Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Molecular Characterization of *Brucella abortus* and *Brucella melitensis* in Cattle and Humans at the North West of Pakistan

Muhammad Zahoor Khan[1,3], Tahir Usman[1,2*], Ummer Sadique[3], Muhammad Subhan Qureshi[3], Mohammad Farooque Hassan[4], Muhammad Shahid[5] and Adnan Khan[1,3]

[1]Key Laboratory of Agricultural Animal Genetics and Breeding, National Engineering Laboratory for Animal Breeding, College of Animal Science and Technology, China Agricultural University, Beijing 100193, PR China; [2]College of Veterinary Science and Animal Husbandry, Abdul Wali Khan University Mardan, Pakistan; [3]Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar, Khyber Pakhtunkhwa, Pakistan; [4]Shaheed Benazir Bhutto University of Veterinary and Animal Sciences, Sakrand, Sindh, Pakistan; [5]Microbiology and Biotechnology Center, Veterinary Research Institute, Khyber Pakhtunkhwa, Peshawar, Pakistan
*Corresponding author: tahircau@gmail.com; tahirusman@awkum.edu.pk

ARTICLE HISTORY (16-309)

Received: November 29, 2016
Revised: February 01, 2017
Accepted: June 03, 2017
Published online: July 24, 2017

Key words:
Brucellosis
Duplex PCR
Prevalence
SPAT
Zoonosis

ABSTRACT

Brucellosis is a fatal zoonotic disease caused by members of the genus *Brucella*, resulting in significant reproductive losses in animals. The present study was designed to evaluate the prevalence of *Brucella abortus* and *Brucella melitensis* by screening the serum of the blood samples through Serum Plate Agglutination Test (SPAT) assay and by duplex PCR. Blood samples were randomly collected from cattle (n=200) and human (n=200), and were placed in two groups with respect to their contact (direct and indirect) with cattle. The overall prevalence of brucellosis in cattle and human through SPAT assay was 15 and 6%, respectively. Amongst human, the prevalence was 10% in female and 2% in the male. The high rate of infection was found in females than males due to their frequent contact with cattle compared to males in the study area. Molecular diagnosis using duplex PCR showed 13 and 4% prevalence of brucellosis in cattle and human, respectively. The duplex PCR revealed 6 and 2% positive cases in female and male, respectively (P>0.05). Collectively, these results suggested a high prevalence of the diseases in humans (females) having direct contact with cattle. Furthermore, the results infer that the optimized PCR approach is more efficacious, specific and reliable compared to the routine conventional SPAT assay.

To Cite This Article: Khan MZ, Usman T, Sadique U, Qureshi MS, Hassan MF, Shahid M and Khan A, 2017. Molecular characterization of *Brucella abortus* and *Brucella melitensis* in cattle and humans at the North West of Pakistan. Pak Vet J, 37(3): 360-363.

INTRODUCTION

Brucellosis is an imperative contagious and zoonotic disease of bacterial origin in cattle possessing a potential health alarm and economic importance worldwide (Mustafa *et al.,* 2016). Both *B. abortus* and *B. melitensis* are the predominant causative agents of brucellosis in animals and humans (Gul *et al.,* 2007, 2013). The causative agents of brucellosis are facultative intracellular gram-negative bacteria belonging to the genus *Brucella*. These are facultative anaerobes, non-spore forming, gram-negative and non-motile coccobacillus bacteria, which are classified into six species based on their preference for the host (O'Callaghan and Whatmore, 2011). The clinical signs of *Brucella* infection in the female cow are the death of the calves, stillbirth, reduced milk yield and predominantly abortion in last trimester, whereas, infertility is the most common sign in male (Abubakar *et al.,* 2010). In humans, the common signs and symptoms of brucellosis are sudden onset of septicemia followed by undulant fever, chills, nocturnal sweating, weaknesses, restlessness and headache (El-Koumi *et al.,* 2014). A common mode of transmission to humans include using unpasteurized milk and its products, direct contact with infected animals, aborted fetuses or their excreta. Brucellosis is recognized as an occupational disease and most commonly affecting veterinary practitioners, farmers, butchers and other workers which have close contact with animals or their products (Dean *et al.,* 2012). The disease was found to be the major health problem

