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A multi-pronged approach for diagnosis of *Brucella melitensis* infection in sheep flocks

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ABSTRACT

Isolation, serological tests and molecular methods were adopted for the diagnosis of brucellosis in sheep flocks with history of reproductive disorders. Three serological tests RBPT, STAT and iELISA were employed to screen 527 sheep from 6 farms. Among serological tests, iELISA detected higher positives (14.80%), than RBPT (5.88%) and STAT (4.17%). *Brucella melitensis* was isolated from 6 of 33 seropositive sheep and none from seronegative animals. All the isolates were identified as *B. melitensis* by biochemical tests and confirmed by genus and species specific - AMOS PCR. None of the isolates yielded products of 285bp, 976bp and 498bp specific for *B. suis, B. ovis* and *B. abortus,* respectively. Of the 33 seropositive sheep, the *bcsp*31genus specific amplicon of 223bp was observed in 26 vaginal, 6 serum and 2 in blood samples. Vaginal samples was found suitable clinical sample for the isolation and PCR, followed by serum and blood. Different diagnostic approaches used in the present study are of value as anti *Brucella* antibodies and antigen were detected confirming the disease in sheep flock. This practically oriented approach is of immense importance, as it enables to institute the control strategies at the earliest.

Key words: Brucellosis, iELISA, PCR, Sheep, Serological tests

Brucellosis is a reproductive disease of most species of domestic animals and responsible for economic losses (OIE 2009). *Brucella melitensis* is a dominant causative agent of brucellosis in sheep and goats in many countries including India and causes severe infection in human beings (Cloeckaert *et al.* 2003).The estimated losses due to abortion and stillbirths in sheep and goat would be up to ₹10 000 million (Gupta and Vihan 2001). Increasing demand for dairy products, change in agricultural methods, frequent mixing of flocks during grazing and inter-state trade of animals contribute to the high prevalence and wide distribution of brucellosis in these animals (Smits and Kadri 2005). Few reports of sheep brucellosis are available (Rumani and Punya 2005, Maher and Venkataraman 2007, Shome *et al*. 2006*).*Diagnosis of brucellosis is always done by multiple tests like isolation, serological tests and molecular approaches. Keeping the epidemiology of brucellosis, a multi-pronged approach for diagnosis of brucellosis in sheep flock with a history of infertility, abortion and repeat breeding by isolation, serological tests and PCR were carried out.

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MATERIAL AND METHODS

Antigens, hyperimmune serum and reference strains: Rose bengal coloured antigen and *Brucella abortus* plain antigen were obtained from the Institute of Animal Health and Veterinary Biological (IAH & VB), Bengaluru, India. Anti *B. abortus* hyperimmune serum raised in sheep and OIE reference sera were obtained from Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS), Bengaluru, India. *B. abortus* S99, S19; *B. melitensis* 16M *and B. suis* 1330 reference strains were procured by PD_ADMAS from Indian Veterinary Research Institute (IVRI), Izatnagar, were used for the bio-chemical tests and PCR.

Collection of sera samples for preliminary study and serological screening: Blood samples (10 ml) without anticoagulant were collected aseptically in sterile vials from 527 sheep from 6 flocks (Table 1). The sera after separation were used for detection of anti *Brucella* antibodies by 3 serological tests, viz. Rose Bengal Plate Test (RBPT), Standard Tube Agglutination Test (STAT) and indirect ELISA (iELISA). The RBPT and STAT (Weybridge technique) were performed as per Alton *et al.* (1975) and titre of 1:80 (160

Table 1. Prevalence of anti *Brucella* antibodies in sheep by serological tests

Farm no.	No. of samples tested	No. of positives		
		RBPT	STAT	iELISA
1	3	0	0	0
2	8	0	0	0
3	53	2 (3.77)	2 (3.77)	2 (3.77)
4	360	0	0	31 (8.61)
5	47	3 (6.38)	2 (4.25)	20 (42.55)
6	56	26 (46.42)	18 (32.14)	25 (44.64)
Total	527	31 (5.88)	22 (4.17)	78 (14.80)

The number in parenthesis is in %.

