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Full Length Article

Application of Serum Based PCR and Fluorescence Polarization Assay for Diagnosis of Brucellosis among People Occupationally at Risk to Disease

Rashid Mahmood[1*], Tayyaba Ali[1,2], Usman Waheed[1,3], Mohammad Asif[1] and Qaiser M. Khan[1]
[1]*Soil and Environmental Biotechnology Division, National Institute for Biotechnology & Genetic Engineering (NIBGE), Jhang Road, Faisalabad, PAKISTAN*
[2]*Department of Zoology, Government College University, Faisalabad, Pakistan*
[3]*Department of Pathobiology, University of Veterinary and Animal Sciences, Lahore, Sub-campus Jhang, PAKISTAN*
*For correspondence: rashid.nibge@hotmail.com; ali.tayyaba@gmail.comTel: + 92 41 265 3398

Abstract

Brucellosis is an important and overlooked zoonotic disease especially in developing countries having great potential to badly affect the people occupationally exposed to disease. In humans, the disease is usually marked with undulant fever, headache, fatigues, back pain, general malaise and arthritis. There have been a number of published studies available focussing on the serological surveillance in Pakistan but very little has been done using serum as clinical specimen for Polymerase Chain Reaction (PCR) and advanced serological techniques like Fluorescence Polarization Assay (FPA). In this study we have used serum as clinical sample for molecular and serological diagnosis of brucellosis in occupationally exposed human population to address the limitations associated with safe handling of field samples and current diagnostic procedures. Sera from 110 volunteers were collected and analysed by employing serology (Rose Bengal Test, competitive ELISA, FPA) and serum PCR assay. Seropositivity was observed in 4.5% (5/110), 9% (10/110) and 31% (34/110) of samples with RBT, cELISA and FPA, respectively. The PCR assay could detect about 38% (42/110) samples ($p<0.05$). Serum PCR and FPA were able to detect more positive cases in each category of human population sampled. We found serum could be used as more dependable and safer clinical specimen for both serological and molecular investigations. Together with serum PCR, this study strongly recommends the introduction of FPA into routine clinical diagnosis for brucellosis. Our findings described here will help to understand and let decision makers to adopt and implement better choices for future brucellosis control measures. © 2016 Friends Science Publishers

Keywords: Brucellosis; Molecular Detection; Serum PCR; RBT; cELISA; FPA

Introduction

Brucellosis is a zoonotic disease of great socio-economic importance caused by Gram -ve, facultative, intracellular bacterial organisms of the genus *Brucella* (O'Callaghan and Whatmore, 2011). Among all known *Brucella* species, *Brucella abortus*, *Brucella melitensis*, *Brucella suis* and *Brucella canis* are the most important contributors to human brucellosis (Young, 1995). The recent discovery of *Brucella* species in marine mammals including dolphins, seals and porpoises presented an emerging hazard to persons working with sea life (Godfroid *et al*., 2005). In humans, the disease usually is marked with undulant fever, headache, fatigues, back pain, general malaise and arthritis. There may be localized complications which may involve the digestive, pulmonary, cardiovascular, genitourinary, osteoarticular and nervous systems (Corbel, 2006). Man can contract disease through consumption of infected and unpasteurized dairy products (Bikas *et al*., 2003), dealing directly with contaminated animal parts, inhalation of contaminated aerosolized particles in laboratories and occupational contagion to highly exposed persons like veterinary professionals, dairy persons and butchers etc (Abo-Shehada *et al*., 1996; Memish and Mah, 2001; Omer *et al*., 2002; Mukhtar, 2010). Without sufficient and timely antibiotic treatment course, some patients develop a "chronic" brucellosis syndrome with many characteristics of the "chronic fatigue" syndrome (Corbel, 2006; Thakur *et al*., 2002).

