Original article

Sero-prevalence of brucellosis in humans population of Charsasdda, Khyber Pakhtunkhwa, Pakistan

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Abstract

Background: Brucellosis caused by *Brucella* spp, is an infectious and zoonotic disease of wild and domestic animals of worldwide distribution.
Aim: The present research was conducted to determine the sero-prevalence of brucellosis in humans, *Homo sapien* (L, 1758) population in Charsadda, Khyber Pakhtunkhwa (KP), Pakistan during August 2014-January 2015.
Methods: Blood samples (n=200) were collected randomly during February-December 2014. They were screened for brucellosis by using Serum Plate Agglutination Test (SPAT) and Polymerase Chain Reaction (PCR).
Results: Two species, *B. abortus* and *B. melitensis* were present separately or in the same sample by SPAT while only separately by PCR. Out of 200 samples of humans 10% and 7.5% were positive by using SPAT and PCR, respectively. The prevalence of the disease in males were, 12% by SPAT while 9% by PCR, in female, 8% by SPAT (males to females' ratio of 3:2) while 6% by PCR(males to females' ratio of 4:3). In different age groups of humans, i.e., 1-20, 21-40, 41-60, and >60 were as 10.38, 9.52, 12.00, and 7.14% by SPAT while 7.79, 8.33, 4.00, and 7.14% by PCR, respectively. In professional groups of humans, i.e., farmers, animal keepers, servants, house-wives and others, the prevalence was 11.42, 12.5, 0, 8.75 and 10.71% by SPAT while 8.57, 6.25, 0, 7.5, 7.14 and 3.75% by PCR, respectively.
Conclusion: The sero-prevalence of brucellosis in humans is existed in the study area.
Recommendation: An effective control programme of the disease is recommended.

Keywords: *Brucellosis, infectious, Polymerase Chain Reaction (PCR), Serum Plate Agglutination (SPAT), zoonotic disease.*

Introduction

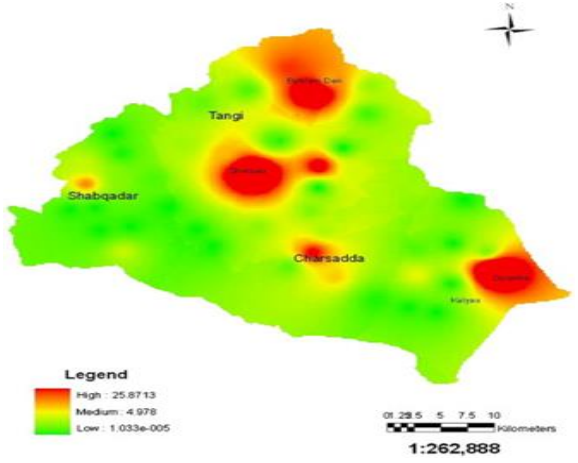
Brucellosis is an infectious disease caused by bacteria of genus *Brucella*. Originally, it is actually a zoonotic disease and is a problem of wild and domestic animals globally; the areas such as the Middle East, Mediterranean, Latin America, and Asia have high prevalence especially in sheep, goats, and cattle etc (1). The evolution of brucellosis as a disease is closely linked with the development of humankind, i.e., when they started to cultivate, domesticate and popularize animals. Brucellosis has further linked with military campaigns being a leading problem at the time of Crimean war. The name *Brucella* was given to the bacteria in honor of David Bruce. Different species of *Brucella* are *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, *B. neotomae*, *B. pinnipediae* and *B. cetaceae* etc (2). Brucellae are microscopic, non-motile, do not produce spores and toxin, facultative,

intracellular, gram-negative coccobacilli parasites (3).

Brucellosis may be present with acute onset, shows symptoms of continue or irregular fever of variable duration, abundant sweating, exhaustion, anorexia, weight loss, headache, arthralgia, generalized aching and swelling etc with a rare complication (4). The spread of the disease is facilitated by eating practices, society, use of untreated milk and milk products, careless husbandry practices, climatic and environmental cleanliness, socioeconomic position etc. However, humans are infected from contaminated pets and very rarely from humans (5).

In Pakistan, sero-prevalence was found to be 6.9% by real time PCR and 21.7% by ELISA respectively in Potohar plateau and in district Lahore in humans at high risk, and in slaughterhouse workers (6). PCR is considered as a standard method for the diagnosis of Brucellosis in humans as compare to other serological methods as these tests gave false positive results due to cross reactivity with other bacterial species (7).

