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Short Communication

Prevalence of Bovine Brucellosis in Islamabad and Rawalpindi Districts of Pakistan

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Authors' Contributions
IK and SHK conceived and designed the project. TA, SR and SHK collected samples, performed the experimental work and analyzed the data. TA, SHK, IK and RA wrote the article.

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ABSTRACT

A study was conducted to determine the prevalence of *Brucella abortus* in cattle and buffaloes in the districts of Islamabad and Rawalpindi, Pakistan. A total of 120 serum samples were randomly collected from buffaloes and cattle (60 per species) at Civil Veterinary Hospitals, animal markets and peri-urban livestock holdings in Rawalpindi and Islamabad. Serum samples were initially screened by the Rose Bengal Plate Test (RBPT). RBPT positive samples were subjected to a *B. abortus* specific indirect enzyme-linked immunosorbent assay (i-ELISA). Serum samples that were confirmed to be positive for *B. abortus* through serology were subjected to an rPCR in order to test its efficacy in detecting *Brucella* in blood of infected animals. Initially a *Brucella* genus-specific bcsp31 genomic region based rPCR was used. This was followed by two species-specific rPCRs that detected IS*711* genomic region of *B. abortus* and *B. melitensis*. Five (8.3%) serum samples from cattle and 1 from buffalo (1.6%) were found to be positive for *B. abortus* by RBPT. Four (6.6%) out of the 5 RBPT positive cattle samples were also found to be positive by i-ELISA, while one was negative in i-ELISA. The single RBPT positive buffalo was also positive in i-ELISA. *Brucella* genus specific rPCR amplification occurred in all the 5 seropositive samples. In the species-specific rPCR *B. abortus* was detected in all the samples.

Brucellosis is an economically important disease of various animal species and is one diseases that have been enlisted by the Office International des Epizooties (OIE). Important animal species that can get this disease include cattle, buffalo, swine, sheep, goats, camels, dogs and being zoonotic can also infect humans. For diagnosis of disease, the bacteria can be isolated from body secretions, tissues and aborted fetuses. However, this is not practiced due to difficulties in culturing the organism. Instead various serological techniques are applied for diagnosis such as Milk Ring Test (MRT), Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT) and ELISA (Godfroid *et al*., 2010). A quicker, inexpensive, sensitive and safe approach is PCR that detects *Brucella* DNA in serum samples of infected animals (Bricker, 2004). The PCR and i-ELISA had been found to have sensitivity levels of 87.5% and 98.2%, respectively, when applied on milk samples for diagnosis of bovine brucellosis. Based upon these results it is recommended that both tests should be used together for screening of herds in epidemiological and surveillance programs (Kattar *et al*., 2007).

Brucellosis has been reported since long in Pakistan and due to its increasing prevalence emphasis has been put on regular screening of livestock herds and of animals brought at abattoirs and at livestock markets (Abubakar *et al*, 2010). In the present study cattle and buffaloes in two districts of Pakistan were screened for antibodies against *B. abortus*. Furthermore, an rPCR assay was tested for its effectiveness to detect *Brucella* in the serum of local breeds of infected animals.

Materials and methods

The study was conducted in the districts of Rawalpindi and Islamabad. A total of 120 blood samples (60 each from cattle and buffaloes) were randomly collected in 10 mL disposable clot activating tubes. The samples were

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collected from animals brought at animal markets and abattoirs and from animals kept in small animal holdings. While collecting samples, history, including abortion, or long calving interval was also recorded. The samples were also collected from animals brought for treatment at various veterinary hospitals in the districts of Rawalpindi and Islamabad. Samples were transported at 4°C to the Department of Animal Sciences, Quaid-i-Azam University, Islamabad. Sera were separated by centrifugation at 10,000 rpm for 1 min to enhance the process of serum separation and samples were stored at -20°C till further use.

The RBPT was performed as described in the Office International des Epizooties (OIE)'s manual (www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.04_Brucellosis.pdf). A drop of test serum was mixed with 30µL of RBPT antigen (obtained from Veterinary Research Institute, Lahore) on a slide and left for 4 minutes. Along with each test sample, a positive control reaction using positive serum (obtained from the University of Agriculture, Faisalabad) was also performed. Each test sample was checked for agglutination by comparison with the positive control.

The serum samples found to be positive by the RBPT were subjected to a *B. abortus* specific i-ELISA using a commercially available kit (Cat. No. C561, IDEXX Switzerland) following manufacturer's protocol. i-ELISA was performed at the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore.