both for livestock and human in North African countries (Jennings *et al.,* 2007). Social and economic factors were also documented to be the most important risk factors for brucellosis in Sub-Saharan and North African regions (Mitka *et al.,* 2007). The prevalence of brucellosis has also been documented in Pakistan with increasing occurrence of the disease in the large dairy farms (Mukhtar, 2010). In Pakistan, this disease is most commonly found in rural women as they are extensively involved in handling the animals and their products (Abubakar *et al.,* 2011). *Brucella* infection is widespread in subcontinent constituency showing severe social and economic threats to both human and livestock (Ali *et al.,* 2013).

Various kinds of serological tests, for example milk ring test (MRT), rose Bengal precipitation test (RBPT), and serum plate agglutination test (SPAT) are appropriate for initial screening of *Brucella* infection in cattle (Gul *et al.,* 2007). Serological tests are the major diagnostic tools for screening of animals and humans brucellosis in the field; they are neither fully sensitive nor specific due to insufficient immunity or serological cross-reactivity. Molecular tests are preferred over the conventional tests because serological tests procedures are laborious for large-scale diagnosis and detection of the pathogen by PCR in DNA samples of the host is a true indication of the presence of a particular pathogen (Gwida *et al.,* 2011). Brucellosis is a complicated disease and cannot be diagnosed by single diagnostic test; therefore, the combination of serological and molecular tests is needed for the identification of *Brucella* species. None the less, these tests carry some drawbacks (Abubakar *et al.,* 2011). In Pakistan, veterinarians mostly depend on conventional methods of screening due to limitations of facilities and financial issues. The RBPT, SPAT and MRT are the most commonly performed tests for identification of this disease at both government and private veterinary laboratories in Pakistan (Gul *et al.,* 2007).

Due to lack of specificity and sensitivity of serological tests and culture techniques, different molecular methods have been optimized both for the diagnosis of bovine and human brucellosis (Al-Dahouk *et al.,* 2007). The PCR has widely been used to detect *Brucella* DNA even at biovar levels (Chimana *et al.,* 2010). Keeping in view the above-mentioned review, the present study was conducted with the objectives, to document the prevalence of brucellosis in cattle and humans at district Karak, situated at the North West of Pakistan and to diagnose the *B. abortus* and *B. melitensis* on a molecular level by duplex PCR.

MATERIALS AND METHODS

Study area and sample size: In the present study, blood samples (n=200) were randomly collected each from cattle and human by simple random sampling method at district Karak, Khyber Pakhtunkhwa, Pakistan. The blood samples were also taken from their owners to check for the public health importance of the disease. The samples were collected from 50 male and 50 female having direct contact with the animal. A 5 mL of blood was taken from each animal and human. Human samples were categorized into two groups on the basis of their contact with animal i.e. direct and indirect contact. Out of 5 mL blood sample collected, 2 mL blood was taken in non-EDTA coated tubes for the collection of serum and 3 mL in EDTA coated tube for DNA extraction. The serum was separated from a blood sample and was screened using SPAT assay.

Diagnosis of brucellosis using SPAT: The serum samples and antigen used for screening of brucellosis were kept at room temperature for 30 to 50 min before use. A transparent glass slide was divided into 1.5" square with a wax pencil. A 20 µL of serum was taken on a glass slide with the help of micropipette and one drop of *Brucella* antigen was added. Similarly, one drop of *B. melitensis* antigen was added in the 20 µL of serum on another square. The slide was manually rotated for 2-3 min. The samples forming visible complex were declared as positive (Alton *et al.,* 1988).