The district Charsadda (KP, Pakistan) is enclosed to district Mardan by south, west, and north by district Malakand and east by Union Council Kaoz Bahram Dheri. It is situated with an altitude of 276 m (908 feet) and 29 Km from the provincial capital Peshawar. It is beautiful, lush green area and provided true natural habitats for development of fauna and flora (Figure 1). The main fruits are pricot, *Prunus Armeniaca* (L, 1758); guava, *Psidium guajava* L, 1758; citrus, *Citrus* spp. L, 1758; plum, *Prunus cerasifera* Ehrh; peach, *Prunus persica* (L, 1758); pear, *Pyrus calleryana* Decne; banana, *Musa acuminate* Decne; apple, *Malus domestica* Borkh, 1803; date, *Phoenix dactylifera* L, 1758; pomegranate, *Punica granatum* L, 1758; mango, *Magnifiera indica* L, 1758; grapes, *Vitis vinifera* L; fig, *Ficus carica* L; avocado, *Persea americana* Mill 1758 and strawberry, *Fragaria ananassa* Duchesne etc are the commercially important fruits of this area. The main crops are wheat, *Triticum aestivum* L; maize, *Zea mays* L; rice, *Oryza sativa* L and sugarcane, *Saccharum officinarum* L. (8) Important wildlife animals and birds are including monkey, *Macaca mulatta* Zimmermann; black bear, *Ursus americanus* Pallas; *Naemorhedus goral*; wolf, *Canis lupus lupus* L; fox, *Vulpes vulpes* (L); jungle cat, *Felis chaus* Schreber; cat, *Felis domesticus* L, Himalayan monal, *Lophophorus impejanus* Latham and koklass pheasant, *Pucrasia macrolopha* Lesson. The disease, brucellosis was neglected in Pakistan, especially in rural areas (9).



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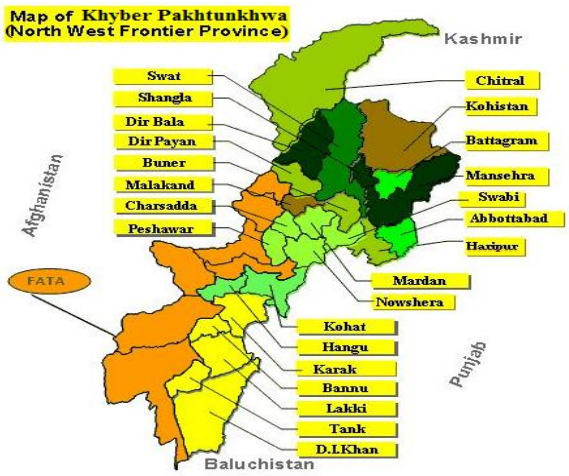

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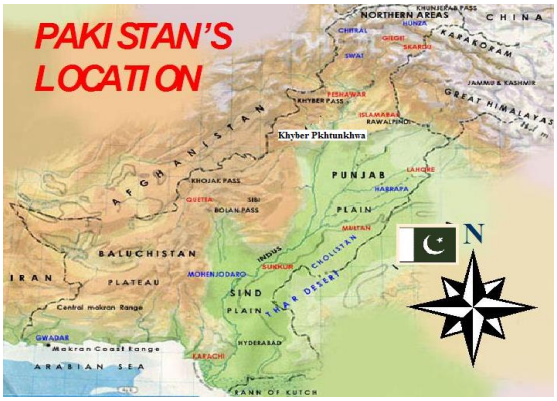

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Figure 1. Map of the study area, district Charsadda (A) is located in Khyber Pakhtunkhwa (KP) (B), one of the province of Pakistan (C); where the present research was conducted for sero-prevalence of brucellosis in humans, *Homo sapien* (L, 1758) during August 2014-January 2015 (10)

The present research is the first report in the present study area, i.e., Charsadda to estimate the sero-prevalence of life threatening disease of zoonotic importance, i.e., brucellosis in general population by using SPAT and PCR.

Materials and methods

District Charsadda is located at 34° 8 43" north and 71° 43 53" east having area of 996 km2 (243753 acres) with a population of 1.7 million, mostly related to agriculture and livestock. About 86% of the total area (729.80 km2) is cultivated.

