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Serological and Molecular Detection of *Brucella abortus* from Cattle by RBPT, STAT and PCR and Sample Suitability of Whole Blood for PCR

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ABSTRACT

Brucella abortus, one of the major pathogen causing abortions in cattle worldwide and a zoonotic agent, need to be detected earlier in order to prevent its spread among animals. The present study aimed at to know the prevalence of *B. abortus* in cattle population of three states (Uttar Pradesh, Uttarakhand and Tamil Nadu) of India by serological (Rose Bengal Plate Test (RBPT) and Serum Tube Agglutination Test (STAT)) and molecular (polymerase chain reaction) detection in sera samples and whole blood (n = 370), respectively. Out of a total of 370 sera samples, 61 (16.49%) were positive by RBPT and 59 (15.94%) by STAT. Screening of the whole blood samples by genus specific *bcsp*31 gene based PCR as well as species specific IS*711* gene based PCR revealed that 56 (15.13%) samples were positive for brucellosis. None of the serologically negative sample showed positivity by PCR; however few positive samples were tested negative by PCR. Sensitivity and specificity of PCR compared with RBPT was 100 and 92.4% while with STAT these were 100 and 95.16%, respectively. Results are promising that whole blood can be used for studying the molecular epidemiology of *B. abortus* in cattle and particularly detecting the active phase of infection and PCR can be well adopted as a valuable test for mass screening of animals for this purpose. The present study adds to the prevalence data available regarding to *B. abortus* infection in cattle population and highlights the usefulness and advantages of molecular tool of PCR over serological tests.

Key words: *Brucella aborus*, cattle, prevalence, RBPT, STAT, PCR, whole blood

INTRODUCTION

Brucellosis, caused by a member of gram negative bacterium, is a well documented disease in animals and also a potential zoonotic agent (Gupta *et al*., 2014). *Brucella abortus* is the major abortion (usually in third trimester) causing agent in cattle and has been reported to affect cattle industry worldwide (Seleem *et al*., 2010). Brucellosis is endemic in most parts of India (Kumar *et al*., 2009; Islam *et al*., 2013) and it was found to be highest in Assam as per the earlier reports by sero-epidemiology and prevalence in Uttar Pradesh was 6.37% in cattle (Islam *et al*., 2013).

Diagnosis of a disease is of prime significance in order to identify, prevent and control a disease. Rapid and confirmatory diagnosis is more important in case of diseases which are highly infectious in nature, have high economical significance and possess zoonotic threats which cause havoc to the mankind (Atluri *et al*., 2011). A diagnostic method combined with appropriate sample for diagnosis at any stage of infection is the requirement to keep brucellosis in check. Conventional cultural isolation and identification of the agent is the gold standard test for *B. abortus* but is time consuming, laborious and also needs skills as well as biosafety measures to prevent zoonosis (Taleski, 2010). Various serological tests like Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Tests (STAT), enzyme linked immuno sorbent assay (ELISA) and others are commonly employed for mass screening of animals for brucellosis at farm level (Yu and Nielsen, 2010; Priyadarshini *et al*., 2013). The major drawback of these assays are they are not always specific, can cross react with other gram negative bacteria (*Yersinia enterocolitica* O:9, *Francisella* spp.) and antibodies are not produced at the acute stage of infection (Moussa *et al*., 2011). In this view, DNA detection methods like Polymerase chain reaction (PCR), versions of PCR (nested PCR, real time PCR, multiplex PCR) and loop mediated isothermal amplification test (LAMP) are now being employed for raid and confirmatory detection of brucellosis in cattle (Yu and Nielsen, 2010; Soleimani *et al*., 2013; Dhama *et al*., 2014; Gupta *et al*., 2014). *B. abortus* remains intracellularly and this poses a problem for selection of a suitable sample (Wattam *et al*., 2009). Only during acute phase of infection it circulates in blood, mostly inside the white blood cells and hides itself in mammary organs, genital organs and lymph nodes (Morgan and MacKinnon, 1979). Hence sample should be selected according to the phase of *Brucella*'s life pattern which is not practically possible to find out.

The present study aimed at to know the prevalence of *B. abortus* in cattle population of three states (Uttar Pradesh, Uttarakhand and Tamil Nadu) of India by serological [RBPT and STAT] and molecular (polymerase chain reaction) detection in sera samples and whole blood. Besides comparing the sensitivity and specificity of serological versus molecular tool of PCR, the suitability of the whole blood as a sample for detection of *B. abortus* in cattle using PCR was also evaluated.