IU/ml) and above was taken as positive titre for sheep (Smits and Kadri 2005). The iELISA was carried out as per Shome *et al.* (2006) and sera sample with more than 54% positivity (PP) was considered positive for anti *Brucella* antibodies.

Clinical materials for detailed study: Blood samples with and without anti-coagulant (EDTA) and vaginal swabs were collected aseptically from 33 seropositive and 60 seronegative sheep from 6 flocks and transported to the laboratory on cold chain. To the vaginal sample, 1 ml of PBS was added, vortexed gently and used for isolation. The blood, serum and vaginal secretions were used for DNA extraction.

Isolation of Brucella *from vaginal swabs*: It was carried out as per the method of Alton *et al.* (1988). Vaginal swab suspensions (250 ml) were inoculated in 2 sets of *Brucella* selective broth with antibiotic supplements nystatin (50,000.0 IU), bacitracin (12,500.0 IU), polymyxin-B (2500.0 IU), cycloheximide (50.0 mg), vancomycin (10.0 mg) and nalidixic acid (2.5 mg). One set was incubated aerobically and another with 10% CO_2 at 37°C for 5 days. A loopful of broth culture from both the sets (broth) were streaked onto *Brucella* selective agar and incubated as described above till the appearance of growth or up to 15 days. The colonies suggestive of *Brucella* were streaked on MacConkey Agar (MA) and isolates producing lactose-fermenting colonies on MA were eliminated considering them as non-*Brucella.* The non-lactose fermenting cultures were subjected to rapid slide agglutination test using OIE international standard (OIEIS) and National (Indian Veterinary Research Institute, India) *B. abortus* positive sera. Gram modified Ziel-Neelsen (MZN) staining, oxidase, urease, triple sugar iron test (TSI) and dye inhibition tests using 1:50,000 and 1:100,000 basic fuchsin and thionin to identify the morphological and biological characters of *Brucella* organisms (Corbel and Banai 2005).

DNA extraction from clinical samples and cultures: Genomic DNA was prepared from both reference strains and field cultures by boiling method. In brief, loop full of 3 days old culture was taken in 0.5 ml of sterile double distilled water (DDW), vortexed and boiled for 10 min. The suspensions were adjusted with sterile DDW to give optical density (OD) of 0.5 at 600 nm, centrifuged briefly at 8,000×g and supernatants were used as templates in PCR. The DNA was extracted from clinical samples such as serum/blood/ vaginal samples using DNA kit.

Amplification of Brucella *genus and species-specific sequence by PCR:* Genus specific primers for amplification of *Brucella* genus specific sequences and 4 different species-specific (AMOS) primers (*B. abortus, B. melitensis B. suis* and *B. ovis*) were used (Table 2). For the amplification of target sequences, the reaction mixture of 25 µl each was prepared and PCR was performed in thermal cycler. The amplified products were electrophoresed on 1.5% agarose gel and image was captured in gel documentation system.

RESULTS AND DISCUSSION

Brucellosis is a reproductive disease of economic and public health importance in most species of domestic animals and human beings. The epidemiology of brucellosis involving various animal species and human beings necessitates detail study of brucellosis in sheep flocks with a history of infertility, abortion, repeat breeding and retained placenta.