The sero-prevalence of brucellosis was determined in humans, Homo sapien (L, 1758) population of Charsadda, KP, Pakistan during August 2014-January 2015. The blood samples (n=200) were randomly collected during February-December 2012 by a venipuncture performed using hypodermic needle® (27 gauge: BD, Karachi, Pakistan) and 5 ml disposables syringe® (BD, Karachi, Pakistan) from humans (male or female: 1: 1). They were transferred to a 3 ml EDTA (anticoagulant: ethylene diamine tetra acetate) tubes. They were kept and stored at –20 ºC in refrigerator for experiments, which were conducted at Veterinary Research Institute (VRI) Peshawar, KP, Pakistan. They were centrifuged at 12000 rpm for 5 min to separate serum, and stored at the same frizzing temperature as mentioned above. They were tested for presence of antibodies of Brucella spp, using serum plate agglutination test (SPAT), which was further confirmed by using PCR.

Serum plate agglutination test (SPAT)

On a glass slide 20 µl of serum was taken with micropipette® (BD, Karachi, Pakistan), then each one drop of antigens of Brucella abortus and B. melitensis were added and rotated clockwise and anticlockwise with tooth picks for proper and complete reaction of antigens with serum. However, slide with agglutination under magnifying glass was considered as positive and vice versa.

DNA extraction

Polymerase Chain Reaction (PCR) Kit® [Shangai Zhijiang Biotechnology Company Limited (SZBCL), Shangai, China] was used. Serum 50 µl was pipetted in a 0.5 ml tube, then 50 µl DNA extraction buffer was added, it was then closed and vertexed for 10 sec. Then it was incubated at 100 °C for 10 min and centrifuged at 13000 rpm for 10 min. The supernatant contained DNA extracted and can be used for PCR template.

Polymerase chain reaction (PCR)

Oligonucleotide primers are specific for the direct amplification of Brucella spp, which were used for the detection of the same in blood by PCR. The master mixed volume for each reaction was pipetted as with 35 µl reaction mixed with 0.4 µl enzyme [10X PCR buffer, MgCl2 (25 mm), dNTPs (500 µM), primer 1 mixed with primer 2] and 9.6 µl PCR water was separately added with 5 µl sample DNA in a volume of 50 µl. The reaction was performed in a thermal cycler® (Multigene Labnet International Inc., Wood Bridge, USA). The cycling conditions were an initial denaturation at 95 °C for 2 min, template denaturation at 93 °C for 15 sec, annealing at 55 °C for 30 sec, and primer extension at 72 °C for 30 sec for a total 35 cycles with a final extension at 72 °C for 10 min.

Agarose Gel Electrophoresis (AGE)

The agarose gel 1.2 gm was taken in a flask and 1 X, TAE (tris acetate EDTA: ethylene diamine tetra acetic acid) buffer 100 ml was added, it was vertexed and heated in microwave oven for 3 min. Then the solution was cooled at -55 °C and ethedium bromide 0.4 µl was added. The gel was poured in gel caster containing comb and allowed to solidify, after about 45 min the comb was pulled out from the gel slots. Then it was placed in gel tank containing 1 X TAE buffer, 5 µl loading dye was added to 10 µl DNA (i.e., Bromophenol Blue) and loaded. Then 5 µl of DNA marker was loaded on both right and left sides of the gel. Voltage (120 V) was applied for 45 min. The gel was examined by UV light and the specific amplified DNA product was determined by identifying the base pair (bp) of DNA bands comparing with 100-bp DNA leader used as DNA marker and was photographed using gel documentation system.

Statistical analysis

Data were analyzed by using one-way ANOVA at $p<0.01$ and un-weighted pair of group arithmetic mean (UPGAM) (11, 12).

Results

The present research was carried out to evaluate the prevalence of brucellosis (n=200) in the human population of Charsadda, KP, Pakistan. However, by using SPAT, 10% samples were positive with brucellosis, i.e., 2.5% with *Brucella abortus*, 3.5% with *B. melitensis* and 4% with both. Moreover, by using PCR, the positive samples were 7.5%, in which 2.5% with *B. abortus* and 5% with *B. melitensis* (Table 1).

Similarly, the gender wise brucellosis (n=100 for each sex) was also determined in the same population. In males, 12% samples were found positive with brucellosis by SPAT, i.e., 3% with *B. abortus*, 4% with *B. melitensis* and 5% with both, respectively. using PCR, 9% samples were found positive in which 3% with *B. abortus* and 6% with *B. melitensis.* Moreover, in females, 8% samples were positive by SPAT in which 2% with *B. abortus*, 3% with *B. melitensis* and 3% with both *B. abortus* and

B. melitensis. Furthermore, by using PCR, 6% samples were found positive in which 2% with *B. abortus* and 4% with *B. melitensis* (Table 2).