MATERIALS AND METHODS

Bacterial strains: Two standard strains of *B. abortus* namely *B. abortus* S99 and *B. abortus* S19, obtained from Division of Veterinary Public Health and Division of Biological Standardization, Indian Veterinary Research Institute (IVRI), Izatnagar, India, were used in the present study. Both the cultures were tested for its family/genus characters and tested to be pure.

DNA template preparation: Bacterial DNA of both the strains was extracted using modified Cetyl trimethylammonium bromide (CTAB) method (Rodriguez *et al*., 1997). Briefly, 2 mL of *Brucella* culture previously inoculated in *Brucella* selective broth (Hi Media, India) was taken in a microfuge tube and centrifuged at 12,000 rpm to settle the pellet. Using 200 µL of 1% TE (Tris EDTA), 50 µL of 10% SDS and 5 µL of proteinase K, the pellet was resuspended and rapid pipetting was carried out to mix the contents and incubated at 37°C for 1 h. The incubated mixture was added with 5M NaCl (500 µL) and CTAB (100 µL) and kept in water bath at 63°C for 10 min, followed by addition of equal volume of phenol:chloroform:isoamylalcohol (25:24:1) and centrifugation at 10,000 rpm for 10 min. Aqueous phase was carefully transferred to another microfuge tube to which 1/10 volume of 7.5 M ammonium acetate and 2 volume of chilled absolute

ethanol followed by centrifugation at 10,000 rpm; final washing was given with 70% ethanol. Finally, 50 µL nuclease free water (NFW) was added to resuspend the DNA and the DNA was stored in -20°C till further use.

Sample collection: The serum samples and whole blood samples (n = 370 each) were collected separately from cattle population of different parts of three states [Uttar Pradesh (220), Uttarakhand (100) and Tamil Nadu (50)] of India, from various sources viz., cattle farms, polyclinics and slaughter houses. The serum samples were collected in vacutainers without anticoagulant; while whole blood samples were collected in heparin coated vacutainers. Most of the samples were collected from areas where there was a history of abortion or suspicion of *Brucella* occurrence. All the serum and whole blood samples were subjected for serological and molecular detection of *B. abortus*.

Serological tests: Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination Tests (STAT) for detection of *B. abortus* antibodies in all the serum samples (n = 370) was carried out according to the standard protocol of Alton *et al.* (1975). RBPT and STAT antigens were procured for Division of Biological Standardization, IVRI, Izatnagar.

Molecular Test

PCR detection of *B. abortus*: DNA from the whole blood was extracted according to protocol of Sambrook and Russell (2001) with slight modifications. Briefly, 300 µL of whole blood along with 700 µL of lysis buffer (1 M Tris-Cl, 5 M NaCl, 0.5 M EDTA, 10% SDS) and 5 µL proteinase K was taken in a 2 mL microfuge tube. The contents were mixed thoroughly and incubated at 56°C in water bath overnight. Tris saturated phenol was added in equal volume to the lysed mixture and centrifuged at 12,000 rpm for 15 min. Phenol: Chloroform: Isoamylalcohol (25:24:1) was added to the supernatant and centrifuged at 12,000 rpm for 10 min. To the aqueous phase, isopropyl alcohol (double the volume) was added to precipitate the DNA. The DNA pellet obtained after centrifugation at 10,000 rpm was washed twice with 70% chilled ethanol; air dried and added with 50 µL NFW to resuspend the DNA. The DNA thus obtained was stored in -20°C till further use.

