysis was used in place of the traditional exact logistic regression analysis to overcome the computational limitations and convergence issues caused by the sparseness (separation) of the data (Heinze and Schemper, 2002).

A manual forward stepwise model-building approach was employed with the Akaike's Information Criterion as the calibrating parameter to select the final model. The model with the smallest Akaike's Information Criterion was considered to be the most appropriate model. The effects of confounding were investigated by observing the change in the estimated coefficients of the variables that remain in the final model once a nonsignificant variable is reintroduced. When the inclusion of a nonsignificant variable led to a change of more than 25% of any parameter estimate, that variable was considered to be a confounder and was included in the model (Dohoo *et al*., 2003).

Multicolinearity was assessed among the independent variables using the Cramer's phi prime statistic, which expressed the strength of the association between two categorical covariates. Values > 0.7 were indicative of colinearity, and in this case only the variable most significantly associated with the response was kept in the model (Holt *et al*., 2011). The models were built using the firthlogit function (Firth, 1993) in STATA, version 12, software (StataCorp LP, College Station, TX).

Table 3. Probes for *Brucella* Genus and Species-Specific Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR type	*Probe (5′ to 3′)*
Brucella genus	6FAM-AAATCTTCCACCTTGCCCTT GCCATCA-BHQ1
B. abortus	6FAM-CGCTCATGCTCGCCAGACTT CAATG-BHQ1
B. melitensis	6FAM-CAGGAGTGTTTCGGCTCAGAA TAATCCACA-BHQ1

Results

A total of the 262 human blood samples were collected from ICT, Rawalpindi and Attock (Table 1). A total of 18 (6.9%, 95% CI: 4.1, 10.6) samples were found to be seropositive

Table 2. Primers for *Brucella* Genus and Species-Specific Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR type	*Forward primer 5′ to 3′*	*Reverse primer 5′ to 3′*
Brucella genus	GCTCGGTTGCCAATATCAATGC	GGGTAAAGCGTCGCCAGAAG
B. abortus	GCGGCTTTTCTATCACGGTATTC	CATGCGCTATGATCTGGTTACG
B. melitensis	AACAAGCGGCACCCCTAAAA	CATGCGCTATGATCTGGTTACG

for brucellosis based on a parallel interpretation of the two serological tests: RBPT and SAT. The distribution of the number of individuals that were tested, the number and percentage of seropositive, and their 95% confidence limits are presented in Table 1. Of the 245 individuals who reported to have had contacts with animals, 18 (7.3%, CI: 4.4, 11.4) were brucellosis seropositive whereas none of the 17 individuals reporting no contact with animals were seropositive. Similarly, all individuals who confirmed drinking raw milk were seropositive whereas only 6 out of the 250 individuals who never drank raw milk were seropositive (Table 1). In addition, there were more brucellosis seropositive men compared to women and those in the lower age categories presented a higher number of seropositives. There appeared to be more seropositives among Abattoir workers and livestock farmers compared to veterinary personnel and milkers. Finally, individuals in ICT presented a higher number of seropositives compared to those in Rawalpindi and Attock (Table 1).

Prior to performing the univariate regression analysis, all levels for the categorical variables with no positive cases were dropped from the analysis. After dropping two categories of occupation—namely, veterinary personal (31 observations) and others (17 observations)—and dropping completely the variable representing contact with animals, the total sample size dropped from 262 to 214. The results of the univariate analysis are based on 214 (Table 4). The samples were similar to those based on 262 samples (Table 1) and indicated that the factors—occupation, locality, and consumption of raw milk—were all statistically significantly associated with human brucellosis seropositivity ($p < 0.05$) (Table 4). Of the potential risk factors initially considered in the multivariable Firth's logistic regression model, only consumption of raw milk was statistically significant at the 5% level and thus was retained in the final model. In addition, the results were confounded by occupational status and locality and were also retained in the final model. The estimated odds ratios and their 95% CIs are presented (Table 5). The Cramer phi prime estimates indicated no important correlations between any pairs of the independent variables (values < 0.7).

Overall, the odds of brucellosis seropositivity were 1758.5 (CI: 48.47, 63799.12) times higher for those who consumed raw milk compared to those who did not consume raw milk. In addition, for abattoir workers and milkers, the odds of brucellosis seropositivity were 5.1 (CI: 0.09, 294.65) and 1.8 (CI: 0.02, 161.45) times higher, respectively, compared to those of livestock farmers (Table 5). Finally, the odds of brucellosis seropositivity for people living in ICT were 3.7 times higher compared to those of people living in Attock, whereas the odds were 0.6 times lower for those in Rawalpindi compared to those of people in Attock.