An rPCR described by Probert *et al*. (2004) was tested for its efficacy in detecting *Brucella* at genus level and then at species level (*B. abortus* and *B. melitensis*) in the serum of infected cattle and buffaloes. For this purpose, the samples confirmed to be positive via both the immunological tests *i.e.* RBPT and i-ELISA were subjected to the rPCR. Genomic DNA was extracted from serum samples using a commercially available kit (Cat No. FABGK001, Favorgen, Taiwan). First a *Brucella* genus-specific rPCR targeting the bcsp31 gene was performed on genomic DNA using the primers: 5'GCTCGGTTGCCAATATCAATGC 3' and 5'GGGTAAAGCGTCGCCAGAAG 3' and the genus specific probe 5' 6FAMAAATCTTCCACCTTGCCCTTGCCATCABHQ1 3' (Tibmolbiol, Berlin, Germany). The reaction contained the following components: 10µL Taq-Man™ Universal Master Mix (Applied Biosystems, New Jersey, Applied Biosystems), 200 nM of each primer, 100 nM of probe, 4µL of template DNA and water up to a total volume of 20µL. Along with each test reaction no-template-control (NTC) and positive control reactions containing *Brucella* DNA were also amplified. The amplification program used was 10 min at 95°C, followed by 45 cycles of 95°C for 15s and 57°C for 1 min. Next the species-specific rPCR that targeted the IS*711* element downstream of the *alkB* gene in *B. abortus* and insertion of the same element downstream of the BMEI1162 locus in *B. melitensis* was performed using the following primers for *B. abortus* and for *B. melitensis* 5'GCGGCTTTTCTATCACGGTATC3' and Reverse 5'CATGCGCTATGATCTGGTTACG3' 5'AACAAG-CGGCACCCCTAAAA3' and reverse CATGCGCTAT-GATCTGGTTACG3'. The probes used for *B. abortus* and *B. melitensis* were HEXCGCTCATGCTCGCCAGACT-TCAATGBHQ1 and CY5CAGGAGTGTTTCGGCT-CAGAATAATCCACABHQ2, respectively.

The reaction mixture was the same as in the genus-specific rPCR, however, the following modified amplification parameters were used: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15s, annealing/extension at 60°C for 60 seconds. Samples having a cycle threshold (Ct) values of ≤ 40 were considered positive for the both the genus specific and species specific rPCRs. Positive samples were recorded by visualization of graphical representation of cycle numbers versus fluorescence values.

Table I.- Prevalence of brucellosis in cattle and buffalo of Rawalpindi and Islamabad.

	Cattle	Buffalo
Total samples (n)	60	60
RBPT positive (n)	5	1
i-ELISA positive (n)	4	1
rPCR positive (n)	4	1

Results and discussion

The results of prevalence of *B. abortus* in cattle and buffaloes based on RBPT, i-ELISA and rPCR are shown in Table I. In RBPT, 5 out of 60 cattle samples and 1 out of 60 buffalo samples were positive (Table I). Upon re-testing of RBPT positive samples by i-ELISA, 4 out of the 5 cattle samples were found to be positive, while the 1 RBPT positive buffalo sample also came up as positive. The 1 RBPT positive cattle sample that was negative by i-ELISA was considered as *Brucella* negative and the prevalence rate was calculated by excluding that sample. The disagreement in the results of the two tests can be attributed to the probability of the RBPT to give false positives as a result of the cross-reaction of the RBPT antigen with other gram negative bacteria. The 4 cattle and 1 buffalo samples were also positive for *Brucella* by the genus specific rPCR. The species specific rPCR showed that *B. abortus* was present in all the 5 samples. Thus the prevalence was calculated as 6.6% for cattle and 1.6% for buffaloes. A previous study was conducted

in the same region in which MRT was used to determine the prevalence. Compared to our results a slightly higher prevalence (6.9%) in cattle and a higher prevalence in buffaloes (6.6%) was noted (Ali *et al*., 2013).

In Pakistan several studies have been conducted to study the prevalence of bovine brucellosis and majority of the reports are based on serological methods. Though earlier studies showed low prevalence rates of 0.33 to 0.65% (Sheikh *et al*., 1967) much higher prevalence (21.05 to 26.1%) has been reported in some of the relatively recent studies conducted in the provinces of Punjab and Khyber Pukhtunkhwa (KPK) (Akhtar *et al*., 1990; Ramzan, 1996). In other studies varying levels of prevalence have been observed. In a study conducted on animals at livestock farms, the incidence of brucellosis was found to be 14.70% in cattle and 15.38% in buffaloes at government livestock farms, and 18.53% in cattle and 35.40% in buffaloes at private livestock farms in various districts of Punjab (Nasir *et al*., 2004). In Quetta, prevalence rates of 8.5% in buffaloes and 3% in cattle have been noted (Shafee *et al*., 2011). At world level, the prevalence of brucellosis varies between different countries and continents of the world (Lopes *et al*., 2010).

