INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
15–326/2016/18–2–311–318
DOI: 10.17957/IJAB/15.0088
http://www.fspublishers.org

Full Length Article

Serological and Nucleic Acid Based Detection of Brucellosis in Livestock Species and Molecular Characterization of *Brucella melitensis* Strains Isolated from Pakistan

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Abstract

This study aimed at molecular detection and characterization of *Brucella* spp from Pakistan. For this purpose, whole blood samples (n=167) were collected from different species of livestock and analysed at Animal and Plant Health Agency (APHA), United Kingdom. Samples were analysed employing Rose Bengal Test (RBT), Competitive Enzyme-linked immunosorbent assay (cELISA), PCR IS*711* and culture examination. We found 1% (2/167) samples positive for infection by culture, 4% (7/167) by RBT, 6% (10/167) by cELISA and 21% (35/167) by PCR IS*711*. Results were found statistically significant using chi-square test (p-value <0.05). The blood culture positive bacterial isolations were further subjected to classical biotyping and molecular techniques for characterization and found as non-vaccine strains of *Brucella melitensis*. Molecular characterization using Multilocus Sequence Analysis (MLSA) and Variable Number of Tandem Repeat (VNTR) analysis demonstrated that although there was some similarity in the patterns generated, the isolations were distinct from each other. These isolates were not the part of geographically confined group but were representative of other *B. melitensis* strains found in the region stretching from southern Europe into South Asia. To our knowledge, this is the first report of molecular characterization of *B. melitensis* isolates from Pakistan. The molecular methods described in the present study will help to understand the disease dynamics and future brucellosis control in Pakistan. © 2016 Friends Science Publishers

Keywords: Brucellosis; Molecular Characterization; *Brucella melitensis*; MLSA; VNTR; Pakistan

Introduction

Brucellosis is a zoonosis of global socio-economic importance caused by Gram negative facultative intracellular bacteria of genus *Brucella*. The disease has been categorized as 'Multiple species disease' and noted in Notifiable Terrestrial and Aquatic Animal Diseases list of the World Organisation for Animal Health (OIE) (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2014/). In developing countries, brucellosis is one of the most neglected endemic zoonotic diseases. Due to its consistent zoonotic nature attributable to human, livestock and wildlife, it ranks among the top 10 diseases of animals that are capable of infecting and putting multiple economic burdens on poor and improvised sections of human population (Perry, 2002). In livestock, brucellosis is the main cause of reproductive complications such as abortion and sterility whilst in human the disease can manifest itself in many ways including fever, general malaise and arthritis (Barbier *et al*., 2011). Genus *Brucella* is classified into different species based on phenotypic differences and host preferences. For host preference, *B. melitensis* is associated with sheep and goats, *B. abortus* with cattle, *B. suis* with pigs, *B. ovis* with sheep only, and *B. canis* with dogs (Verger *et al*., 1987). However, since 1994, several new *Brucella* species have been isolated from marine mammals (Foster *et al*., 2007) and voles (Scholz *et al*., 2008). More recently, several species of *Brucella* have been isolated from human (Scholz *et al*., 2010), non-human primates, foxes and frogs which determines the expanding nature of the genus (Schlabritz-Loutsevitch *et al*., 2009; Eisenberg *et al*., 2012; Hofer *et al*., 2012). It should, however, be noted that there are circumstances where *Brucella* species have been found to infect other host species in addition to their preferred host

To cite this paper: Mahmood, R., U. Waheed, T. Ali, K.K. Gopaul, A.C. Dainty, J.K. Muchowski, M.S. Koylass, S.D. Brew, L.L. Perrett, A.M. Whatmore and Q.M. Khan, 2016. Serological and nucleic acid based detection of brucellosis in livestock species and molecular characterization of *Brucella melitensis* strains isolated from Pakistan. *Int. J. Agric. Biol*., 18: 311–318

such as *B. abortus* in sheep and goats (Elzer *et al*., 2002; Ocholi *et al*., 2005) or *B. melitensis* in cattle (Alvarez *et al*., 2011). Moreover, some of these species can be further subdivided into biovars based upon dye uptake (Farrel and Robertson, 1967), metabolic processes, susceptibility to phage (Corbel *et al*., 1988), hydrogen sulphide (H_2S) production, CO_2 requirement and specific surface antigens (Alton *et al*., 1988).