Of the 18 individuals who were positive for the two serological tests, only 15 were found to be positive in genus-specific BCSP31 and species-specific IS711 for *B. abortus* qRT-PCRs. Randomly selected serologically negative samples ($n = 20$) were all negative in the BCSP31 genus-specific qRT-PCR.

Discussion

Brucellosis is a disease that is hard to control in developing countries such as Pakistan due to enormous costs related to surveillance and culling. The need for its control is not only the improvement of animal production but also the reduction of human morbidity and harm. This study was carried out to collect preliminary data on human brucellosis in occupational risk groups. Males were found to be more seropositive as compared to females, even though the observed difference was not statistically significant. Male gender-related higher seroprevalence has been reported from neighboring countries such as India and Bangladesh (Thakur and Thapliyal, 2002; Rahman *et al*., 2012). The explanation for this is that in these countries, high-risk professions for brucellosis are male dominated.

Individuals under 30 years of age were found to have a higher number of seropositives compared to those in the older age groups. Most of these are slaughterhouse workers and are not well informed on how to protect themselves from diseases during handling of animals or their products. Consequently, there is a need to educate them before letting them start work. Our findings are in accordance with

Table 4. Potential Risk Factors for Human Brucellosis Seropositivity in the Regions of Islamabad Capital Territory (ICT), Rawalpindi, and Attock of Pakistan Based on a Parallel Interpretation of the Serum Agglutination Test and Rose Bengal Plate Test

Variable	*Category*	*Sera tested*	*Positive (%)*	*CI*	p-*Value*
Locality	ICT	60	10 (16.7)	(8.3, 28.5)	0.024
	Rawalpindi	99	4 (4.0)	(1.1, 10.0)	
	Attock	55	4 (7.3)	(2.0, 17.6)	
Occupation	Milker	53	1 (1.9)	(0.0, 10.1)	0.003
	Abattoir worker	54	10 (18.5)	(9.3, 31.4)	
	Livestock farmer	107	7 (6.5)	(2.7, 13.0)	
Gender	Female	29	2 (6.9)	(0.8, 22.8)	0.548
	Male	185	16 (8.6)	(5.0, 13.7)	
Age	≤30	80	9 (11.3)	(5.3, 20.3)	0.545
	31–50	80	6 (7.1)	(2.7, 14.9)	
	≥51	50	3 (6.0)	(1.3, 16.5)	
Consumption of raw milk	No	202	6 (3.0)	(0.9, 5.2)	< 0.0001
	Yes	12	12 (100)	(73.5, 100)	

CI, confidence interval.

Table 5. Final Model of Risk Factors Associated with Human Brucellosis Seropositivity Among People in High-Risk Occupational Groups in Pakistan Based on a Multivariate Firth's Logistic Regression Analysis

Risk factors	*OR*	p-*Value*	*95% CI*
Consumption of raw milk			
No	1	—	—
Yes	1758.5	<0.001	(48.47, 63799.12)
Occupational hazard group			
Livestock farmer	1.0	0.993	(0.0, 557.7)
Milker	1.8	0.803	(0.02, 161.45)
Abattoir worker	5.1	0.432	(0.09, 294.65)
Locality			
Attock	1	—	—
Rawalpindi	0.6	0.797	(0.01, 384.16)
Islamabad capital territory	3.7	0.970	(0.01, 27.81)

OR, odds ratio; CI, confidence interval.

findings from a study conducted in slaughterhouse workers in Pakistan and studies from other developing countries such as Algeria (Habib *et al*., 2003; Mukhtar, 2008). However, the observed variations in seroprevalence across age groups were not statistically significant.

In this study, the same occupational groups were found to be seropositive as were reported from different countries such as Jordan and Georgia (Abo-Shehada *et al*., 1996; Havas *et al*., 2012). Among abattoir workers, persons working as butchers/throat cutters, blood collectors, digesta collectors, helpers, and leather cleaners were highly affected. The sources of infection are most probably the direct contact with animals and their body secretions/fluids such as blood. Moreover, poor hygienic conditions of slaughterhouses make professional people vulnerable to disease. Variation in seroprevalence of brucellosis related to the nature of work in slaughterhouses has been demonstrated previously (Mukhtar, 2008). Our findings that seroprevalence was highest among abattoir workers (18.5%) and noted among all occupational groups, especially in persons engaged in animal slaughtering and cleaning (i.e., digesta or blood), are in line with investigations from Tanzania (Swai and Schoonman, 2009), but an amazing variation is noted when various countries are compared: 4.0% in Saudi Arabia, 4.1% in Brazil, 9.2% in Lebanon, 25.45% in India, 37.6% in Algeria and 63.3% in Nigeria (Al-Sekait *et al*., 1993; Araj and Azzam, 1996; Kumar *et al*., 1997; Habib *et al*., 2003; Cadmus *et al*., 2006; Ramos *et al*., 2008).