Table 1 Prevalence of brucellosis in the human population of Charsadda, Khyber Pakhtunkhwa, Pakistan during August 2014-January 2015

S No	Tests for presence of antibodies	Species	Human samples (n/%)
		n[1]	200
1.	SPAT[2]	*B*[3]*. abortus*	5
2.		*B. melitensis*	7
3.		Both[4]	8
4.		%[5]	10
1.	PCR[6]	*B. abortus*	5
2.		*B. melitensis*	10
3.		Both	0
4.		%	7.5

[1]n: total number of samples; [2]SPAT: Serum Plate Agglutination Test; [3]*B: Brucella*; [4]Both: sample positive for both *B. melitensis* and *B. abortus* antigens; [5]%: Percentage of total positive samples for SPAT and PCR; [6]PCR: Polymerase Chain Reaction

Table 2 Gender wise prevalence of brucellosis in the human population of Charsadda, Khyber Pakhtunkhwa, Pakistan during August 2014-January 2015

S No	Tests for presence of antibodies	Sex	Male	Female	Total (n/%)
		n[1]	100	100	200
1.	SPAT[2]	*B*[3]*. abortus*	3	2	5
2.		*B. melitensis*	4	3	7
3.		Both[4]	5	3	8
4.		%[5]	12	8	11.5
1.	PCR[6]	*B. abortus*	3	2	5
2.		*B. melitensis*	6	4	10
3.		Both	0	0	0
4.		%	9	6	8.5

[1]n: total number of samples; [2]SPAT: Serum Plate Agglutination Test; [3]*B: Brucella*; [4]Both: sample positive for both

Table 3 Age wise prevalence of brucellosis in the human population of Charsadda, Khyber Pakhtunkhwa, Pakistan during August 2014-January 2015

SNo	Tests for presence of antibodies	Age	1-20	21-40	41-60	>60	Total (n/%)
		n[1]	77	84	25	14	200
1.	SPAT[2]	*B*[3]*. abortus*	2	2	0	1	5
2.		*B. melitensis*	3	2	2	0	7
3.		Both[4]	3	4	1	0	8
4.		%[5]	10.38	9.52	12	7.14	12.50
1.	PCR[6]	*B. abortus*	2	2	0	1	5
2.		*B. melitensis*	4	5	1	0	10
3.		Both	0	0	0	0	0
4.		%	7.79	8.33	4.00	7.14	9.00

[1]n: total number of samples; [2]SPAT: Serum Plate Agglutination Test; [3]*B: Brucella*; [4]Both: sample positive for both

Further, the age wise brucellosis was determined in the same population. In age group ranging from 1-20 years (n=77), in which 10.38% were positive for brucellosis, however, by using SPAT, 2.5% with *B. abortus*, 3.9% with *B. melitensis* and 3.9% with both. Moreover, by using PCR, 7.79% samples were positive, 2.5% with *B. abortus* and 5.29% with *B. melitensis.* In age group ranging from 21-40 years (n=84), 9.52% were positive for brucellosis; however, by SPAT, in which each 2.4% with *B. abortus* and *B. melitensis* while 4.8% with both. Moreover, by using PCR, 8.33% samples were positive, in which 2.4% with *B. abortus* and 6.0% with *B. melitensis.* In age group 40-60 years (n=25), in which 12% were positive by using SPAT, however, no sample with *B. abortus*, but 8% with *B. melitensis* and 4% with both. Moreover, by PCR 4% samples were positive in which no sample with *B. abortus*, but 4% with *B. melitensis.* Age group >60 years (n=14), in which 7.14% were positive, however, by using both SPAT and PCR, each 7.14% with *B. abortus*, moreover, no one with *B. melitensis*, and both. Likewise, different professions of population of Charsada were also considered for the prevalence of brucellosis. Therefore, samples collected from farmers (n=70), in which 11.42% were positive for brucellosis, while by using SPAT, each 2.86% with B. abortus, B. melitensis and 5.71% with both. However, by using PCR, 8.57% samples were positive for brucellosis, in which 2.86% with B. abortus and 5.71% with B. melitensis. Moreover, samples collected from animal keepers (n=16), 12.5% of them were positive for brucellosis, in which each 6.25% with B. abortus and B. melitensis, while no with both. Further, from government servants (n=6), no sample was found positive for brucellosis. In addition, most of the women were house-wives (n=80), their 8.75% samples were positive for brucellosis, while by using SPAT in which each 2.5% with B. abortus, B. melitensis and 3.75% with both. Furthermore, by using PCR, 7.5% samples were positive for brucellosis, in which 1.25% with B. abortus and 6.25 with B. melitensis. Additionally, rest of the samples were grouped as others (n=28), 10.71% of them were positive for brucellosis, while by using SPAT, no with B. abortus, 7.14% with B. melitensis and 3.57% with both. Furthermore, by using PCR, 7.14% samples were positive for brucellosis, in which each 3.57% with B. abortus and B. melitensis, respectively (Figure 2). The presence of antibodies of *Brucella* spp were confirmed by using Polymerase Chain Reaction (PCR) for prevalence of brucellosis in human population in Charsadda, Pakistan. PCR slide of blood samples (n=200) humans, *Homo sapien* was made in Veterinary Research Institute (VRI), Peshawar, Pakistan on 19 December 2011 which shows DNA Marker (1), positive sample with *B. abortus* (2), positive with *B. melitensis* (3) and negative samples (4 and 5) (Figure 3).