DNA extraction and PCR: The DNA was extracted from all the blood samples using DNA extraction kit (Bio Basic Inc, Canada) as per the manufacturer's instructions. For PCR assay, two sets of oligonucleotide primers, BM-f (forward) and BM-r (reverse) for *B. melitensis*, BA-f (forward) and BA-r (reverse) for *B. abortus* were used. These primers were synthesized at the Invitrogen (Carlsbad, California) and were specific for IS711. The primer pairs used in the present study for *B. melitensis* and *B. abortus*, were: BM-F, 1) 5'-AAA TCG CGT CCT TGC TGG TCT GA3' and BM-R, 5'-TGCCGA TCA CTT AAG GGC CTT CAT-3'. 2) BA-F, 5'-GAC GAA CGG AAT TTT TCC AAT CCC-3' and BA-R 5'-TGCCGA TCA CTT AAG GGC CTT CAT-3', respectively *(*Azar *et al.,* 2006). The PCR product consisted of 731bp for *B. melitensis* and 500bp for *B. abortus*. PCR reactions were performed in a 50 µL reaction volume, containing 19.5 µL 1x PCR Master Mix, 2 µL of each primer (Forward and Reverse), 5 µL template DNA and 21.5 µL nuclease free water according to the manual provided by the company (Shanghai ZJ Bio-Tech Co., Ltd. China). The amplification was performed in a thermal cycler (Multi–gene Labnet International Inc. USA), PCR products were visualized by gel electrophoresis using 1.5% of agarose and the photograph was taken on photo–documentation system (Infinity Vilber Lourmat, France). The clear bands of *Brucella* species DNA were considered as positive results (Fig. 1).

Statistical analysis: The data were analyzed using Chi-square test in order to record the prevalence status through SPAT and PCR tests and student t-test was used to compare the differences in the prevalence between different groups. Difference was considered statistically significant when $P \leq 0.05$.

RESULTS

In examining the occurrence of brucellosis in cattle, all collected samples were analysed through SPAT for initial screening followed by PCR for confirmatory diagnosis of the disease. The overall incidence of brucellosis documented in cattle was 15 and 13% through SPAT and PCR test, respectively. The percentage ratio of *B. abortus* and *B. melitensis* was 9 and 6%, respectively through SPAT, whereas, 7.5% of *B. abortus* and 5.5% of

B. melitensis was predicted through PCR (P>0.05; Table 1). Regarding the distribution of *Brucella* infection in humans, the samples showed 6 and 4% prevalence of the *Brucella* infection subjected to SPAT and duplex PCR, respectively. Moreover, the prevalence of *B. meltensis* was higher than *B. abortus* in human (P>0.05; Table 1). In order to determine whether the sex has any role in brucellosis, the infection was also assessed sex wise and it was established that the infection was 10 and 2% in females and males, respectively. The percentage ratio of *B. melitensis* was 8% while that of *B. abortus* was 2%. In males, 2% of *B. melitensis* and no case of *B. abortus* were documented (Table 2; P>0.05). To understand the zoonotic importance of *Brucella* infection, the prevalence of brucellosis in humans having direct contact with animal through SPAT and duplex PCR were documented (Table 3). The samples were screened through SPAT, showing 20% positive samples in case of females and only 4% in males (Table 3). The results showed the high ratio of brucellosis in female compared to male. Regarding duplex PCR results, a higher percentage ratio of 16% was documented in female compared to male 4% (P>0.05).

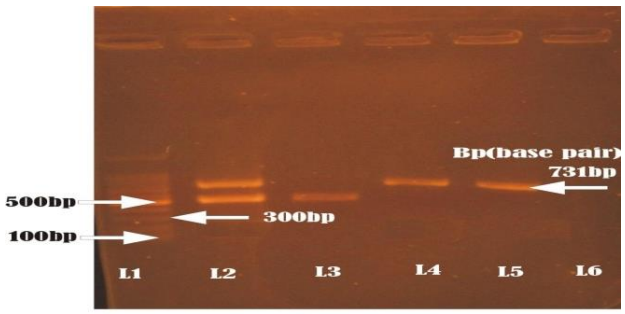

Fig. 1: Agarose gel electrophoresis of multiplex PCR-amplified products from purified DNAs of *B. abortus* and *B. melitensis*. Lane 1, DNA marker. Lane 2, *B. abortus* and *B. melitensis.* Lane 3, *B. abortus.* Lane 4, *B. melitensis*. Lane 5, *B. melitensis*. Lane 6, negative control (PCR buffer).

