

Original Research Article

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Isolation, Identification and Molecular Confirmation of *Brucella melitensis* from Ovine and Caprine Flocks in Karnataka, India

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ABSTRACT

Sheep and goat brucellosis caused by *Brucella melitensis*, one of the most virulent *Brucella* species accounting for economic losses through abortion, stillbirths, reduction of milk yield and infertility. Disease has wide socioeconomic impact, in countries where, livestock sector is the major source of rural income. Early diagnosis is essential to minimise the spread of the disease besides public health importance. The present study reports the isolation, identification, biotyping and molecular confirmation of *Brucella* spp. in 18 different sheep and goat farms in Karnataka suspected to have brucellosis. A total of 550 serum samples, 25 aborted fetuses, uterine discharges and placental tissues were collected. The serum samples were subjected to Rose Bengal Plate Test (RBPT) and Competitive ELISA (c-ELISA). The clinical samples were processed for cultural isolation on Brucella Agar Media with selective antibiotic supplements. A total of 200 (36.36%) and 260 (47.27 %) serum samples were positive by RBPT and c-ELISA, respectively, further 195 (35.45 %) of them being positive by both the tests. Five *Brucella* isolates were recovered from 100 clinical samples. The isolates were characterized to their species by growing them on *Brucella* specific medium, biochemical reactions, CO₂ requirement, H₂S production, agglutination with A and M mono-specific antiserum, dye sensitivity to basic fuchsin and thionin. Further, molecular confirmation of the isolates was done by amplification of *B. melitensis* 16S (rRNA) sequence analysis by genus specific PCR and species specific IS711 repetitive DNA fragment by *Brucella* AMOS PCR. The present study envisages seroprevalence of at least 35.45 per cent and isolation rate of 25 per cent for *B. melitensis* warranting the need for institution of strict control measures.

Keywords

Brucellosis,
Brucella melitensis,
RBPT and *Brucella*
AMOS PCR

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Introduction

Brucellosis still remains, a highly contagious, infectious, endemic worldwide zoonosis especially in the Mediterranean and Middle-East regions of the world involving many countries including India where it constitutes occupational and public health hazard (Shakerian *et al.*, 2005 and Khamesipour *et al.*, 2014).

Caprine and ovine brucellosis caused by *B. melitensis* is widespread in India due to frequent mixing of flocks while grazing and trading (Smits and Kadri, 2005). Transmission of *B. melitensis* among caprine and ovine herds is rapid *via* contact, by fomites, contaminated feed and water, introduction of adult males into the flock for breeding (Khamesipour *et al.*, 2013). The human brucellosis results mainly due to consumption of contaminated milk, meat, exposure to animal reservoirs. Due to high rates of brucellosis in sheep and goats, socioeconomic status of the risk group and higher pathogenicity of *B. melitensis* among all the *Brucella* species, makes this organism being most frequently isolated from humans (OIE, 2007).

Although currently employed serological and molecular methods are rapid, they are often non-specific leading to false positives and do not provide direct evidence for the presence of the pathogen. Hence isolation of *Brucella* spp. from the clinical samples is considered to be the gold standard for confirmatory diagnosis of infection and to institute eradication programs.

The present study was undertaken to diagnose brucellosis in sheep and goats having the history of infertility, abortion and repeat breeding; by isolation, identification, biochemical characterization and PCR confirmation.

Materials and Methods

The reference bacterial strains viz., *Brucella abortus* S99 and *Brucella melitensis* 16M, *B. suis* 1330, mono-specific A and M antiserum were procured from Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh.

History

The study was undertaken in 18 different brucellosis suspected sheep and goats farms located in four different districts of Karnataka, India, consisting of 400 sheep of Rambouillet and Bannur breeds and 150 goats of Beetal and Jamanapari breeds. These animals were reared in semi-intensive system with good managerial and feeding practices and vaccinated annually against sheep pox, enterotoxaemia (ET), peste-des-petis of ruminants (PPR) and hemorrhagic septicaemia (HS).

Samples

A total of 550 serum samples and 100 clinical samples from 25 aborted fetuses, including internal organs (lung, spleen, heart and liver), abomasal contents, uterine discharges and placental tissues were collected. Fetuses were necropsied with all biosecurity measures to obtain clinical sample.

Bacterial isolation, identification and biochemical characterization

A aborted animals included in the present study were screened for anti-brucella antibodies using RBPT (Alton *et al.*, 1988) and c-ELISA (Svanovir Brucella Ab c-ELISA kit) based on S- LPS antigen which detects *B. abortus*, *B. melitensis* and *B. suis*. PI values of >40 were considered as negative and that of <40 were declared as positives as per the manufacturers instructions.