The disease is endemic in many countries particularly belonging to the Arabian Gulf, Mediterranean basin, the Indian subcontinent and in parts of Central and South America and Mexico (Young, 1995; Pappas *et al*., 2006). Globally, the incidence of brucellosis in human population in endemic disease regions varies widely with more than 500000 cases reported annually (Corbel, 1997; Pappas *et*

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al., 2005). It can affect all age groups of people and the occasional human to human transmissions are also reported (Mantur *et al*., 1996; Sauret and Vilissova, 2002). The disease is still prevalent in varying trends both in USA and in some Western countries (Pappas *et al*., 2006). Yet, the true incidence is unknown for most of the countries and it is expected to be much higher than reported incidences due to under-diagnosis and low levels of surveillance and reporting. Occupationally, persons related to veterinary profession, dairy farming and animal husbandry, butcher shops and slaughter house operations and individuals working in microbiological laboratories are at high risk of getting brucellosis amongst all general human population categories (Abo-Shehada *et al*., 1996; Memish and Mah, 2001; Asif *et al*., 2014).

Whilst the clinical picture of brucellosis in human is not specific and misleading, the diagnosis requires proper laboratory support. Bacterial isolation and identification through culture from infected materials (blood, milk or afterbirth) is the gold standard for the confirmatory diagnosis of brucellosis (Alton *et al*., 1988). Presence of viable bacteria is sufficient to detect brucellosis, yet the lack of a positive culture from clinical specimens especially in chronic cases and neurobrucellosis does not rule out the disease since a very low number of bacteria may be present in an infected person (Araj *et al*., 1988). Moreover, given the infectious nature of *Brucella* organisms, specialist handling facilities (BSL-3) are required (Gilsdorf, 2005). It is for these reasons that alternative strategies including serological and molecular methods have been developed for the diagnosis of brucellosis.

In the case of serological choices, a number of different tests available for diagnosis including Rose Bengal Test (RBT), Serum Agglutination Test (SAT), Standard Tube Agglutination Test (STAT), Enzyme Linked Immuno Sorbent Assay (ELISA), Milk Ring Test (MRT) and Fluorescence Polarization Assay (FPA) (Godfroid *et al*., 2010). Fluorescence Polarization Assay is based on principle of spinning of molecules in a liquid medium correlating with their mass. The technique is relatively a new, quick and reliable serological tool that offers an important alternative to conventional serology yet to be included in routine testing schedule (McGiven *et al*., 2003). On contrary, the use of conventional serological tests both as a confirmatory and screening tool has been popular means for the diagnosis of disease for a number of years. However, whilst they are being regularly used, the diagnostic value of these tests is questionable. These screening assays are inexpensive but do have problems with specificity through cross reactions with non *Brucella* organisms such as *Yersinia enterolitica* which share common epitopes in their lipopolysaccaride (LPS) structure (Kittelberger *et al*., 1998).

To overcome the pitfalls associated with serological tests, there has been a drive to develop molecular techniques like Polymerase Chain Reaction (PCR) based on the amplification of pathogen DNA. As molecular diagnostic methods are directed towards the pathogen, they represent an important breakthrough due to their improved specificity over serological assays and are not variable with host response. For conventional PCR, a number of targets available for the detection of *Brucella* DNA with the most popular being 31 kDa immunogenic outer membrane protein *bcsp*31 (Baily *et al*., 1992) and a multiple copy insertion sequence IS*711* (O'Leary *et al*., 2006). In addition to the rapidity of molecular techniques in terms of result generation, PCR have been directly applied to a variety of clinical specimens without the need for culture thereby speeding up identification times (Yu and Nielsen, 2010). Presence of *Brucellar* circulatory DNA in sera is believed to be due to degradation of bacterial cells during infection with the liberation of free DNA in serum (Elfaki *et al*., 2005). There remains a bone of contention concerning the relevance of PCR testing of serum, however, in brucellosis; the serum PCR has shown exceptional sensitivity level in comparison with blood for the diagnosis of acute human cases (Zerva *et al*., 2001; Vrioni *et al*., 2004; Elfaki *et al*., 2005).