Table 1: Overall prevalence of brucellosis in cattle and humans through SPAT and duplex PCR

Test used	Positive			Negative
	Total	*B. abortus*	*B. meltensis*	
Cattle				
SPAT	30(15)	18(9)	12(6)	170(85)
PCR	26(13)	15(7.5)	11(5.5)	174(87)
P value				0.83
Humans				
SPAT	12(6)	2(1)	10(5)	188(94)
PCR	8(4)	-	8(4)	192(96)
P value				0.32

There were 200 samples in each test. Values in parenthesis indicate percentage.

Table 2: Sex-wise overall prevalence of brucellosis in human through SPAT and PCR

	Species	Positive			Negative
		Total	*B. abortus*	*B. meltensis*	
SPAT	Human(F)	10(10)	2(2)	8(8)	90(90)
	Human(M)	2(2)	-	2(2)	98(98)
PCR	Human(F)	6(6)	-	6(6)	94(94)
	Human(M)	2(2)	-	2 (2)	98(98)
P value					0.32

There were 100 samples in each test. Values in parenthesis indicate percentage.

DISCUSSION

Brucellosis is a complex infection in provisos of epidemiology, diagnosis and control. Though several serological and cultural tests are used to detect the disease, yet considerable problems remain in the diagnosis of this disease (Gul *et al*., 2014, 2015). Here, we report a series of studies supporting the more efficacious role of the two main serological and molecular tests commonly used in the screening of brucellosis in cattle and human at district Karak, Pakistan. The present study acknowledged high positive cases on SPAT (18%) compared to PCR (13%). In the case of specificity for PCR and SPAT, our reported data is in line with Mahmood *et al.* (2015), who observed that PCR is a more specific diagnostic tool in the identification of brucellosis in comparison to other conventional serological methods. Saleha *et al.* (2014) also concluded that PCR is more specific compared to SPAT and reported a prevalence of 37.1 and 25.7% of the disease on SPAT and PCR, respectively, in cattle of district Peshawar, Pakistan. The variation might be due to sample size, different habitat, poor management and free grazing of animals. However, the study of Bakhtullah *et al*. (2014) supported our reported study; the authors noticed 18% prevalence of brucellosis on SPAT at Bannu and Lakki Marwat districts of Khyber Pakhtunkhwa, Pakistan. The similarity is due to the same environment and livestock management practices prevailing in the southern Khyber Pakhtunkhwa. The more positive cases through SPAT might be due to cross-reactions with other Gram-negative bacteria such as Salmonella and *E. coli* (Saleha *et al.*, 2014).

Regarding sex-wise prevalence, we demonstrated a higher ratio of brucellosis in females as compared to males. Our research supported the study of Din *et al.* (2013), who reported 5.33% and 9.33% seroprevalence of brucellosis in human males and females, respectively through SPAT. Imad *et al*. (2011) screened high ratio of *Brucella* infection in females (41.5%) compare to males (29.5%), furthermore, the authors documented the higher occurrence of the disease in rural areas which show the potential zoonotic importance of brucellosis in rural areas. A series of studies reported a high prevalence of brucellosis in females compared to males and also investigated that PCR is a method of choice for the diagnosis of brucellosis (Tibesso *et al.*, 2014). Noticeably, females are more commonly affected by this disease because of their extensive involvement in the handling of animals and their products in rural areas of third world countries and are unawareness of proper handling of livestock and their product, safety procedures and livestock diseases. We noticed a higher incidence of *B. meltensis* than *B. abortus* in human, which is in line with previous studies (Tiller *et al.*, 2010; Catharina *et al.*, 2015), who described that morphologically all species of *Brucella* are identical yet each species has affinity for specific host i.e. *B. abortus* was more commonly found in cattle while *B. melitensis* predilection hosts are human, sheep and goats. Din *et al.* (2013), also observed 6 and 3% cases of *B. melitensis and B. abortus* in human at district Bhimber, AJK, Pakistan. The reason might be due to the host-pathogen interaction affinity. However, a solid reason behind the predilection affinity of *Brucella* species is yet unknown.