Primers targeting for the *bcsp*31 gene were used for *Brucella* genus specific PCR (Baily *et al.*, 1992) and primers targeting IS*711* gene were employed for *B. abortus* species specific PCR (Doust *et al.*, 2007). The primer sequences used and the expected product sizes are presented in Table 1. The *bcsp*31 gene based genus specific PCR was optimized with initial denaturation at 95°C for 10 min followed by 35 cycles of denaturation (94°C for 1 min), annealing (55°C for 1 min) and extension (72°C for 1 min). Final extension was carried out at 72°C for 7 min. Similarly, IS*711* gene based species specific PCR was optimized with the same conditions used for *bcsp*31 PCR, except for the annealing condition which was kept at 58°C for 1 min. All the 370

Table 1: Details of PCR primers

Name	Sequence (5'-3')	Base pair	Reference
***Brucella* spp. (*bcsp*31) PCR primers**			
B4	TGGCTCGGTTGCCAATATCAA	21	Baily *et al.*, 1992
B5	CGCGCTTGCCTTTCAGGTCTG	21	
***B. abortus* (IS*711*) PCR primers**			
BAF	GACGAACGGAATTTTTCCAATCCC	24	Doust *et al.*, 2007
BAR	TGCCGATCACTTAAGGGCCTTCAT	24	

whole blood samples were screened for the presence of *Brucella* genus first with the *bcsp*31 primers based PCR and the samples which were found positive were screened further by IS*711* primers based PCR for detecting the *B. abortus* at species level.

RESULTS

Serological testing of the 370 serum samples revealed that 61 (16.49%) samples were positive by RBPT and 59 (15.94%) samples were positive by STAT. Screening of the whole blood samples by genus specific *bcsp*31 gene based PCR as well as species specific IS*711* gene based PCR revealed that 56 (15.13%) samples were positive for the *Brucella* genus as well as the *B. abortus* species, showing expected 223 bp (Fig. 1) and 498 bp sized amlpicons (Fig. 2), respectively. None of the serologically negative sample showed positivity by PCR; however few positive samples were tested

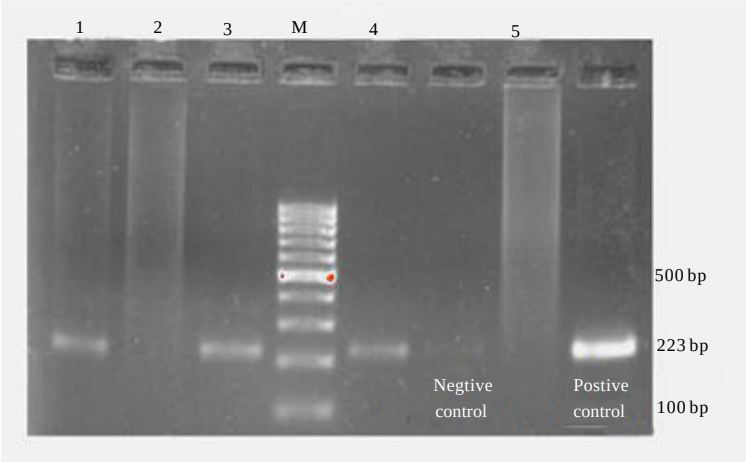

Fig. 1: *bcsp*31 gene based PCR for detection of *Brucella* spp. at genus level. Lane 1-5 DNA samples from whole blood, Lane M-100 bp ladder

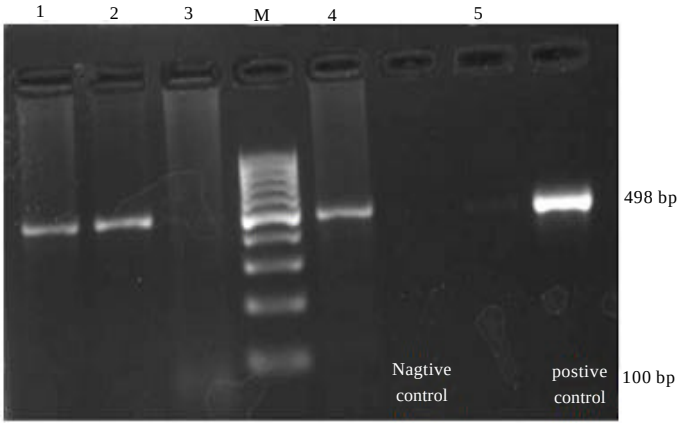

Fig. 2: IS*711* gene based PCR for detection of *B. abortus* at species level. Lane 1-5 DNA samples from whole blood, Lane M-100 bp ladder

Table 2: Serological and molecular detection of *Brucella abortus* in clinical samples by RBPT, STAT and PCR

		No. of samples positive					
Sampling region	No. of samples	RBPT	Prevalence (%)	STAT	Prevalence (%)	PCR	Prevalence (%)
Uttar Pradesh	220	38	17.27	37	16.82	35	15.90
Uttarakhand	100	15	15.00	15	15.00	14	14.00
Tamil Nadu	50	8	16.00	7	14.00	7	14.00
Total	370	61	16.48	59	15.94	56	15.13

negative by PCR. The clinical sensitivity and specificity of PCR compared with RBPT was 100 and 92.4% while with STAT these figures were 100 and 95.16%, respectively. The results of the serological and molecular detection and the prevalence rate of *B. abortus* in different areas under study are presented in Table 2. Among the three states, Uttar Pradesh showed a higher prevalence rate of 15.9% followed by Uttarkhand and Tamil Nadu both having 14% prevalence rate as detected using PCR.