Worldwide, millions of people are at risk particularly in areas where improper treatment procedures of milk and poor hygienic conditions are of main concern. Control of disease in animal reservoir is a pre-requisite for the prevention of brucellosis in human population. In Pakistan, most of the diagnosis revolves around aforesaid old serological tests whereas classical diagnosis of the organism by culturing from clinical samples is rare and the use of new and sophisticated serological and molecular techniques for identification such as PCR and FPA even more so. In terms of a diagnostic tool, there is a need to both develop and apply better serological and molecular techniques in the control of brucellosis. In present study, we have evaluated the PCR using serum instead of whole blood for *Brucella* spp. detection along with recently introduced Fluorescence Polarization Assay (FPA) and compared the results with conventional serological testing. We have also addressed the lack of knowledge as to the extent of brucellosis in occupationally exposed people in Pakistan. The data generated from this work has given a guide to the prevalence of brucellosis among people at risk and performance of different diagnostic procedures which be helpful in shaping future strategies for both the diagnosis and control of disease in exposed human population.

Materials and Methods

Sample Collection

The peripheral blood samples (n=110) were collected aseptically from mostly asymptomatic volunteers occupationally at high risk of contracting the disease from animal reservoir and currently working in parts of Hazara, Peshawar and Charsada districts of Khyber Pakhtunkhwa

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(KPK) Province, Pakistan. The individuals stratified by geographical location and occupation include veterinary professionals, livestock farmers/animals handlers, village house wives and butchers. The purpose of the study and sampling procedure were explained to the persons regarding collection of blood samples and necessary information before study. Their relevant data and past medical history was recorded in a structured data collection *pro-forma*. For serum separation, 5 mL of blood was taken aseptically in vacutainer® (Beckton, Dickinson and Company, New Jersey, USA). Serum was separated by spinning the whole blood samples for 5 min at 1790 x g (Mukhtar and Kokab, 2008). The serum samples were then refrigerated and transferred to laboratory where they were tested and analysed with Rose Bengal Test (RBT), cELISA, Fluorescence Polarization Assay (FPA) and serum PCR assay.

Rose Bengal Test (RBT)

Samples were tested by Rose Bengal Test (RBT) as described earlier (Alton *et al*., 1988; Stack *et al*., 1999) using the kits (APHA Weybridge, UK) according to manufacturer's guidelines. One drop (0.03 mL) of serum was taken on a white tile followed by the mixing of an equal measure of homogenous suspension of purified antigen. A visible clumping/agglutination was recorded positive after thorough mixing for 3-4 min.

cELISA

The cELISA as explained previously by Stack *et al*. (1999) and Alton *et al*. (1988) is based on detection of smooth *Brucella* strains' lipopolysaccharide (LPS) antigen. The cELISA kits by APHA Weybridge, UK were used according to kit's instructions. Polystyrene plates coated with *B. melitensis* lipopolysaccaride antigen were labelled followed by the addition of test serum samples to the plate. Labelled anti-*Brucella* antibodies (MAb) were added followed by adding up of substrate and chromogen solution. The wells which were exposed to positive serum did not show a colour change as the antibodies from positive serum bound the antigenic sites in the wells thereby preventing the labelled antibodies from binding. Whereas in *Brucella* negative serum samples, those wells that had bound labelled antibodies (MAb) to the antigenic sites on the plate were detected by a colour change due to the reaction of substrate and chromogen. The optical density (OD) was read at 450 nm whereas the % OD cut-off of 50% was used for the analysis of results.

Fluorescence Polarization Assay (FPA)