Usually, brucellosis in animals can be diagnosed by clinical symptoms (abortions), culture from infected materials (blood, milk or afterbirth) and serology. In the case of serological tests, there are a number of different methodologies available for diagnosis including Rose Bengal Test (RBT), Serum Agglutination Test (SAT), Standard Tube Agglutination Test (STAT), Milk Ring Test (MRT) and Enzyme-linked immunosorbent Assay (ELISA) (Godfroid *et al*., 2010). However, more recently molecular tools, such as conventional and real-time PCR assays have been developed for both detection and characterization of *Brucella* (Yu and Nielsen, 2010). For conventional PCR, a number of targets such as *bcsp*31 (Baily *et al*., 1992) and multiple copy number insertion sequence IS*711* (O'Leary *et al*., 2006) are available for the specific detection of *Brucella*. These assays can be applied directly for detection from clinical samples, without the need for setting up of cultures (Yu and Nielsen, 2010). Moreover, the use of molecular techniques like Multilocus Sequence Analysis (MLSA) and Variable Number of Tandem Repeat (VNTR) genotyping have allowed for more precise *Brucella* identification and brucellosis epidemiology (Whatmore, 2009). A number of recent isolates of *Brucella* have been identified using MLSA (Scholz *et al*., 2008, 2010; Schlabritz-Loutsevitch *et al*., 2009). Usefulness of VNTR in determining strain diversity in a population has been proven through literature (Cespedes *et al*., 2011) along with the determination of transmission between wild and domestic fauna (Abril *et al*., 2011) and possible zoonotic transmission (Gwida *et al*., 2012).

Pakistan is an agriculture based country and most of the population is involved in land cultivation and animal husbandry. A large number of human populations are exposed every day to a variety of animals and their excreta. This is particularly the case with rapid and continuous increasing dairy production units in the country and farmers/dairy men and their families are bound to work in the poor hygienic environments (Asif *et al*., 2014). The exact prevalence of brucellosis is not yet established in the country but it is believed to be endemic in nature depending on various factors like climatic conditions, host species, sex and age of the host animals. There are several published reports; however, no detailed data or proper reporting centres to outline the prevalence of brucellosis in man and animal is available (Nasir *et al*., 1999; Gul and Khan, 2007). Lack of proper diagnosis and extensive surveillance systems are the main reasons in documentation of exact picture of brucellosis in the country. Moreover, the use of sophisticated and robust techniques are still not fully validated. By developing and validating modern high throughput techniques, we can pave the way to address the disease control issues on scientific basis. In the present study, blood samples were collected from different regions; serum was separated and tested using a number of serological and molecular techniques. Phenotypic and molecular characterization of *Brucella* isolates using classical biotyping and molecular epidemiological tools are also provided. The data generated from this study will word as a guide to the future brucellosis studies and will be helpful in devising strategies for diagnosis, characterization, and control of brucellosis in livestock and human population in Pakistan.

Materials and Methods

Sample Collection

The blood samples (n=167) were collected from selected areas of Hazara division, Peshawar and Charsada districts of Khyber Pakhtunkhwa (KPK) Province, Pakistan. Samples were collected from different farms/house hold animals having the history of reproductive problems in past. Five mL of blood from different species of livestock i.e. cattle, buffalo and goat was collected in duplicate vacutainers® (Beckton Dickinson, New Jersey, USA) for culture and serum separation. Serum was obtained by spinning whole blood samples for 15 min at 1790 x g. The samples were then refrigerated and transported to Animal and Plant Health Agency (APHA), Surrey, United Kingdom where they were analysed at Department of Bacteriology, FAO/WHO Collaborating Centre for Reference and Research on Brucellosis. Gold standards for isolation and identification of *Brucella* and the diagnostic work were carried out according to OIE Manual of Standards for Diagnostic Tests and Vaccines (http://www.oie.int/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals/).