Discussion

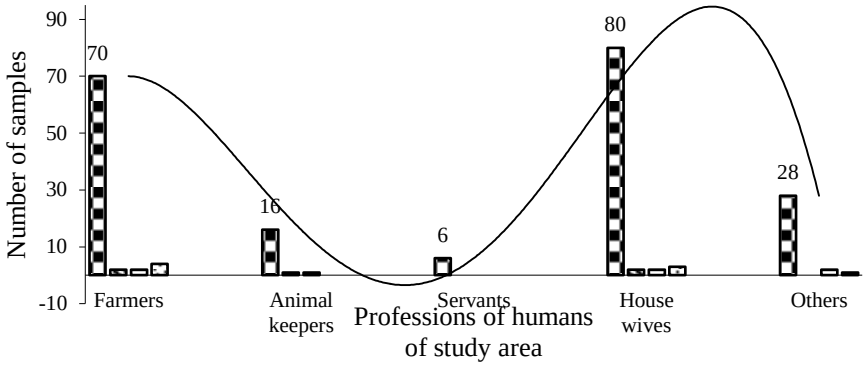
In the present research, out of 200 samples of humans 20 (10%) samples were found positive with brucellosis by using SPAT. Karimi et al. (13) reported a prevalence rate of 2% in general population by using SPAT in Islamic Republic of Iran. Adam and Hussan (15) in Sudan reported a prevalence of 8.9% by Serum Tube Agglutination Test (STAT). The findings of the above 1st study are much contrast with the present research, i.e., this study was conducted in 2003 and the disease being infectious is spreading with time. The finding of the 2nd study is more or less similar slight difference might be due the use of different test for screening, also the environmental conditions of 2 regions are different.

At the present, by using PCR the positive samples were 15 (7.5%). A study conducted by Jobran et al. (14) in Najran, Southwestern Saudi Arabia found 38 (11.17%) prevalence of brucellosis in patients having clinical characteristics of brucellosis by using PCR in 340 samples. The prevalence of brucellosis is more in the above study as compare to the present research may be due to the difference in sampling population and environmental conditions.

At the present, out of 100 samples of males and females each, 12% and 9% of males and 8% and 6% of female samples were found positive on SPAT (males to females' ratio of 3:2) and PCR (males to females' ratio of 4:3), respectively. Adam and Hussan (15) reported a male to female ratio of 2.4:1 in Khartoum State, Sudan. Ahmad et al. (7) noticed that sero-positivity in the Yafran municipality, Libya was higher among males than females, with 66% of samples from males and 34% from females positive for *Brucella* infection. From above 2 studies, it is clear that brucellosis is more prevalent in males as compare to females which is more or less similar to the present research. It might be due to the reason that males are more exposed to the source of infection (16, 17).