The FPA is a procedure for measuring antigen/antibody interaction based on random rotation of molecules in a solution. In principle, the smaller sized molecules spin faster and depolarize a polarized light beam more, while bigger sized molecules spin more slowly and, consequently, depolarize light less. The test was conducted as described by McGiven *et al*. (2003) using 96 well microtitre plate layout. Test buffer used was prepared in 1 L of distilled water by adding 0.836 grams of Sodium monophosphate monohydrate ($Na_2HPO_4.H_2O$), 1.49 grams Sodium triphosphate dodecahydrate ($Na_3PO_4.12H_2O$), 9 grams Sodium chloride (NaCl) and 0.50 grams lithium Dodecyl sulphate (lauryl sulphate) $CH_3(CH_2)11OSO_3Li$ with a final pH of 7.5. Microtitre plates were labelled followed by the adding up of 180 µL of test buffer and 20 µL of test/control serum in duplicate replica to appropriate wells according to plate layout. Buffer and serum samples were mixed by repeated pipetting and incubated for 3 min on rotary shaker (125 rpm) at room temperature. To compute the background reading for each sample, plates were measured on a Tecan Polarion Fluorescence Polarization microplate reader after the initial incubation. This step was followed by the addition of 10 µL of *Brucella* O-polysaccharide conjugated isothiocyanate fluorophore (Diachemix TM antigen; supplied by Diachemix, Whitefish Bay, WI, USA) to all wells with the exception of buffer and Fluorescein controls. After an incubation of further 2 min, the plates were read again on microplate reader to take raw parallel as well as perpendicular data for each sample and measured the final intensities in millipolarization units (mP). To compute the 'mP', the following formula was used: mP=1000 x ((Iv-Ih)/(Iv+Ih)), where Iv & Ih are parallel and perpendicular light intensities, respectively. For positive human sera, samples that read 15 mP above the mean negative control were recognized as positive and those below were negative.

DNA Extraction and PCR Testing for Presence of *Brucella* from Serum Samples

To test directly from a clinical specimen, DNA was isolated from 200 µL of serum by using DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the kit's protocol. Conventional PCR based on the *Brucella* spp. specific target 31 kDa outer membrane protein *bcsp*31 (Baily *et al*., 1992) was carried out for the detection of *Brucella* DNA. The primers Forward (TGGCTCGGTTGCCAATATCAA) Reverse (CGCGCTTGCCTTTCAGGTCTG) were used for amplifying 223bp DNA fragment within the *bcsp*31 gene. The PCR amplification reaction mixture was set up as follows in a reaction volume of 25 µL (final concentrations): FastStart 1x PCR buffer with MgCl2 (Roche), 800 nM of both forward and reverse primers, 0.4 mM dNTPs, 1 unit FastStart Taq DNA polymerase (Roche) with 5 µL DNA as template. The *B. melitensis* 16 M reference strain was used as positive control while in negative control no DNA was added in amplification reaction mixture to check for false positives or possible inhibition during the PCR reaction. Thermocycler parameters used were as follows: initial denaturation at 94°C for 5 min followed by 40 cycles of

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94°C for 30 sec for template denaturation, 60°C for 30 seconds for primer annealing and 72°C for 1 min for primer extension and a final extension step of 72°C for 7 min. The amplified PCR products corresponding to desired fragment size of 223bp were mixed with 3 µL of Invitrogen's Blue Juice (gel loading buffer) and visualised using a 2% agarose gel in 1X TAE buffer containing ethidium bromide and a of 1 kb size marker. Amplifications of desired fragment i.e., 223 were then photographed under the UV transilluminator at a wavelength of 254 nm with Eagle Eye Gel Documentation System (Stratagene, USA). Each sample was tested in duplicate manner and only those that produced a product of the correct size on both occasions were identified as *Brucella* positive.

Analysis of the Diagnostic Testing

The diagnostic data were statistical analysed with chi-square test ($\chi 2$) (Steel *et al*., 1997) using software GraphPad Prism (version 6.05, La Jolla, CA 92037 USA, www.graphpad.com). A P-value <0.05 was regarded as statistically significant.