For isolation, the aborted foetal internal organs, uterine discharges and placental tissues were inoculated on to *Brucella* selective broth (Himedia) containing antibiotic supplements (nystatin, bacitracin, polymyxin-B, cycloheximide, nalidixic acid) and incubated with and without 10 per cent CO₂ at 37 °C for 72 hours. The broth culture from both sets were plated onto Tryptic soya agar (TSA) (Becton Dickinson, Franklin Lakes, USA) with supplements and with and without 10 per cent CO₂ at 37 °C until the appearance of growth or up to one week. Colony morphology and opacity were recorded under stereomicroscope (Olympus SZ61®). To differentiate smooth and rough colonies, a solution of neutral acriflavine (Sigma® A 8126) was freshly prepared at 1:1000 dilution and mixed with a small loop of culture on a microscopic slide and examined under a low power stereomicroscope. The agglutination features were examined against anti-*Brucella* polyclonal serum.

Pure cultures of all the five isolates along with three reference strains (*B. abortus* S 99, *B. melitensis* 16M and *B. suis* 1330) were confirmed by their Grams reaction, requirement of added carbon dioxide for growth, H₂S production, catalase, urease, oxidase tests, inhibition of growth by basic fuchsin and thionin dyes and agglutination with monospecific anti-A, anti-M sera for biotyping (Alton *et al.*, 1988).

Molecular confirmation of isolates

Confirmation of isolates was done by PCR assays. The isolates were grown on TSA at 37 °C for 48 hours, inactivated for 2 hours at 80 °C, and DNA was extracted with the QIAamp DNA mini kit (Qiagen, Dusseldorf, Germany). The genus and species specific *Brucella* AMOS (*abortus-melitensis-ovis-suis*) PCR were carried out using published genus specific BCSP31 (Baily *et al.*, 1992) and

species specific IS711/AB and IS711/BM (Bricker and Halling, 1994) primers. The amplification products were examined by agarose gel electrophoresis in 1.5 per cent agarose gel stained with ethidium bromide.

Results and Discussion

Ovine and Caprine brucellosis caused by *B. melitensis* is widespread in India and its relative importance varies with the geographical region influenced by husbandry practices, availability of susceptible animal population, frequent mixing of flocks during grazing and inter-state trade of animals. Among all the *Brucella* species, *B. melitensis* is mostly responsible for human brucellosis and prevalent in Southeast Asian countries including India. The present study reveals the occurrence of *B. melitensis* infection in an organised farm.

Serodiagnosis

Serological tests are the most suitable methods employed for diagnosis of sheep and goat brucellosis while isolation remains gold standard to confirm the disease (Maged Ahmed *et al.*, 2011)

A total of 200 (36.36%) and 260 (47.27 %) of 550 serum samples were positive by RBPT and c-ELISA respectively whereas 195 (35.45 %) of them were positive by both the tests.

All the serum samples from aborted animals were positive by RBPT and c-ELISA (Table 1).

These two tests continue to be principal serosurveillance tools for bovine, ovine, caprine and human brucellosis across the India. (Isloor *et al.*, 1998; Mehra *et al.*, 2000; Singh *et al.*, 2000; Sen *et al.*, 2002; Henk *et al.*, 2005; Saleem *et al.*, 2004; Sharma *et al.*, 2007 and Vivekananda *et al.*, 2012).

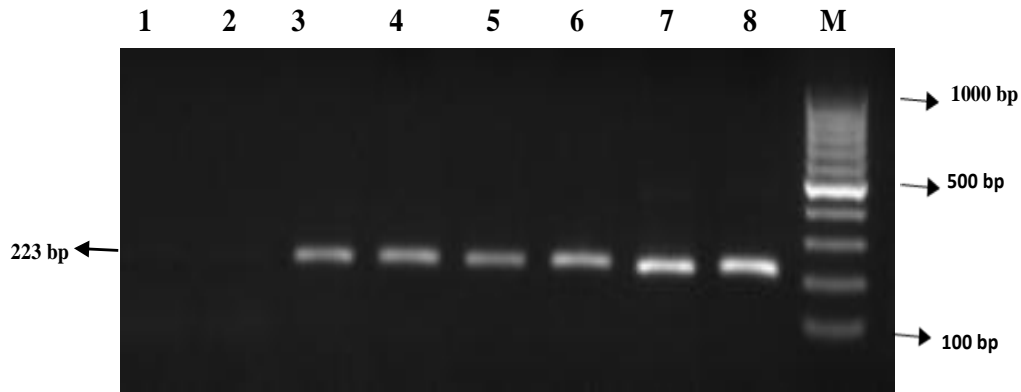


Fig 1: Genus specific PCR

Lane 1 - No template control; Lane 2 - non *Brucella* control; Lane 3 to 7 - *B. melitensis* field isolates; Lane 8 - *B. abortus* S99; Lane M - 100 bp ladder