Table 3: Prevalence of brucellosis in humans having direct contact with animal using SPAT and PCR

	Species	Positive			Negative
		Total	*B. abortus*	*B. meltensis*	
SPAT	Human(F)	10(20)	2(4)	8(16)	40(80)
	Human(M)	2(4)	-	2(4)	48(96)
PCR	Human(F)	6(12)	-	6(12)	44(88)
	Human(M)	2(4)	-	2 (4)	48(96)
P value					0.31

There were 50 samples in each test. Values in parenthesis indicate percentage.

In the case of humans, our results raise the consideration of a high prevalence of *Brucella* infection in those people having direct contact with animals and also a high occurrence was noticed on SPAT in contrast to PCR test. These findings are in consistent with the study of Elfaki *et al.* (2005), who conducted similar kind of study in Saudi Arabia. Asif *et al.* (2014) also investigated higher ratio of *Brucella* cases in people who were more closely engaged in livestock practices and the frequency was administered at higher fraction through SPAT. The higher number of positive cases by SPAT as compared to PCR may be due to lack of specificity of SPAT. Secondly, PCR is more precise in judgment compare to other serological tools in use for the diagnosis of brucellosis.

Conclusions: The results of the present study showed that the disease is prevalent in both human and cattle in the North West of Pakistan. The outcome infer that the disease has zoonotic importance and the incidence was found higher in individuals who were in direct contact with animals compared to those who have indirect contact with animals. Collectively, these results suggest that the benefit may be obtained from the addition of SPAT for initial screening however duplex PCR is more accurate, sensitive and specific for the diagnosis of brucellosis in comparison to SPAT test. Using PCR for the accurate diagnosis of *Brucella* species is recommended as a tool of choice in both humans and cattle. The further in-depth study is recommended to evaluate the host immune response to *B. melitensis and B. abortus.*

Acknowledgments: Authors are thankful to the University of Agriculture Peshawar and Microbiology and Biotechnology Center, Veterinary Research Institute, Khyber Pakhtunkhwa, Peshawar, 25000, Pakistan for providing the chemicals and equipments for current research.

Authors contribution: TU and MSQ designed the experiment and supervised the project. MZ and MS collected the samples and carried out the lab work. US and MFH performed data analyses and contributed in writing the manuscript. All authors read and approved the paper.

REFERENCES

Abubakar M, Mansoor M and Arshed MJ, 2011. Bovine Brucellosis. Old and new concepts with Pakistan perspective. Pak Vet J 32:1-9.

Ali S, Ali Q, Neubauer H, *et al.,* 2013. Seroprevalence and risk factors associated with brucellosis as a professional hazard in Pakistan. Foodborne Pathog Dis 10:500-5.

Alton GG, Jones LM, Angus RD, *et al.,* 1988. Techniques for the brucellosis laboratory, Institute National de la recherché Agronomique (INRA), Paris, France pp:63-129.

Asif M, Waheed U, Farooq M, *et al*., 2014. Frequency of brucellosis in high risk human groups in Pakistan detected through polymerase chain reaction and its comparison with conventional slide agglutination test. Int J Agric Biol 16:986-90.

Azar DK, Effat A and Seyed MA, 2006. Isolation of *Brucella melitensis* and *Brucella abortus* from brucellosis patients by conventional culture method and polymerase chain reaction technique. Pak J Med Sci 22:396-400.

Bakhtullah, Parveen F, Shahid M, *et al*., 2014. Sero–prevalence of brucellosis in cattle in southern area of Khyber Pakhtunkhwa, Pakistan. Res J Vet Pract 2:63-6.