It was reported that age constituted an important epidemiological risk factor for human brucellosis (18). In the present research, the highest prevalence was found in age group ranging from 40-60, i.e., 12% samples were positive by using SPAT. The age group having the lowest prevalence of brucellosis is >60 years, i.e., 7.14% samples were infected by using

SPAT and PCR. Adam and Hussan (15) reported that the average age of brucellosis patient was 43.9 years



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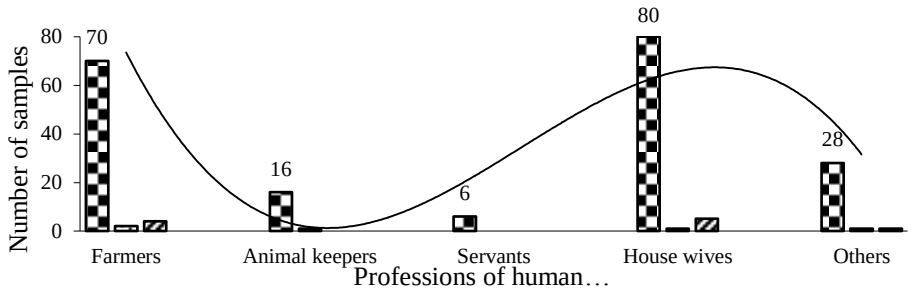

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Figure 2 Prevalence of brucellosis in different professions of human population in Charsadda, Khyber Pakhtunkhwa, Pakistan during August 2014-January 2015 by using Serum Plate Agglutination Test (SPAT: **a**) and Polymerase Chain Reaction (PCR: **b**); : total number of samples; : samples positive with *Brucella abortus*; : samples positive with *B. melitensis*; : sample positive with both *B. melitensis* and *B. abortus* (only for SPAT); data were analyzed by two factor without replication Anova; degree of freedom (df): 3, 4 (for SPAT) and 2, 4 (for PCR); F test value: 7.299 were significantly different when compared only rows (for SPAT) and 10.703 were significantly different when compared rows and columns

The presence of antibodies of *Brucella* spp were confirmed by using Polymerase Chain Reaction (PCR) for prevalence of brucellosis in human population in Charsadda, Pakistan. PCR slide of blood samples (n=200) humans, *Homo sapien* was made in Veterinary Research Institute (VRI), Peshawar, Pakistan on 19 December 2011 which shows DNA Marker (1), positive sample with *B. abortus* (2), positive with *B. melitensis* (3) and negative samples (4 and 5) (Figure 3).

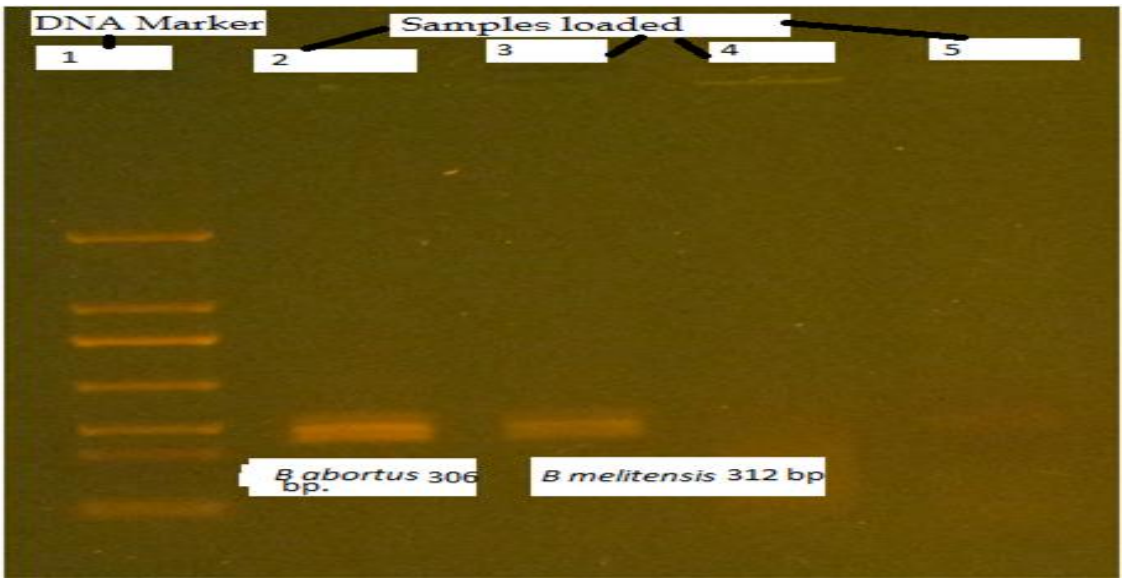

Figure 3 Confirmation of presence of antibodies of *Brucella* spp by using Polymerase Chain Reaction (PCR) for prevalence of brucellosis in human population in Charsadda, Khyber Pakhtunkhwa, Pakistan: positive PCR slide of blood samples (n=200) humans, *Homo sapien* was made in Veterinary Research Institute (VRI), Peshawar, Pakistan on 19 December 2014; 1: DNA Marker; 2: positive sample with *B. abortus;* 3: positive with *B. melitensis*; 4, 5: negative samples

in Khartoum, Sudan. The people in this age are more strictly attached to their work and its place, however, *Brucella* infects individuals of any age group.