Results

In this study, sera from 110 volunteers involved in various livestock related activities were tested with serum based *bcsp*31 PCR assay along with 3 serological methods for presence of antibodies against *Brucella*. A PCR product with a molecular size of 223bp (Fig. 1) was obtained from 38% (42/110) samples while seropositivity was observed in 4.5% (5/110), 9% (10/110) and 31% (34/110) of samples with RBT, cELISA and FPA, respectively ($p<0.05$) (Table 1). The PCR and serological results were compared: Of 42 PCR positive samples 14.5% (16/110) were also detected positive by serological tests. FPA was found to be more sensitive than rest of the serology and detected antibodies in overall 31% (34/110) samples, of which 13.6% (15/110) were also positive by PCR. Competitive ELISA detected 3.6% (4/110) with PCR whereas none of the sample detected positive by both PCR and RBT. Furthermore, PCR was able to detect *Brucella* spp. DNA in additional 23.64% (26/110) that were negative by serological methods. Likewise, FPA and cELISA identified 12.73% (14/110) and 1.82% (2/110) positive samples, respectively which were negative by all other tests. However, in overall, only 0.90% (1/110) sample was found positive by all the testing during the study. The differences between PCR assay results and serological methods were found to be statistically significant ($p<0.05$). Table 2 and Fig. 2 summarize the comparative analysis of positive samples with both *bcsp*31 PCR and serology.

All the sets of investigated population were found to be vulnerable to brucellosis during the study. The breakup of positive cases by profession detected by single or multiple tests are presented in Table 1 and 3.

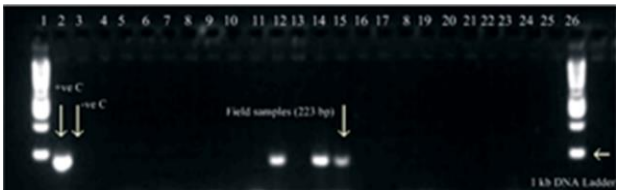

Fig. 1: Electrophoretic analysis (2% agarose gel stained with GelRed^{TM} solution) of *bcsp*31 based PCR products for diagnosis of brucellosis. Amplified product of 223 bp was obtained using Baily's primers, template DNA from *B. melitensis* 16M reference strain as positive control and sera of animals from field areas. Lane 1 & 26, 1 kb DNA marker; Lane 2, positive control (*B. melitensis* 16M DNA); Lane 3, negative control (no DNA was added); Lane 12, 15 & 15, serum DNAs from sera samples positive for brucellosis; Rest of the blank wells are negative sera samples. The image was scanned initially followed by editing for better view

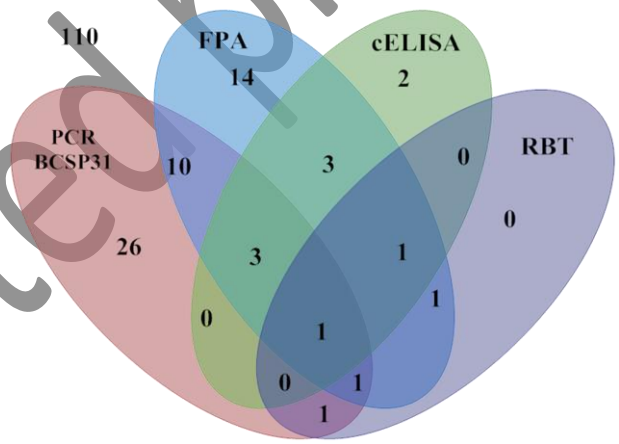

Fig. 2: Venn diagram showing a summary of RBT, cELISA, FPA and serum PCR results of human samples (n=110) describing the number of positive samples with single and multiple tests during the study

It can be observed that PCR and FPA were able to detect more positive cases in each category of human population sampled. When the subjects were asked for their past clinical history, the male respondents were found generally asymptomatic except for having few clinical signs such as fever and arthritis. These problems were usually ignored or treated with conventional pain killers or analgesics etc. It was found that none of the respondents have approached any laboratory regarding brucellosis infection. The females selected during the study, though in smaller number, showed a higher prevalence of brucellosis among all the groups studied. All had the abortion history in past and a few of them have more than once happened between 4th-5th months of their pregnancy along with other clinical sings such as headache etc.