Serological Testing of Blood

The serum samples were tested by Rose Bengal Test (RBT) and cELISA as described earlier (Alton *et al*., 1988; Stack *et al*., 1999) using the kits (APHA Weybridge, UK) according to manufacturer's instructions.

i. For RBT, 30 µL of serum was taken on a white tile followed by the addition of equal measure of homogenous suspension of purified antigen. A visible clumping/agglutination were considered positive after thorough mixing for 3–4 min.

ii. The cELISA was performed using polystyrene plates coated with *B. melitensis* lipopolysaccaride antigen. Plates were labelled and the test serum samples were applied to the plate. Enzyme Labelled anti-*Brucella* antibodies (monoclonal antibody, MAb) were added followed by the addition of substrate and chromogen solution. The enzyme

reacts with substrate and catalysed the reaction to detectable rate by the transforming the colourless chromogen into pigmented compound in those wells that had bound the enzyme labelled antibodies to the antigenic sites on the plate. Detectable quantities of bound enzyme labelled antibodies were not available in those wells which were exposed to positive serum as the antibodies from the positive sample bound to the antigenic sites in the wells preventing the enzyme labelled antibodies from binding. The optical density (OD) was read at 450 nm and the % OD of 50% was used as basic criteria for the interpretation of results.

Isolation and Identification of *Brucella* through Conventional Biotyping Techniques

For isolation, 1 mL of blood was added to 10 mL of Brodie and Sinton broths (Bordie, and Sinton, 1975) followed by sub-culturing on Serum Dextrose Agar (SDA) (Jones and Morgan, 1958) and Farrell's media plates (Farrell and Robertson, 1972). Suspected isolates were subjected to various biochemical tests for typing which include serotyping, urease production, H_2S production, dye-plate inhibition, CO_2 dependency, and phage typing. The World Animal Health (OIE) recognised methodology and APHA Standard Operating Procedures (SOPs) were strictly followed.

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

To test directly from clinical material, DNA was extracted from 200 µL of serum using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the kit protocol. Conventional PCR based on the *Brucella* spp. specific target IS*711* (O'Leary *et al*., 2006) was performed for the detection of *Brucella*. The PCR reaction mixture was prepared with a final volume of 25 µL. FastStart 1x PCR buffer with $MgCl_2$ (Roche), 0.4 mM dNTPs, 800 nM of both forward and reverse primers, 1 unit FastStart Taq DNA polymerase (Roche) with 5 µL template DNA (from DNeasy extraction). The following oligonucleotide forward (5´-GACGAACGGAATTTTTCCAATCCC-3´) and reverse (5´-TGCCGATCACTTAAGGGCCTTCAT-3´) primers were used during the reaction. Thermocycler parameters used were as follows: pre-incubation at 94°C for 5 min, 40 cycles of 94°C for 30 sec for heating, 63°C for 30 sec for annealing and 72°C for 1 min for extension and final extension step of 72°C for 5 min. We spiked an aliquot of each sample with 1 µL of a 1 $ngmL^{-1}$ dilution of *B. abortus* (strain 544) genomic DNA obtained through phenol chloroform extraction (Sambrook *et al*., 1989) to check for possible inhibition or false negatives through PCR. The products of amplification were visualised using a 2% agarose gel and a size marker of 1 kb, with a fragment size of 498 bp corresponding to the desired IS*711* target (O'Leary *et al*., 2006). Each sample was tested in duplicate and only those that generated a product of the correct size on both occasions were identified as *Brucella* positive.

