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

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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.

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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-Kouni et al., 2014).
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-Dahout 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, 2) 5'-TGCCCA TCA CTT AAG GGC CTT CAT-3'. 2) BA-F, 3) 5'-GAC GAA CGG AAT TTT CCT AAC CCT-3' and BA-R, 4) 5'-GCGCA 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<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. melitensis 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).

**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 Bakhullah 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%) compared 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 (Tibbesso 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. melitensis 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 1:** Overall prevalence of brucellosis in cattle and humans through SPAT and duplex PCR

<table>
<thead>
<tr>
<th>Test used</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>Total</td>
<td>B. abortus</td>
</tr>
<tr>
<td>SPAT</td>
<td>30(15)</td>
<td>18(9)</td>
</tr>
<tr>
<td>PCR</td>
<td>26(13)</td>
<td>15(7.5)</td>
</tr>
<tr>
<td>P value</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>Humans</td>
<td>SPAT</td>
<td>12(6)</td>
</tr>
<tr>
<td>PCR</td>
<td>8(4)</td>
<td>-</td>
</tr>
<tr>
<td>P value</td>
<td>0.32</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPAT</td>
<td>Total</td>
<td>B. abortus</td>
</tr>
<tr>
<td>Human(F)</td>
<td>10(10)</td>
<td>2(2)</td>
</tr>
<tr>
<td>Human(M)</td>
<td>2(2)</td>
<td>-</td>
</tr>
<tr>
<td>PCR</td>
<td>Human(F)</td>
<td>6(6)</td>
</tr>
<tr>
<td>Human(M)</td>
<td>2(2)</td>
<td>-</td>
</tr>
<tr>
<td>P value</td>
<td>0.32</td>
<td></td>
</tr>
</tbody>
</table>

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

![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).](image)
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.

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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.

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