Among RBPT, STAT and iELISA used to screen 527 sheep**,** iELISA detected higher positives (14.80%), than RBPT (5.88%) and STAT (4.17%) (Table 1). Similar observations were made while comparing ELISA with conventional tests to screen brucellosis in goats (Renukaradhya *et al.* 2002, Maher and Venkataraman 2007). The most compelling direct evidence of infection is obtained by isolation of the causative agent. *Brucella* organisms were isolated from 6 of 33 seropositive and none from 60 seronegative sheep. Vaginal swab from bovine abortion due

Table 2. Genus and species-specific (AMOS) primers

Name of the primer	sequence 5′ % 3′	product length (bp)	reference
bcsp 31 F	CGC GCT TGC CTT TCA GGT CTG	223	Baily *et al.* (1992)
bcsp 31 R	TGG CTC GGT TGC CAA TAT CAA		
B. abortus F	GAC GAA CGG AAT TTT TCC AAT CCC	498	Bricker and Halling (1994)
B. melitensis F	AAA TCG CGT CCT TGC TGG TCT GA	731	
B. suis F	GCG CGG TTT TCT GAA GGT TCA GG	285	
B. ovis	CGG GTT CTG GCA CCA TCG TCG	976	
IS 711 R	TGC CGA TCA CTT AAG GGC CTT CAT (Common to all the above forward primers)		

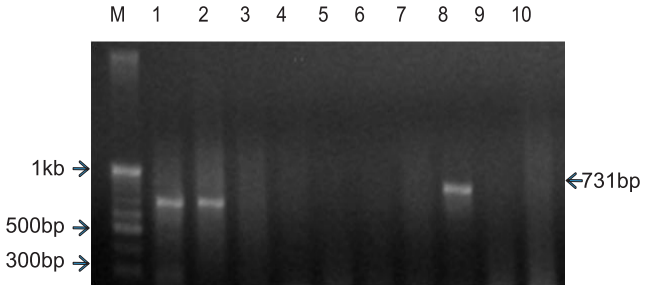
Table 3. Comparison of isolation and PCR in clinical samples

No. of samples		No of samples positive by isolation		No of samples positive by PCR		
				serum	blood	vaginal swabs
93	Seropositive	33	6	6(18.18%)	2 (6.00)	26 (78.78)
	Seronegative	60	0	0	0	2 (3.33)

The number in parenthesis is in %.

to brucellosis was reported to be an excellent clinical sample for the recovery of *Brucella* and a material of choice at any stage of infection (Ilhan *et al.* 2007). Occurrence of more number of organisms in foetal stomach contents, placental membrane and vaginal discharge of infected animals and mostly in vaginal secretion in chronic cases of brucellosis was reported (Keid *et al.* 2007, Leyla *et al.* 2003). Based on these earlier findings, vaginal samples from sheep were used for isolation.

The isolates showed strong agglutination with 2 reference sera in the slide test, Gram negative, urease and oxidase positive. Isolate showed poor growth in the presence of 5% CO_2, failed to produce H_2S in TSI and grew well in 1:50,000 thionin and basic fuchsin dyes. Morphological and biochemical test results identified the 6 isolates as *B. melitensis*. Though enrichment in *Brucella* selective broth for 48 h followed by culturing on *Brucella* selective agar were adopted, the lesser number of isolates were obtained from serologically positive animals. This is attributed to fastidious nature of *Brucella* organisms, lesser number of viable organisms, stage of the infection at which samples were collected or over growth of other fast growing organisms (Amit 2007). The amplicons of 223bp and 731bp (Fig. 1) in genus and species-specific PCR assays respectively confirmed the isolates as *B. melitensis*. None of the isolates yielded products of 285bp, 976bp and 498bp specific for *B. suis, B. ovis* and *B. abortus,* respectively. This finding is consistent with the *Brucella* species identified by bacteriological examination with that of species-specific PCR.