Catharina S, José AT, Aldenir S, *et al*., 2015. Prevalence of *Brucella* spp in humans. Rev Latino-Am Enf 23:919-26.

Chimana HM, Muma JB and Samui KL, 2010. A comparative study of the seroprevalence of brucellosis in commercial and small scale mixed dairy beef cattle enterprises of Lusaka province and Chibombo district, Zambia. J Trop Anim Health Prod 42:1541-5.

Dean AS, Crump L, Greter H, *et al*., 2012. Global burden of human brucellosis: A systematic review of disease frequency. PLoS Negl Trop Dis 6:e1865.

Din AMU, Khan AS, Ahmad I, *et al.,* 2013. A study on the seroprevalence of brucellosis in human and goat populations of District Bhimber, Azad Jammu and Kashmir. J Anim Plant Sci 23:113-8.

Elfaki MG, Uz-Zaman T, Al-Hokail AA, *et al*., 2005. Detection of *Brucella* DNA in sera from patients with brucellosis by polymerase chain reaction. J Diagn Microbiol Infect Dis 53:1-7.

El-Koumi MA, Afify M and Al-Zahrani SH, 2014. A prospective study of brucellosis in children: Relative frequency of pancytopenia. Iran J Pediatr 24:155-60.

Gul ST and Khan A, 2007. Epidemiology and epizootology of brucellosis: A review. Pak Vet J 27:145-51.

Gul ST, Khan A, Ahmad M, *et al*., 2015. Epidemiology of brucellosis at different livestock farms in the Punjab, Pakistan. Pak Vet J 35:309-14.

Gul ST, Khan A, Ahmad M, *et al*., 2013. Seroprevalence of brucellosis and associated hemato-biochemical changes in Pakistani horses. Pak J Agric Sci 50:745-50.

Gul ST, Khan A, Rizvi R, *et al*., 2014. Sero-prevalence of brucellosis in food animals in the Punjab, Pakistan. Pak Vet J 34:454-8.

Gwida MM, El-Gohary AH and Melzer F, 2011. Comparison of diagnostic tests for the detection of Brucella spp. in camel sera. BMC Res Notes 4:525.

Jennings G, Hajjeh R and Grgis F, 2007. Brucellosis as a cause of acute febrile illness in Egypt. Trans R Soc Trop Med Hyg 101:707-13.

Mahmood R, Ali T, Waheed U, *et al.,* 2015. Application of serum based PCR and fluorescence polarization assay for diagnosis of brucellosis among people occupationally at risk to disease. Int J Agric Biol 18:377-84.

Mitka S, Anetakis C, Souliou E, *et al.,* 2007. Evaluation of different PCR assays for early detection of acute and relapsing brucellosis in humans in comparison with conventional methods. J Clin Microbiol 45:1211-8.

Mukhtar F, 2010. Brucellosis in a high risk occupational group: seroprevalence and analysis of risk factors. J Pak Med Assoc 60:1031-4.

Mustafa K, Shahid HA, Rani A, *et al.,* 2016. Occurrences of *Brucella abortus* in serum and milk samples of cattle in Loralai, Balochistan. Sci Int Lahore 28:1183-6.

O'Callaghan D and Whatmore MA, 2011. Brucella genomics as we enter the multi- genomic era. Brief Funct Genomics 10:334-41.

Saleha S, Basit A, Rahim K, *et al.,* 2014. Comparison of milk ring test; serum plate agglutination test and polymerase chain reaction for the detection of bovine brucellosis. Res J Vet Pract 2:5-8.

Tibesso G, Ibrahim N and Tolosa T, 2014. Sero-Prevalence of bovine and human brucellosis in Adami Tulu, Central Ethiopia, World Appl Sci J 31:776-80.

Tiller RV, Gee JE, Frace MA, *et al.,* 2010. Characterization of novel Brucella strains originating from wild native rodent species in North Queensland, Australia. Appl Environ Microbiol 76:5837-45.