DISCUSSION

Brucellosis is a zoonotic disease which needs to be diagnosed at the earliest and PCR is an exceptional tool which can diagnose a disease within 3 h accurately (Habtamu *et al*., 2013). Various diagnostic assays are used for the detection of *B. abortus* from cattle which includes basic bacteriological, serological and molecular assays (Da Silva Mol *et al*., 2012). Basic bacteriological techniques though remain as the standard tests they are laborious and involve risk to the laboratory person as the organism is zoonotic (Sam *et al*., 2012). This study was aimed at the diagnosis of *B. abortus* in three different states of India using serological test like RBPT, STAT and molecular assay of PCR and also the whole blood was evaluated as a sample of choice for mass screening of cattle for *B. abortus*. Serological tests recorded more positivity in the present study compared to PCR but these are not specific always because there are chances of cross reaction with other bacterial species (Moussa *et al*., 2011). The status of animal with respect to *Brucella* infection was not known during the time of sample collection and hence organism might have lodged in organs like lymph node, mammary glands or uterus and so DNA was not recovered from whole blood sample (O'Leary *et al*., 2006; Moussa *et al*., 2011). During acute phase of infection when the circulating organisms are higher, good quantity of DNA can be extracted and used for PCR (Gemechu *et al*., 2011). The prevalence of *B. abortus* from whole blood of cattle using PCR was 15.13% showing that *B. abortus* can be detected from whole blood sample. Similar results that DNA can be extracted from whole blood and used as a sample for screening animals for brucellosis were reported by Guarino *et al*. (2000) in buffaloe, Keid *et al*. (2010) in dogs and Khamesipour *et al*. (2013) in cattle and sheep. Lot of studies demonstrated that *Brucella* can be detected by PCR from whole blood of human samples but there are some contradictory findings in case of cattle (O'Leary *et al*., 2006; Bhanu Rekha *et al*., 2013). Al Nakkas *et al*. (2002) and Leal-Klevezas *et al*. (2000) used buffy coat instead of whole blood for DNA extraction as the macrophages take up the *Brucella* organisms. But buffy coat separation needs additional steps (Mitka *et al*., 2007) and hence the use of whole blood as such was tried in this study and it showed good results. Conventional DNA extraction protocol was carried out during the study which might be another reason for lower quantity and quality of DNA. Use of commercially available kits can improve the quantity and quality of extracted DNA (Queipo-Ortuno *et al*., 2008; Keid *et al*., 2010).

The prevalence of brucellsois in India in this study was lesser as compared to earlier study by Trangadia *et al.* (2010) but higher in Uttar Pradesh compared to Kumar *et al.* (2009). Various factors like time of sample collection, infection status of the animal, condition of the farm, number of samples collected can all influence on the detection results. In order to know the exact scenario, an extensive epidemiological survey should be conducted state wise which can help in planning the control and eradication measures for this important pathogen. The specificity and sensitivity of the PCR assay was compared with RBPT and STAT which was promising that PCR can be employed as a test for rapid screening of herds.

CONCLUSION

In conclusion, PCR can be used as a rapid and confirmatory diagnostic tool for mass screening of herds with brucellosis. The whole blood samples can be used as a valuable field sample of choice which can be collected easily unlike aborted foetal stomach contents needing aborted foetus or lymph node samples. Also, whole blood samples have utility in knowing the active/acute phase of infection *B. abortus* using PCR. This study adds to the available prevalence data regarding *B. abortus* infection in cattle. Extensive molecular epidemiological studies are suggested for knowing the magnitude of *B. abortus* infection in the cattle population of the country which would help to devise and adopt appropriate disease prevention and control measures against this economical important animal pathogen possessing public health concerns.

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