Though the gold standard for diagnosis of brucellosis is the isolation and culture of the causal organism, the procedures for isolation and cultivation of *Brucella* are tedious, time-consuming and expensive. Furthermore, working with *Brucella* is risky and biosafety level-3 is required. Therefore, molecular tests like rPCR are a quick and safe way of detecting *Brucella*. Another advantage of rPCR is that it can detect DNA of damaged bacteria, the cultivation of which is not possible (Hinic *et al*., 2009). Several different single-primer and multiplex PCRs and rPCRs have been developed for detecting *Brucella* species (Yu and Nielsen, 2010). Probert *et al*. (2004) described a multiplex PCR for simultaneous detection of *B. abortus* and *B. melitensis* in a single tube. They tested it on genomic DNA extracted from *Brucella* and found that it accurately detected both *Brucella* species. We used the same assay on genomic DNA extracted from serum in order to measure the efficacy of this assay in identifying the organism directly in the serum of infected animals. The i-ELISA positive samples were also positive by the rPCR, which showed this rPCR assay was suitable for detecting *Brucella* in the serum of infected animals that belong to the local breeds of cattle and buffaloes.

Among 60 cattle that were sampled, 35 had a history of abortion, and out of these 35 samples 4 were positive for brucellosis (Table II). Out of 60 buffalo serum samples, 20 were from animals that had a history of abortion. In these 20 samples, only 1 was positive for *Brucella*. In case of 60 cattle samples, 30 were from animals that had long calving intervals and 3 of the 30 samples were positive for brucellosis. The 1 buffalo positive for brucellosis had been found to have a history of long calving interval. In order to find out if brucellosis was a risk factor associated with abortions and long calving intervals univariate analysis using the software Minitab 12.22 (Minitab Inc, PA, USA) was applied (Table II). Using risk analysis, at 95% confidence interval upper bound was found to be 0.02 and lower bound was found to be 50.939. Odd ratio was calculated to be 1. These results indicate that abortion and long calving interval were risk factors associated with brucellosis in animals tested in this study.

Table II.- Prevalence of brucellosis in animals with risk factors.

	Cattle	Buffalo
Total samples (n)	60	60
Animals with abortion history	35	20
Brucella positive samples	4	1
Brucella positive samples (%)	11.42%	5%
Animals with long calving interval	30	18
Brucella positive samples	3	1
Brucella positive samples (%)	10%	5.5%

There are also other causes of abortion in bovines. The non-infectious causes include genetic factors, vitamin A deficiency, heat stress and trauma *etc.* Infectious causes other than brucellosis include *Neospora caninum* infection, bovine viral diarrhea, infectious bovine rhinotracheitis, leptospirosis, mycotic abortion, *Trueperella pyogenes* infection, trichomoniasis, listeriosis, chlamydiosis, and Bluetongue *etc.* Any of the above mentioned factors could have been responsible for abortion in the rest of the 31 cattle and 29 buffaloes with a history of abortion. Infection with Brucella can also lead to long calving intervals. Thirty of the cattle and 1 of the buffaloes, from which samples had been taken, had a history of long calving interval. Among these, 3 cattle and the 1 buffalo were found to be positive for brucellosis, which may be the cause of the long calving interval in these animals. Some nutritional or managemental factor, or some other infection might have been the cause of long inter-calving interval in the rest of the animals with long calving intervals.

In order to control the disease proper diagnosis, vaccination and screening of animals at farms, at livestock markets and at abattoirs have been emphasized. Furthermore, quarantine measures have also been suggested. Mixing of infected and susceptible animals should also be prevented. Awareness about the disease in

farmers and in the livestock and public health authorities is also important.

Conclusion

In the present study, prevalence of brucellosis in cattle and buffaloes in the districts of Rawalpindi and Islamabad was ascertained. The prevalence was found to be 6.6% in cattle and 1.6% in buffaloes. The RBPT can be used as an initial screening test. However, the results must be verified by i-ELISA. The rPCR tested in this study accurately detected *Brucella* in the serum of all the animals that were positive by serology. Thus this rPCR is an accurate, fast and safe method for detecting *Brucella* in local breeds of cattle and buffalo.

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Statement of conflict of interest

The authors declare no conflict of interest for this research work.

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