A previous study in slaughterhouse workers (Mukhtar, 2008), shows 21.7% prevalence. Also, livestock farmers often were identified as being seropositive (6.5%). This group has contact with body secretions and excretions of infected animals. It is well known that milk and aborted material from brucellosis-infected animals is contaminated by a significant number of brucellae (Nikokar *et al*., 2011). Drinking of unpasteurized milk was identified as an important risk factor among slaughterhouse workers from Pakistan (Mukhtar, 2008). Seroprevalence of brucellosis in this professional group was also reported from other countries (i.e., 3.2% in Iran and 14.22% in Turkey [Salari, 2002; Otlu *et al*., 2008]).

Among milkers, only one individual was seropositive. Hygiene is not a factor that contributes to the spread of disease. The installation of community boiling centers for pasteurization of raw milk will contribute to reducing spread of the infection. Similar findings are reported from Jordan where farmers, veterinary workers, and meat and milk handlers were found to be at high risk (Abo-Shehada *et al*., 1996). Interestingly, no seropositive case was identified among veterinary workers and veterinary students. A possible reason could be that veterinary students are not yet exposed to livestock except an occasional contact during clinical education (e.g., via rectal palpation). Veterinary workers (i.e., veterinary doctors and assistants) are using protective gear such as gloves and have a profound knowledge of spread of zoonotic diseases. Very low seroprevalence (0.4%) of brucellosis was also recorded in veterinarians from Korea (Lim *et al*., 2011). A high seroprevalence of brucellosis was also found in high-risk-group individuals (8.2%) as compared to those from a control group (0.5%) in Jordan (Abo-Shehada *et al*., 1996). However, the variations observed in our study were not statistically significant.

Symptoms of brucellosis were noted among the seropositive cases in this study (Table 6), which is in accordance with findings in high-risk professionals in other countries (e.g., Eritrea and Iran [Omer *et al*., 2002; Rezaee *et al*., 2012]). Considerable variation in the number of seropositive cases was seen in the three study regions. Higher prevalence (12.2%) was presented in samples collected from ICT. This finding can be attributed to the heterogeneous or unequal distribution of samples in the sampling regions, since most abattoir workers were from ICT. Similarly, variations in seroprevalence from different regions were found in a study in Georgia (Havas *et al*., 2012). However, the observed variations were not statistically significant.

It is worth noting that the total sample size for the analysis in this study was 214 with only 18 positive samples, leading to very low or sometimes zero-frequencies across subgroups for the risk factors with respect to seropositivity. These observations lead to the use of exact univariate methods such as the Fisher's exact test and multivariable methods such as Firth's logistic regression analysis. The estimated odds ratios should be interpreted with care, since the small number of observations means low precision for the estimates as evidenced by the wide 95% CIs for the estimated odds ratios. In future studies, the sample size should be increased to obtain more precise estimates.

Table 6. Clinical Symptoms of Brucellosis Among Seropositive Individuals (N=18) of Different High-Risk Occupation Groups in Pakistan

	Occupational groups		
Symptoms	*Abattoir workers* (n=*10*)	*Animal keepers* (n=*7*)	*Milkman* (n=*1*)
Fever	8	5	1
Sweating	6	7	0
Joint pain	1	0	0
Back pain	6	2	0
Body pain	0	2	0
Abdominal pain	7	2	1
Joint swelling	0	0	0
Fatigue	8	3	1
Lack of appetite	2	0	0
Chills	1	0	0

Conclusions

This study reveals that brucellosis is an important public health issue in Pakistan, especially among high-risk professionals and among those who consume unpasteurized milk and milk products. Because brucellosis is a zoonotic disease, control or eradication of this disease in animals is strongly advised.

Acknowledgments

Shahzad Ali is recipient of an Indigenous PhD Fellowship and International Research Support Initiative Program (IRSIP) from the Higher Education Commission, Pakistan at Friedrich-Loeffler-Institut, Jena, Germany.

Disclosure Statement

No competing financial interests exist.

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