Molecular Characterization of Bacterial Isolations

Molecular characterization was carried out for classification of *Brucella* into species and biovars. In brief, genomic DNA was extracted from field isolates which grew on blood culture through procedures as explained earlier by Whatmore *et al.* (2005). Based on single nucleotide polymorphism (SNP) identified by Whatmore *et al.* (2007), species identification of field isolates and determination of possible vaccine markers were undertaken using previously described multiple outcome real-time PCR assays (Goupal *et al*., 2008, 2010). These competition assays were set up in final reaction volume of 12.5 µL containing 6.25 µL TaqMan genotyping mix (Applied Biosystems, Warrington, United Kingdom) using Agilent MX3005p platform (Agilent, La Jolla, CA) with working concentrations of primers, probes and cycling conditions as mentioned in above citation. MxPro software provided with Mx3005p was used for analysis of results.

Multilocus Sequence Analysis (MLSA) was performed using nine unique genome sequences amplified through PCR according to the procedure illustrated by Whatmore *et al*. (2007). The amplified PCR products were purified using the QIA quick 96 PCR purification kit (QIAGEN, cat. no. 28183) followed by sequencing using Big Dye terminator cycle sequencing kit (version 3.1, Applied Biosystems) according to manufacturer's guidelines. Sequenced data was edited by using DNA Star® Lasergene 8 software and Phylogenetic analysis was performed using MEGA 6.0 (Tamura *et al*., 2013). For VNTR analysis, an MLVA-16 approach as used by Al Dahouk *et al*. (2007) was adopted for typing and species identification of *Brucella*. Fragment sizes were determined using an ABI3130 x l sequencer (Applied Biosystems, Warrington, UK) as per manufacturer's instructions. Fragment sizes were imported into GeneMapper® v3.7 software (Applied Biosystems, Warrington, UK) and VNTR allele calls (number of DNA tandem repeats) generated. VNTR allele calls were analysed in Bionumerics v6.6 (Applied Maths) as character data.

Analysis of the Diagnostic Testing

The diagnostic data were statistically analysed with chi-square test (χ^2) (Steel *et al*., 1997) using GraphPad Prism, version 6.05 (La Jolla, CA USA), (www.graphpad.com). P-value<0.05 was considered statistically significant.

Results

Serological, Molecular and Culture Diagnosis of Samples

Serological testing of the 167 serum samples found 7 (4%) samples positive by RBT and 10 (6%) by cELISA for presence of antibodies against *Brucella* whereas molecular testing using IS*711* PCR and blood culture identified 35

(21%) and 2 (1%) positives, respectively (χ^2=52.30, P<0.05) (Table 1). Species-wise occurrence of disease was found higher in goats as compared to bovine (cattle and buffalo) (Table 1). Results from different methods used for brucellosis diagnosis are plotted in Venn diagram for comparative analysis showing 2 (1%) samples out of 167 as positive by all serological, molecular and culture detection methods remarking the discrepancies of different diagnostic procedures used during the study (Fig. 1).

Culture and Classical Biotyping

Traditional microbial tests phenotypically identified the characteristics of *Brucella* positive in two blood cultures (Table 1) and were referred to as S26 and S27. These field isolates grew on Serum Dextrose Agar (SDA) showed urease activity but did not produce H_2S. Culture plates containing Thionin and Basic Fuchsin dyes did not inhibit the growth of *Brucella*. Both isolates showed better growth on CO_2 dependency plates without any supplementary CO_2 presence (Table 2). The isolates were subjected to agglutination tests for their predominant agglutinogen (A or M, indicating *B. abortus* and *B. melitensis* antigens respectively) with mono-specific A and M antisera. They were agglutinated with M mono-specific serum only. Phage typing showed the Partial Lysis (PL) with Berkeley (Bk2) and Izatnagar (Iz) (Table 2). Based on these phenotypic characteristics and phage typing, S26 and S27 field isolates were identified as *B. melitensis* biovar 1.