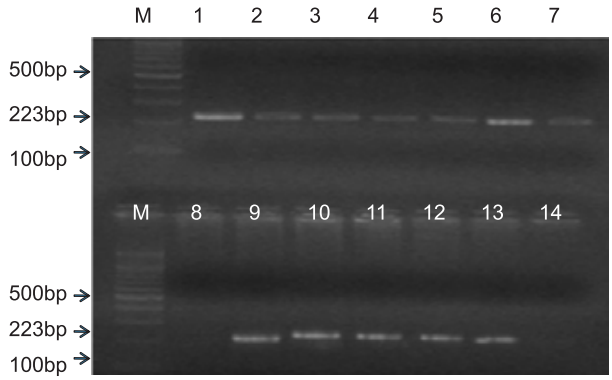


Lane M, Molecular size marker (100bp ladder); Lane 1, Positive control (*B. melitensis* 16 M); Lane 2 & 8, PCR amplified 731bp product from vaginal secretion; Lane 3 7 & 9, Vaginal secretion negative for *B. melitensis;* Lane 10, Negative control (NFW).

Fig. 1. Amplification of 731bp specific for *Brucella melitensis* in clinical samples of sheep

The 223bp genus specific amplicon was observed in 26 vaginal, two in blood and six in serum samples of 33 seropositive sheep blood, serum and vaginal samples (Table 3; Figs 1, 2). The *bcsp* 31 primer was found highly sensitive and suitable for detection of *Brucella* DNA and these observations are in accordance with those of Baily *et al.* (1992), Morata *et al.* (1998), Zerva *et al.* (2001) and Amit (2007). Further, amplicon product size being very short (223bp) and it can be easily amplified in the clinical materials (Baily *et al*. 1992). All the 6 vaginal samples which were positive by genus PCR yielded *Brucella* cultures confirming the active excretion of the organism in vaginal discharge.

There are reports of direct detection of *Brucella* DNA by PCR in blood, vaginal secretions and other clinical materials (Zerva *et al.* 2001). However, from the same animal, serum, blood and vaginal secretion have not been evaluated in the previous studies for the selection of suitable clinical material for PCR. Our results indicated that the best clinical sample where the highest positives were detected is of vaginal samples (78.78%), followed by serum (18.18%). Blood samples preferred least (6%) because of low PCR positivity which may be due to PCR inhibitors (Morata *et al.* 1998). It is also observed that some of the animals, which are PCR negative in vaginal samples, were positive in blood or serum. This would be acceptable fact because presence of high bacterial load in the blood and low in vaginal discharge, especially in the early stage of infection, prior to the onset of



Lane M, Molecular size marker (100bp ladder); Lane 1, Positive control (*B. melitensis* S99); Lane 2–7 & 9–13, PCR amplified 223bp product from vaginal secretion; Lane 8, Negative for *Brucella* DNA; Lane 10, Negative control (NFW).

Fig. 2. PCR amplification of 223bp *bcsp* 31 gene of *Brucella* species in vaginal secretions from sheep.

clinical manifestation. However, it is difficult to identify the stage of brucella infection at the time of sample collection.

In AMOS PCR, 731bp amplicon specific for *B. melitensis* was observed in 5 vaginal samples out of 34 genus positive clinical samples (3.2). AMOS PCR detected all the 3 biovars of *B. melitensis* and only strain of *B. ovis* and hence AMOS primers were preferred in the sheep which is usually infected by *B. ovis* and *B. melitensis* species. The less number of species specific PCR positives observed in clinical samples and these PCR assays were used only for *Brucella* pure cultures and reference strains (Bricker and Halling 1994). Absence of cross-amplification among different *Brucella* species by 3 primer pairs strongly indicated their specificity of primers for particular species of *Brucella.* Vaccination of sheep against brucellosis using Rev-1 vaccine strain is not practiced in India and hence differentiation of vaccinated and infected strains is not relevant. Similarly, Godfroid *et al.* (2010) have stated PCR specificity closer to 100% and recommended both culture and their detection by PCR on clinical samples.

In conclusion, brucellosis in sheep flocks was confirmed by serological tests and isolation of *B. melitensis* from vaginal samples of seropositive sheep. In PCR, *bcsp* 31 genus specific primer was found specific for *Brucella* DNA in clinical samples and *B. melitensis* species-specific primers were used for confirmation of isolates. Vaginal swab was found suitable clinical sample for isolation and PCR than the blood and serum samples.

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