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Table 1: Results of different tests for diagnosis of Brucellosis using sera from human population at-risk to disease

Test population	No. of sera tested	Tests applied				χ^2 (p-value)
		RBT	cELISA	FPA	PCR	
Vet Professional	55	5 (9%)	10 (18%)	19 (35%)	20 (36%)	15.41 (0.001)
Farmers/Animal Handlers	39	-	-	9 (23%)	12 (31%)	25.26 (<0.05)
Village house wives	9	-	-	3 (38%)	7 (78%)	18.28 (<0.05)
Butchers	7	-	-	3(43%)	3 (43%)	7.64 (=0.05)
Total	110	5 (5%)	10 (9%)	34 (31%)	42 (38%)	50.01 (<0.05)

Table 2: Frequency of RBT, cELISA, FPA and PCR tests on 110 sera samples for the detection of *Brucella* infection in human

Positive samples	RBT	cELISA	FPA	PCR	%
1	+	+	+	+	0.90
3	-	+	+	+	2.73
1	+	+	+	-	0.90
1	+	-	+	+	0.90
10	-	-	+	+	9.09
3	-	+	+	-	2.73
1	+	-	+	-	0.90
1	+	-	-	+	0.90
26	-	-	-	+	23.64
14	-	-	+	-	12.73
2	-	+	-	-	1.82
47	-	-	-	-	42.73
110					

Table 3: Analysis of different tests results among various categories of occupationally exposed population

Test applied	Test population				χ^2 (p-value)
	Vet. Professional % ,+ve	Dairy Farmers % ,+ve	Village Housewives % ,+ve	Butchers % ,+ve	
RBT	9% (5/55)	-	-	-	15.35 (p<0.05)
cELISA	18% (10/55)	-	-	-	31.43 (p<0.05)
FPA	35% (19/55)	23% (9/39)	38% (3/9)	43% (3/7)	1.95 (p>0.05)
PCR	36% (20/55)	31% (12/39)	78% (7/9)	43% (3/7)	7.02 (p>0.05)

During the study, the possible risk factors related to brucellosis were also studied. Obviously, all the people were found having direct link with livestock in one way or other. The people belonging to dairy production were found having the habit taking raw milk streams directly into their mouth while milking their animals just for getting pure milk ingredients of for fun some times. We also found that though all the vet professions were aware of zoonotic infections to varying extents, but only a very few of them were found observing biosecurity measures like during artificial insemination of animals, parturition and related gynaecological problems. Similarly, women living as house wives in villages were found actively participating in livestock husbandry practices i.e., handling and processing of raw milk and its products, cleaning animals' houses, animal feeding and making dung cakes etc. Similarly, the persons related to meat industry, were found dealing with healthy and sick animals while slaughtering and processing of meat and other by-products without having any protective clothing etc. However, all the groups except veterinarian were totally unaware of the zoonotic infections like brucellosis which was found very alarming in this case.

Discussion

Brucellosis is endemic in subcontinent region representing serious social and economic problems in both human and livestock population especially in developing countries like Pakistan. There are a number of published reports regarding the prevalence of brucellosis in different human categories with reference to their occupation particularly (Mukhtar and Kokab, 2008; Mukhtar, 2010; Ali *et al*., 2013). Similarly, many of these previous studies have generally focussed on the diagnosis of disease through conventional serological response and not the direct detection of specific antibodies or pathogen through advanced serological, culture or molecular means.

Ideally, any diagnostic procedure should be inexpensive, specific and sensitive thereby capable of detecting all stages of the infection. At present, no such test exists up to date for brucellosis. Although this is established now that PCR testing of sera can generate more positive results than serology and recommended that PCR from serum samples together with serological testing could be used as rapid screening and confirmation strategy for human brucellosis (Zerva *et al*., 2001; Elfaki *et al*., 2005).

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However, LPS based serological diagnosis should be viewed carefully due to cross reactivity concerns with other bacteria (Kittelberger *et al*., 1998). The fact that *Brucella* isolation and identification from culture is "gold standard", it should be noted that these classical methods are undertaken in a time frame of minimum 3-4 days in highly specialized management facilities. On contrary, the molecular identification is carried out from a crude extraction in a general laboratory setup within 3-4 h signifying safety advantage over culture.