At the present, of 70 farmers 11.42% and 8.57% samples were found positive for brucellosis by using SPAT and PCR, respectively. Apan et al. (19) that reported 32 (13%), 35 (14.22%) and 44 (17.88%) by RBPT, SAT and ELISA, respectively out of 246 samples, collected from the farmers in Kars district, Turkey between 2004 2006. In a study conducted in Middle Anatolia, the sero-prevalence of brucellosis was determined as 3.2% in farmers. In another study performed in the same region, 4.8% sero-positivity of *Brucella* was reported for humans living in the rural area (20). The finding of the above 1st study is higher as compare to the present research, this is due to different time period and study design, while the findings of the next 2 studies are less as compare to the present study, it might be due different living styles of the people in different study areas.

In the preset research, in animal keepers, the prevalence rate of brucellosis was 12.5% and 6.25% by using SPAT and PCR, respectively, in 16 samples. Musa et al. (21) reported from Nyala, Southern Darfur, Western Sudan, a brucellosis prevalence of 18% among febrile patients with history of animal contact. This is in contrast with the findings of the present research where the samples were collected randomly from normal population.

At the present, most of the women were house wives, and were having a frequent animal contact, of 80 samples prevalence of brucellosis was 7 (8.75%) and 6 (7.5%) by using SPAT and PCR, respectively. Otlu et al. (22) reported that 20.68% prevalence of brucellosis in females with animal contact using ELISA in Kars, Turkey from 2004-2006. Adam and Hussan (15) reported that a prevalence rate of 8.9% in febrile patients having animal contact by using STAT. the finding of the 1st study is in contrast with the present research. The study was conducted in different time with different experimental design. The finding of the 2nd study is little bit difference, is due to different samples of population that is febrile patients.

In the present research, from servants 6 samples were collected in which no sample was found positive for brucellosis. Rest of the people were grouped as others and having 28 samples, 3 (10.71%) of them were found positive for brucellosis by using SPAT, 2 (7.14%) by PCR. It becomes eminent that people having no animal contact (23) are at minimum risk of getting brucellosis. An organized obliteration programme may be comprised public information and government should inaugurate educational seminars or symposium for public awareness. Routine or regular requests for laboratory diagnosis is being done for other endemic disease like typhoid-fever and malaria etc should be commenced to augment proper diagnosis.

Conclusion

The sero-prevalence of brucellosis (n=200) was 10% and 7.5% positive by using SPAT and PCR, respectively. The males to females' ratio was 3:2 by SPAT and 4:3 by PCR. In animal keepers, the prevalence was the highest, i.e., 12.5 by SPAT compared with other professional group. Two species, *B. abortus* and *B. melitensis* have been identified; they were present weather separately or in the same sample by SPAT while only separately by PCR. The present study indicated that the prevalence of brucellosis was found in humans to some extend in Charsadda, Pakistan.

Recommendations

Environmental, personal, and domesticated animals' hygiene should be maintained in the study area. A regular checkup of humans and their cattle should be suggested for the presences of the *Brucella* spp. All measured for the prevention of illness and maintenance of health should be applied. An effective control programme of the brucellosis is recommended.

Acknowledgments

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References

1. Rijpens O, Nancy P, Jannes G, Van Asbroeck M, Rossau R, Herman LM. Direct detection of *Brucella* spp in raw milk by PCR and reverse hybridization with 16S-23S rRNA spacer probes. Appl Environ Microbiol. 1996; 62(5): 1683-1688.

2. Sohn AH, Probert WS, Glaser CA, Gupta N, Bollen AW, Wong JD, Grace EM, Donald MWC. Human neurobrucellosis with intracerebral granuloma caused by a marine mammal *Brucella* spp Emerg Infect Diseas. 2003; 9: 485-488.

3. Morad AA, Moreno E, Moriyon I. *Brucella melitensis*: a nasty bug with hidden credentials for virulence. Proc Natl Acad Sc USA 2002; 99: 1-3.