Molecular Analysis of Bacterial Isolates

The samples identified and characterized as *B. melitensis* biovar 1 through standard and classical biotyping techniques were further subjected to molecular characterization. Using single nucleotide polymorphism (SNP) based multiple outcome real-time PCR and vaccine strain identification assays, it was possible to confirm the identity of the two *Brucella* isolates as non-vaccine *B. melitensis* strains (Fig. 2a and b). The 9-loci MLSA profiles generated by the isolates were identical and comparable with other *Brucella* MLSA profiles held at the APHA internal database. It was determined that both profiles fall in sequence type (ST) 8, which is a type associated with *B. melitensis* (Fig. 3). Within this ST, there are strains from each of the three accepted *B. melitensis* biovars (including the *B. melitensis* biovar 2 strain 63/9). There were a number of strains associated with geographical areas like India, Middle East and Southern Europe.

Subsequently MLVA-16 was performed on the isolates, which showed that there were similarities at a number of VNTR loci, but they were unique to each other based on differences at two loci (Table 3). Using a weighted analysis, the isolates were compared with 108 *B. melitensis* profiles from a global database held at the Institut Pasteur, Paris (http://mlva.u-psud.fr/*brucella*/) using

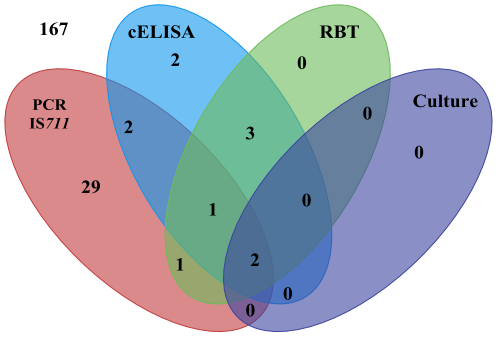

Fig. 1: Venn diagram showing a summary of serological (RBT, cELISA), molecular and cultural testing results of animal samples (n=167) illustrationg the number of positive samples with single and multiple tests

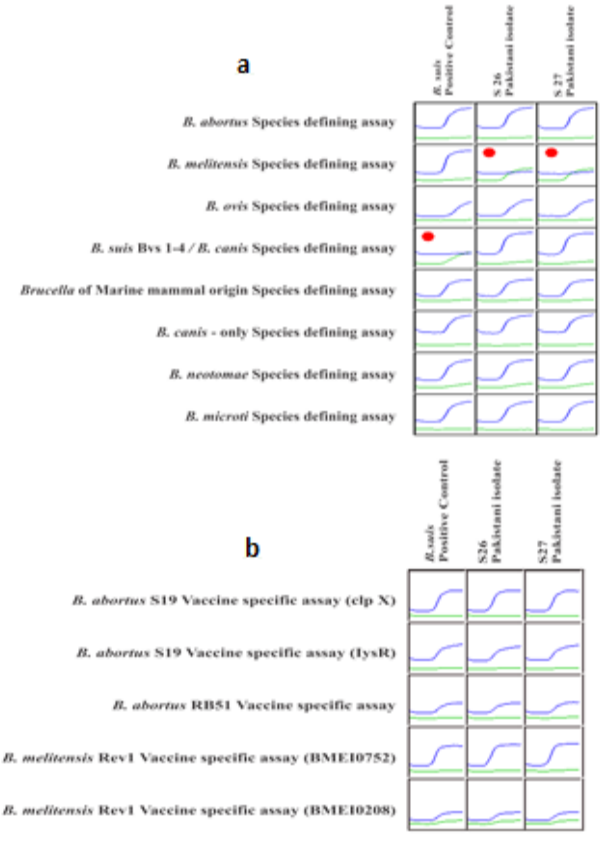

Fig. 2a, b: Results of real time PCR identification of caprine isolates based on species (a) and vaccine (b) specific SNPs. From these tests both S26 and S27 isolates were positively identified as non-vaccine *B. melitensis*

the Bionumerics Version 6.6 software (Applied Maths, Sint-Martens-Latem, Belgium) for further characterization. The placement of these strains in the current global scheme showed that these strains grouped with *B. melitensis* isolates

