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Short Communication

Molecular Identification of Bovine Brucellosis Causing Organisms at Selected Private Farms in Pothohar Plateau, Pakistan

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SA, IK and SU collected the samples and analysed the data. SA, HN and FM wrote the article. TJ helped in preparation of the article.

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Brucellosis, Brucella abortus, Real-time PCR, Cattle, Pothohar Plateau.

ABSTRACT

Brucellosis is a worldwide zoonotic infection in animals including Pakistan. A study was conducted to identify the potential cause of infection in three different cattle farms located at Potohar Plateau in Punjab, Pakistan. A total of 399 serum samples from indeginous and exotic cattle were tested for brucellosis by RBPT, SAT and multiplex real-time PCR for *Brucella* sp., *Brucella abortus* and *Brucella melitensis*. A total of 20 (5.01%) samples were found seropositive by RBPT and 19 (4.76%) by SAT, however real-time PCR showed 13 (3.25%) samples positive for both *Brucella* (sp.) and *Brucella abortus*. None of the seropositive samples were found positive for *Brucella melitensis* by real-time PCR. Exotic cattle tended to be more positive for the infection as compared to indigenous cattle. Routine screening and strict biosecurity measures are recommended for prevention of infection in animals. Humans must consume milk after pasteurization.

Brucellosis is a worldwide zoonotic infection in a variety of animals *e.g.* cattle, buffaloes, sheep, goats and camels. It is primarily caused by *Brucella abortus* in bovines and *Brucella melitensis* in small ruminants whereas cross-species transmission of this infection is possible (Aparicio, 2013; Ali *et al*., 2015; Wareth *et al*., 2015). Characteristic signs include abortion in last trimester and retention of fetal membranes in female animals whilst orchitis and epididymitis in males, resulting overall in infertility (Corbel, 2006). Once infected, the bacteria are heavily shed in vaginal secretions and milk (Aparicio, 2013; Ebrahimi *et al*., 2014). In animals, this infection transmits directly via contact with infected animals or indirectly through ingestion of contaminated feed or water whereas in humans; it is through ingestion of contaminated raw milk or milk products (Mukhtar, 2010; Wareth *et al*., 2014; Ali *et al*., 2016). This infection is considered as endemic in Pakistan (Munir *et al*., 2011; Ali *et al*., 2014). The country bears a huge number of livestock *i.e.* 186.2 million among which ruminants (bovines, small ruminants and camels) make share of 180.5 million (96.9 %) (Anonymous, 2016). Current study was designed to investigate the infection status at three private farms located in Pothohar Plateau region by serological and molecular diagnostic techniques.

Materials and methods

Pothohar Plateau is located in north east of Pakistan consisting of four districts; Rawalpindi, Chakwal, Jhelum, Attock and Islamabad Capital Territory (ICT). It is surrounded by Jhelum River in the east, Indus River in the west, Margalla hills in north and the Salt range in south. This area falls under arid climate zone of Pakistan (Khan, 2004; Kazmi and Rasul, 2012). Livestock are mainly fed by grazing on range lands (Chaudary *et al*., 2007). Both indigenous and exotic cattle are prevalent with exotic animals raised primarily for milk production. However, intensive husbandry practices are only seen by exotic cattle.

A total of 399 blood samples (250 from indigenous and 149 from exotic cattle) were collected from three different livestock farms in the region. Blood samples (3 ml) were collected directly from jugular vein with a 5 ml syringe. Each blood sample was transferred into a non-EDTA coated vacutainer, placed at room temperature for 30 min, and centrifuged at 4,000 rpm for 15 min. Serum was discharged into a 1.5 ml Eppendorf tubes and stored at

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Table I.- Primer/Probes Sequence for *Brucella* genus and species-specific real-time-PCR.

Serial No.		Sequence
Brucella (genus)	Forward primer	5'-GCTCGGTTGCCAATATCAATGC-3'
	Reverse primer	5'-GGGTAAAGCGTCGCCAGAAG-3'
	Probe	FAM-AAATCTTCCACCTTGCCCTTGCCATCA-BHQ1'
Brucella abortus	Forward primer	5'-GCGGCTTTTCTATCACGGTATTC-3'
	Reverse primer	5'-CATGCGCTATGATCTGGTTACG-3'
	Probe	FAM-CGCTCATGCTCGCCAGACTTCAATG-BHQ1
Brucella melitensis	Forward primer	5'-AACAAGCGGCACCCCTAAAA-3'
	Reverse primer	5'-CATGCGCTATGATCTGGTTACG-3'
	Probe	FAM-CAGGAGTGTTTCGGCTCAGAATAATCCACA-BHQ1

Table II.- Presence of *B. abortus* antibodies in different breeds of cattle using Rose Bengal Plate Test (RBPT).