There have been several studies describing the use of serum PCR for the diagnosis of human brucellosis (Zerva *et al*., 2001; Vrioni *et al*., 2004; Elfaki *et al*., 2005; Queipo-Ortuno *et al*., 2005; Debeaumont *et al*., 2005). In the present study, we have also successfully examined the utility of PCR method using the serum instead of whole blood for the detection of *Brucella* spp. DNA from volunteers involved in livestock practices. The detection of 223 bp segment in 38% positive cases showed that serum PCR assay is highly sensitive in the diagnosis of human brucellosis (Pilar *et al*., 1999; Stella *et al*., 2007). We also analysed the usefulness of FPA which is still to be included in routine clinical testing for screening and confirmation of brucellosis in Pakistan alongside PCR. Remarking the discrepancies among serological results, there was also a significant superiority of FPA observed which detected 31% positive samples in comparison to RBT and cELISA identifying only 4.5% and 9% positives samples, respectively. This is in accordance with another study comparing the Fluorescence Polarization Assay with three conventional serological methods (Rose Bengal, Standard Agglutination Test, iELISA) and concluded that FPA is a valuable diagnostic method could be replaced by already established methods for the diagnosis of human brucellosis (Konstantinidis *et al*., 2007). Nevertheless, there was a poor correlation by comparing the results of serology and *bcsp*31 PCR assay in present study. Twenty six (23.64%) out of 110 samples were tested as negative for brucellosis by serology suggesting these were probably acute or chronic cases having antibodies beyond detectable limits (Elfaki *et al*., 2005; Stella *et al*., 2007). On the other hand, 1.81% (2/110), 5.45% (6/110) and 17.27% (19/110) serological positives by RBT, cELISA and FPA, respectively were PCR negative which may be due to either the lack of sensitivity of PCR assay or serological cross-reactivity with other bacteria (Kittelberger *et al*., 1998) (Fig. 2; Table 2). With this and the PCR results in mind it is recommended that combination of diagnostic tests is required especially in the absence of gold standards.

For PCR, whole blood or serum samples are the specimen of choice due to their easy availability. The rationale of preferring serum over blood is because serum has got several advantages in terms of assay standardization, reproducibility, inhibition by anticoagulants, haemoglobin, human DNA or other substances and minimized risks to laboratory staff. Risks associated with handling of contaminated blood specimens in filed areas could be minimized making it more convenient and safe to transport the samples easily from remote areas to the main laboratory. The whole procedure is simplified right from on spot collection of samples from field areas, their safe handling, initial processing, shipment to laboratory and reducing the risks of getting infection to laboratory workers through aerosolization and pipetting mishaps. In literature, the use of serum PCR has been documented by number of published studies where serum has shown excellent sensitivity as compared to whole blood samples for diagnosing infection (Zerva *et al*., 2001; Elfaki *et al*., 2005). Our data also validates these findings and supports the introduction of serum PCR for routine diagnosis of *Brucella* infection in laboratories.

Conventional serological methods are being used by laboratories and investigators both for confirmation and screening since from decades for the diagnosis of infection. During the study, the three serological tests i.e. RBT, cELISA and FPA were conducted in parallel for detecting the antibodies against *Brucella* in serum. Whilst the RBT is a rapid agglutination test used for diagnosis of brucellosis in human (Ruiz-Mesa *et al*., 2005), competitive ELISA (cELIA) has the advantage of its use in different species and its potential to produce results in poor quality or exhausted serum samples as well (Perrett *et al*., 2010). In contrast, FPA is a relatively new technique first developed in 1996 (Nielsen *et al*., 1996) and since then been authenticated for the diagnosis of brucellosis (Bahn and Nockler, 2005). The procedure of FPA is quite simple and accurate, easy to perform, does not require lengthy procedures and can be easily adopted by diagnostic laboratories (Lucero *et al*., 2003). The facts presented here strongly advocate that FPA is significantly a superior diagnostic assay in terms of its versatility by producing excellent results in relatively shorter period of time. It can be used as alternative to RBT for initial screening of sera even for small panel of samples and can offer an excellent replacement to ELISA as a confirmatory method as well. The methodology of FPA clearly suggests its advantages over other serological testing and may be included as regular diagnostic tool in clinical laboratories.