Table 1: Overall and species-wise results of different methods used for brucellosis diagnosis from blood and serum samples

Animal Species	Number of samples	Positive samples detected			
		RBT	cELISA	IS*711* PCR	Culture
Cattle	92	2 (2%)	2 (2%)	13 (14%)	0
Buffalo	45	0	1 (2%)	11 (24%)	0
Goat	30	5 (16%)	7 (23%)	11 (36%)	2 (6%)
*Total	167	7 (4%)	10 (6%)	35 (21%)	2 (1%)

*The differences among IS*711* PCR assay results, serology and culture were found to be statistically significant ($\chi^2 = 52.30$; $p < 0.05$)

Table 2: Biochemical, agglutination and Phage lysis test results of bacterial isolations

Sample	Growth Characteristics					Monospecific Sera		Phages						Interpretation
	Urea	H_2S	CO_2	BF	TH	A	M	Wb	Tb	BK	Fi	Iz	R/C	
S26	++	-	-	+	+	-	+	NL	NL	PL	NL	PL	NL	*B. melitensis* 1
S27	++	-	-	+	+	-	+	NL	NL	PL	NL	PL	NL	*B. melitensis* 1

BF = Basic fuchsin at 20µl/ml (1/50,000 w/v)
TH = Thionin at 20µl/ml (1/50,000 w/v)
CL = Confluent Lysis
PL = Partial lysis
NL = No lysis

Phages : Wb: Weybourne
Tb: Tbilisi
Bk: Berkeley
Fi: Firenze
Iz: Izatnagar

Table 3: MLVA16 profiles for isolated strains showing that both strains are similar but have differences in two loci (highlighted)

Sample	Bruce06	VNTR26	Bruce11	Bruce12	Bruce42	Bruce43	VNTR7	VNTR24	VNTR17	Bruce19	Bruce21	HP6	VNTR5a	HP8	VNTR2	HP2
S26	1	5	3	13	2	2	3	2	4	40	8	5	4	**7**	**2**	6
S27	1	5	3	13	2	2	3	2	4	40	8	5	4	**8**	**7**	6

from the "East Mediterranean" group (Fig. 4).

Discussion

Comparison of Culture, Serological and Molecular Diagnostic Methods

There are a number of published studies regarding prevalence of brucellosis in different areas of Pakistan, in livestock (Nasir *et al*., 1999), human population (Mukhtar and Kokab, 2008; Mukhtar, 2010; Ali *et al*., 2013) and the food chain (Hassan *et al*., 2010; Shafee *et al*., 2011). However, it should be noted that the work by Hassan *et al.* (2010) described the isolation of *Brucella* organisms by culture, which was the part of a wider study into the microbial contamination of meat. In this way the emphasis on many of these previous studies has been the diagnosis of infection through serological response and not on the direct detection of the causative agent through either culture or molecular methods.

Recently, Ali *et al*. (2014) described the isolation of 30 *B. abortus* strains in aborted foeti, vaginal swabs and milk samples collected from bovids that had either aborted or produced antibodies to *Brucella* in milk. Strains were confirmed as *B. abortus* by cultural and molecular methods (Bricker and Halling, 1994). A more holistic approach was adopted by Akhtar *et al.* (2010) who used a combination of serological, culture and molecular methods to screen milk and serum samples from 100 cattle and 100 buffalo. The same PCR test was used to determine the presence of pathogens in milk and serum samples (O'Leary *et al*., 2006) with results being compared with culture outcomes.