Cattle Breeds	Samples examined	Positive sample	Positive (%)
Achai	13	0	0
Cholistani	24	1	4.17
Dhani	56	1	1.79
Lohani	98	6	6.12
Red Sindhi	24	1	4.17
Sahiwal	35	2	5.71
Holstein Frisian	74	5	6.76
Jersey	75	4	5.33

-20 °C until further analysis.

Serum samples were screened initially by Rose Bengal Plate Test (RBPT) and then confirmed by Serum Agglutination Test (SAT) as described by Alton *et al.* (1988). Standard reagents were provided by Veterinary Research Institute (VRI), Lahore, Pakistan. A sample of 1:160 titers or higher was considered positive for SAT.

For molecular detection DNA was extracted from seropositive serum samples by using High Pure PCR Template preparation kit (Roche Diagnostic, Germany) as per manufacturer's recommendations. DNA samples were stored at -20ºC till further analysis. Multiplex real-time PCR technique was used for molecular detection of the etiology as described by Probert *et al.* (2004).

Table I shows primers used for amplification of *Brucella* genus (*bcsp31*) and species (*IS711* for *B. abortus* and *B. melitensis*). The cut-off cycle threshold (Ct) value for a positive sample was set as ≤ 40.

Results and discussion

A total of 20 (5.01%) serum samples were found seropositive by RBPT and 19 (4.76%) by SAT (Table II). Out of these sero-positive samples, 13 (overall 3.25%) were found positive only for both *Brucella* genus (BCSP31) and species *Brucella abortus* (IS711) by real time-PCR (Table III). None of these seropositive samples were found positive for *Brucella melitensis*. Only one sample, found positive by RBPT but negative by both SAT and real-time PCR, could be attributed to the low positive predictive value of RBPT (Gul and Khan, 2007).

Brucella abortus is a common etiology of brucellosis in bovines as well as in camels and small ruminants in the country (Ali *et al.*, 2014, 2015; Fatima *et al.*, 2016). Although open grazing, mixed farming and nomadic rearing pose brucellosis risk to animals, intensive farming also does not guarantee absolute protection (Gul and Khan, 2007; Abubakar *et al.*, 2012; Ullah *et al.*, 2015; Munir *et al.*, 2011). Infected semen, breach in biosecurity and latent infection could be the main reasons. Recently, felines and canines have been reported to disseminate the infection at dairy farms (Wareth *et al.*, 2016). Our study revealed the exotic animals tending to be more seropositive (6.04%) than indigenous animals (4.4%). Same results have been observed elsewhere in the country (Ali *et al.*, 2013; Mangi *et al.*, 2015; Kaleem *et al.*, 2016) but one study has shown opposing results (Khan *et al.*, 2016). These observations however, need to be studied further through intense inter-breed comparison of seropositive animals.

Table III.- Comparison of serological and molecular identification for detection of bovine brucellosis.

Sample No	Cattle Breed	Serological Tests		qRT-PCR		*B. melitensis* (IS711)
		RBPT	SAT	*Brucella genus* (BCSP31)	*B. abortus* (IS711)	
1	Cholistani	+	+	+	+	-
2	Dhani	+	+	-	-	-
3	Lohani	+	+	+	+	-
4	Lohani	+	+	+	+	-
5	Lohani	+	-	-	-	-
6	Lohani	+	+	+	+	-
7	Lohani	+	+	-	-	-
8	Lohani	+	+	-	-	-
9	Red Sindhi	+	+	-	-	-
10	Sahiwal	+	+	-	-	-
11	Sahiwal	+	+	+	+	-
12	Holstein Frisian	+	+	-	-	-
13	Holstein Frisian	+	+	+	+	-
14	Holstein Frisian	+	+	+	+	-
15	Holstein Frisian	+	+	+	+	-
16	Holstein Frisian	+	+	+	+	-
17	Jersey	+	+	+	+	-
18	Jersey	+	+	+	+	-
19	Jersey	+	+	+	+	-
20	Jersey	+	+	+	+	-

Statement of conflict of interest

The authors declare no conflict of interest regarding this paper.

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