4. WHO guidance Geneva (World Health Organization) () Brucellosis in humans and animals. Saudi Med J. 2005: 20 (4): 311-315.

5. Tikare NV, Mantur BG, Bidari LH. *Brucella meningitis* in an infant evidence for human breast milk transmission. J Tropic Pediat. 2008; 54: 272-274.

6. Mukhtar F. Brucellosis in a high risk occupational group: Sero-prevalence and analysis of risk factors. J Pak Med Associ. 2010; 60: 1031-4.

7. Ahmed MO, Elmeshri SE, Abuzweda AR, Blauo M, Abouzeed YM, Ibrahim A, Salem H, Alzwam F, Abid S, Elfahem A, Elrais A. Sero-prevalence of brucellosis in animals and human population in the western mountainous region in Libya, December 2006-January 2008. Europ Surve. 2010; 15(30): 619-625.

8. Perveen F, Ahmad A. Exploring butterfly fauna of Kohat, Khyber Pakhtunkhwa, Pakistan. Sign Open Access J. Entomol Stud. 2012; 1(2): 94-107;

9. Perveen F, Rehman FU. Occurrence of Indian flying fox, *Pteropus giganteus* (Bunnich) (Chyroptera: Pteropodidae) in district Charsada, Khyber Pakhtunkhwa, Pakistan. Intl Fauna Biol Stud. 2014; 1(5): 61-64.

10. OCHA Pakistan. Pakistan: North West Frontier Province District, Tehsil and Union 2009; Online: http://upload.wikimedia.org/wikipedia/commons/7/7f/Phalantha_phalantha.png (Accessed, 12/5/2014).

11. Perveen F, Kashif M. Frequency of infestation of gastrointestinal helminthes parasites in horse, *Equus ferus* in Peshawar, Pakistan. Res Opin Anim Vet Sc. 2012a; 2(3): 145-147

12. Perveen F, Kashif, M. Comparison of infestation of gastrointestinal helminth parasites in locally available equines in Peshawar, Pakistan. Res Opin Anim Vet Sc. 2012b; 2(6): 412-417

13. Karimi A, Alborzi A, Rasooli M, Kadivar MR, Nateghian AR. Prevalence of antibody to *Brucella* sp in butchers, slaughterers and others East Mediterranean. Heal J. 2003; 9: 178-81.

14. Jobran A, Miree A, Alqahtani A. Serological and molecular diagnosis of human brucellosis in Najran, Southwestern Saudi Arabia. **J Infec Public Heal.** 2012; 5(2): 189-194.

15. Adam AAM, Hassan SH. Human brucellosis in Khartoum state: A commonly under diagnosed disease. Sudan J Med Sc. 2010; 5(3): 223-228.

16. Perveen F, Kashif M, Bilqees FM. Frequency of infestation of gastrointestinal helminthes parasites in hinny/mule, *Equus mulus* in Peshawar, Pakistan. Proc Parasitol. 2012a; 54: 65-74.

17. Perveen F, Ullah N, Shabbir I, Shah R. Infestation of gastrointestinal nematode parasites in sheep, *Ovis aries* L in Peshawar, Pakistan. Proc Parasitol. 2012b; 53: 25-36.

18. Youssef, A., Eissa, A. Brucellosis in Saudi Arabia: past, present and future. Ann Saudi Medic. 1999; 19 (5): 403-404.

19. Apan T, Yildirim M, Istanbulluoglu E, Ozarslan B. The studies on brucellosis seroprevalence at

humans, sheep and cattle populations in Kirikkale. Turk J Vet Anim Sc. 2007; 31: 75-78.
20. Cetinkaya Z, Aktepe OC, Ciftci IH, Demirel R. Sero-prevalence of human brucellosis in a rural area of Western Anatolia, Turkey. J Heal Popu Nutri. 2005; 23: 137-141.
21. Musa MT, Shigidi MT, Saeed O M. Brucellosis in humans associated with animals. Saudi Med J. 2000; 20(4): 311-315.
22. Otlu S, Sahin M, Atabay HI, Unver A. Serological investigations of brucellosis in cattle, farmers and veterinarians in the Kars District of Turkey. Turkey Acta Vet. 2008; 77: 117-121.
23. Perveen F, Kashif M, Bilqees FM, Shah R. Frequency of infestation of helminthes parasites in donkeys, *Equus asinus* in Peshawar, Pakistan. Proc Parasitol. 2011; 52: 55-63;