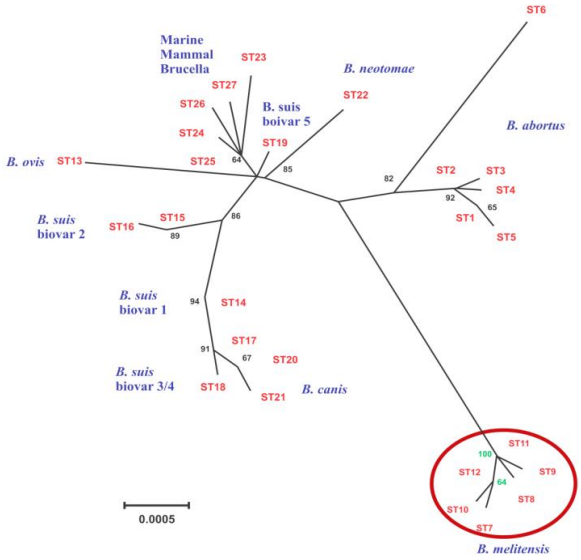

Fig. 3: This figure was adapted from Whatmore *et al.* (2007) and showed the placement of ST8 within the three main groups associated with *B. melitensis* (circled)

It was established that PCR testing of sera and milk

generated more positive results than serology. It was suggested that PCR of serum samples along with serological testing could be used as rapid screening method for bovine brucellosis (Akhtar *et al*., 2010). These observations are similar to those seen in the present study, where PCR identified 35 positive samples, whereas RBT and cELISA detected 7 and 10 positive, respectively. However, there was poor correlation between the results of PCR and serology. In present study, 29/35 PCR positive samples were tested as negative for brucellosis by serology. Similarly, 3/7 and 5/10 serological positives by RBT and cELISA, respectively, were PCR negative (Fig. 1). This is in contrast to another study comparing molecular and serological diagnosis of brucellosis in Malaysian goats where it was found that 70/288 goats tested were positive by complement fixation test (CFT) and conventional PCR, whereas 200/288 were negative by both of these tests (Al-Garadia *et al*., 2011). This observation is confusing as it has been shown that cELISA is a robust serological test that can be used to detect samples unsuitable for CFT (Stack *et al*., 1999) and is also as sensitive as CFT in goats (Perret *et al*., 2010). With this and the PCR results in mind, it was expected to see more serological positives than seen in this test. However, the fact that both culture confirmed samples in present study were also positive by RBT, cELISA and PCR, which at least showed that using a combination of diagnostic methods was as good as culture test.

Molecular Characterization of *B. melitensis* Isolates

This study, to our knowledge, is the first one to describe the molecular characterization of *B. melitensis* strains isolated from Pakistan using MLSA profiles and VNTR loci. Although host specificity in *Brucella* is not a rigid determinant of species, in the current study, the isolates were identified by both classical and molecular methods as *B. melitensis*; the species associated most strongly with caprine brucellosis (Elzer *et al*., 2002) and considered the most significant human pathogen within the genus *Brucella* (Colmenero *et al*., 1996). Regarding *Brucella* identification, it should be noted that classical methods were performed in three days with specialized handling facilities. On contrary, the molecular identification was performed from a crude extraction in a general laboratory setting within 3 h. To determine if the strain is a vaccine (Rev1) by culture, drug resistance profiles for streptomycin and penicillin would require an additional 3 days, whereas the molecular methods can provide the same level of information within 3 h of the preparation of a crude extraction. After preliminary identification of *Brucella*, the techniques of MLSA and VNTR (MLVA-16) were carried out to further characterize the isolates and to place them in a global *Brucella* context using nucleotide databases. It should be noted that the MLVA profiles of the isolates were quite distant from the only other available Pakistani *B. melitensis* isolate which was obtained from a human (Al Dahouk *et al*.,