The present study highlights a serious occupational hazard of brucellosis among people at risk due to their occupation. These include veterinary professionals, farmers, butchers and women living in rural areas dealing routine livestock husbandry directly or indirectly. The study is in agreement with the observations described by several investigators (Mukhtar and Kokab, 2008; Mukhtar, 2010; Ali *et al*., 2013; Asif *et al*., 2014). All the male respondents during the study were found generally asymptomatic except for having few clinical signs such as fever and arthritis. The rest were apparently normal or did not reveal their clinical history due to ignorance or may be due to some social reasons or shyness. However, the females selected during the study, though in smaller number, showed a higher

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prevalence of brucellosis among all the groups studied. The reason we have chosen few selected village house wives as our study subjects because of their direct or indirect involvement in livestock activities in terms of handling and processing of dairy products, animal feeding and dumping animal wastes in their daily life. These females were having an abortion history in recent past in their 4-5 months of pregnancy. They also had the headache problem which is one of the misleading clinical features of brucellosis. Our findings are in accordance with previous published reports for *Brucella* causing in abortion in human (Khan *et al*., 2001). However, based on our observations, we strongly suggest the further demographic investigations for understanding disease pattern in women during pregnancy.

Our study also significantly emphasizes the brucellosis as an occupational problem in veterinary practitioners especially the persons who are frequently engaged in veterinary obstetrics and gynaecology practices. The results are in concurrence with similar findings in Jordon where veterinarians were having high prevalence of brucellosis and had been frequently in contact with animals while in labour (Abo-Shehada *et al*., 1996). In our study, only a few positive cases showed clinical signs i.e. undulant fever and arthritis. In this regard, a general medical practitioner should be careful with respect to occupational exposure of disease because due to varied clinical manifestations or deceptive nature of disease physicians usually overlook brucellosis.

The prevalence of disease in butchers revealed the significance of contact infections dealing with dead carcases and raw meat of infected animals. The infections may get entry thorough cuts in skin or splashing of infected fluids like blood or materials into the eye may lead to infection in healthy human being. This is more cautious as high carelessness is observed while slaughtering followed by processing of meat by butchers and abattoir workers (Mukhtar and Kokab, 2008). Farmers and animal handlers were also found to be at risk during study which explains the wide spread of *Brucella* infection in human population. Hygienically poor livestock practices by farmers, consumptions of raw milk or dairy products and contaminated environmental conditions with *Brucella* especially during parturition further aggravate the conditions favourable for disease transmission to human (Abo-Shehada *et al*., 1996; Ali *et al*., 2013). These results are comparable with our findings described previously (Asif *et al*., 2014) showing the prevalence of brucellosis among the various occupationally exposed human groups thereby emphasizing the need for more accurate and specific diagnostic facilities to combat this important zoonotic infection in Pakistan.

In conclusion, conventional serological techniques along with modern serological methods followed by molecular tools provide a handful mechanism for the detection of *Brucella* infection. The *bcsp*31 serum PCR was found to be more sensitive and accurate during the study. Moreover, we strongly recommend the introduction of FPA in routine clinical investigations for brucellosis. We found serum could be used as more dependable and safer clinical specimen than whole blood or other foetal tissue for serological and molecular diagnosis posing minimum hazards to laboratory workers. It is also concluded that a battery of tests having more than one test should be practiced for getting consensus results. The reported human cases do not actually predict the actual prevalence of disease in human population, as revealed in this study leading to an underestimation of disease burden in exposed people in particular. Also, the consistent prevalence of infection in animal reservoir in developing countries like Pakistan requires much more efforts to estimate the frequency of brucellosis in both general and occupationally at risk human population in the country. Having these data will let decision makers to adopt and implement better choices for future brucellosis control measures.

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