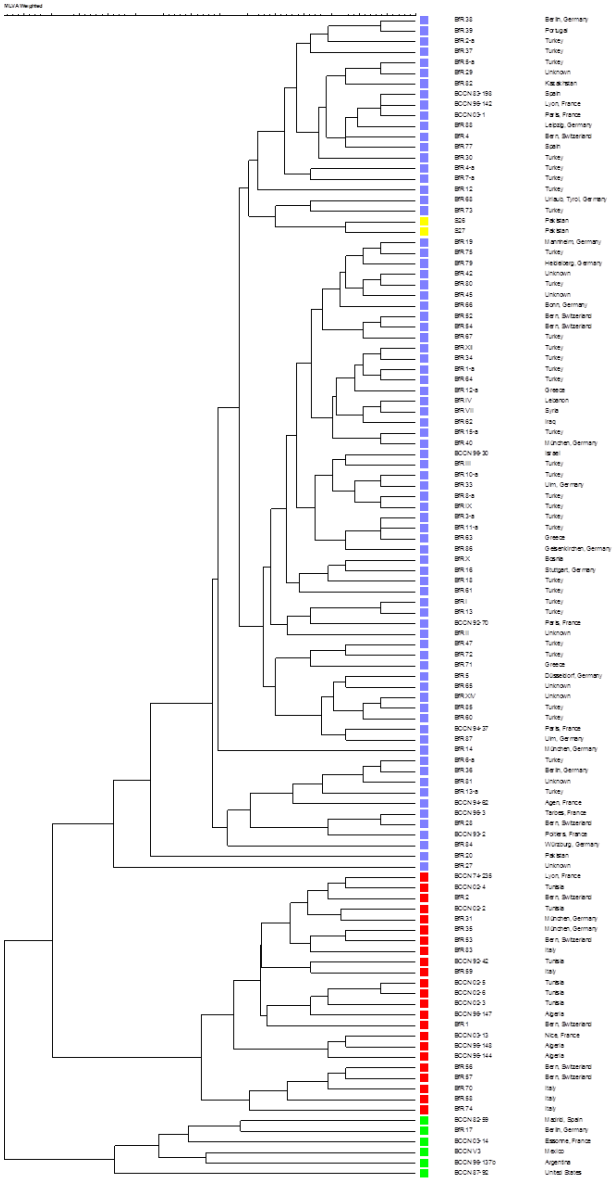

Fig. 4: Weighted dendrogram of isolates taken from Al Dahouk *et al*. (2007) with isolates of present study (in yellow) to determine MLVA16 "grouping" within *B. melitensis*. The groups are East Mediterranean (blue), West Mediterranean (Red) and American (Green). The isolates of present study belong to the East Mediterranean group

2007) suggesting the circulation of diverse genotypes. Further, the results from both molecular methods suggest that these Pakistani isolates were not part of a geographically confined group but were representative of other *B. melitensis* strains found in a region stretching from southern Europe into South Asia. The isolation of *B. melitensis* from caprines in Pakistan along with previous *B. abortus* in bovids (Ali *et al*., 2014) suggests that there is *Brucella* strain diversity within Pakistan.

Conclusion

In conclusion, classical biotyping techniques followed by molecular tools provide a handful mechanism to characterize the genus *Brucella* and to understand the genetic diversity of organism. The IS*711* PCR was found to be more sensitive and accurate method of identification. It was found that serum could be used as dependable and safer clinical sample for serological and molecular diagnosis than whole blood or other foetal tissue posing less hazards to laboratory workers. It is also observed that a battery of tests containing more than one test should be followed for getting consensus results. However, more systematic studies will be required to establish the prevalence of brucellosis and the significance of each species in the livestock of Pakistan. Having these data will allow decision makers to make informed choices for future brucellosis control measures.

Acknowledgements

The authors would like to acknowledge the technical assistance provided by Mr. Andrew Taylor and Ms. Emma-Jane Dale for the serological work and Mr. Steven Shankster and Ms. Pauline Groussaud for their work in the initial VNTR analysis of strains. This work was funded by Higher Education Commission of Pakistan (HEC) and the Department of Environment, Food and Rural Affairs (Defra), United Kingdom as part of ROAME projects SE0311 and SE0313.

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(Received 27 March 2015; Accepted 07 August 2015)