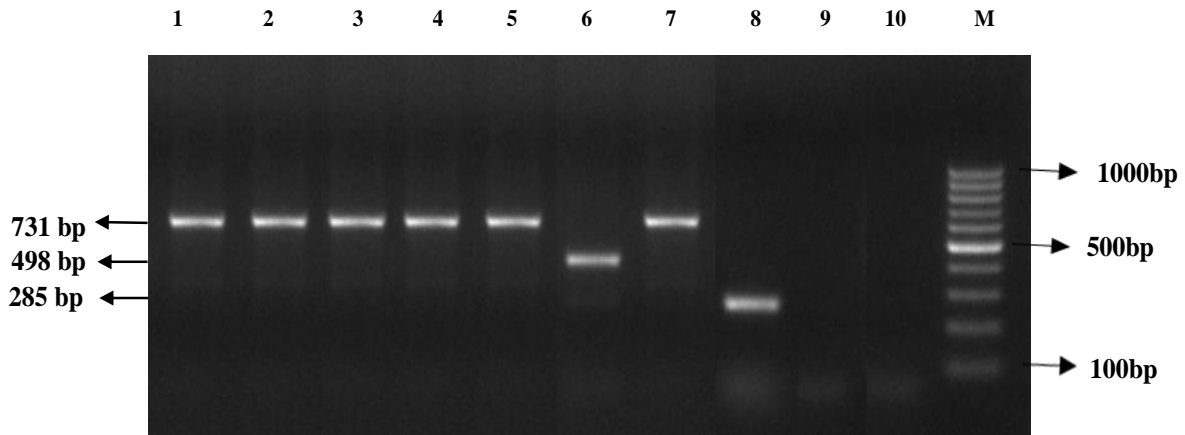


Fig. 2: Species specific AMOS PCR

Lane 1 to 5 - *B. melitensis* field isolates, Lane 6- *B. abortus* S99; Lane 7- *B. melitensis* 16M; Lane 8- *B. suis* 1330; Lane 9- non *Brucella* control; Lane 10- No template control; Lane M- 100bp ladder.

Table 1: Results of serological diagnosis of brucellosis by RBPT and c-ELISA and isolation of *Brucella* in aborted foetuses.

No. of flocks	No. of samples collected			No. of positives by RBPT			No. of positives by c-ELISA			No. of positives by RBPT and c-ELISA			No. of aborted foetuses examined	
	Total	M	F	Total	M	F	Total	M	F	Total	M	F	Total	No. of positives
12 sheep	400	40	360	165	10	155	205	20	185	155	10	145	20	4 25%
6 goats	150	20	130	35	3	17	55	8	47	40	3	37	5	1 20%
18 flocks	550	60	490	200	13	187	260	28	232	195	13	182	25	5 20%
		9.23%	69.34%	36.36%	21.66%	38.16%	47.27%	46.66%	47.34%	35.45%	21.66%	37.14%		

Table.2 Biochemical and antigenic profile of isolates and reference strains

Tests	Isolates					Brucella reference strains		
	1	2	3	4	5	<i>B. abortus</i> S99	<i>B. melitensis</i> 16M	<i>B. suis</i> 1330
Added CO ₂ requirement	Not required	Not required	Not required	Not required	Not required	Required	Not required	Not required
Oxidase test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Catalase test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
H ₂ S production	-ve	-ve	-ve	-ve	-ve	+ve	-ve	-ve
Urease test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Methyl red	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Indole	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Growth in presence of thionin 1:50000 (20µg/ml)	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve
Growth in presence of Basic fuchsin 1:50000 (20µg/ml)	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
Agglutination with <i>B. melitensis</i> mono-specific antiserum	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
Agglutination with <i>B. abortus</i> mono-specific antiserum	-ve	-ve	-ve	-ve	-ve	+ve	-ve	+ve
Motility	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Grams reaction	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Colony morphology	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Agglutination in presence of acriflavin	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Nucleotide sequences of *Brucella* genus specific primers

Name of the primer	Sequence 5'– 3'	Product length (bp)	Reference
<i>bbsp31</i> F	CGC GCT TGC CTT TCA GGT CTG	223	Baily <i>et al.</i> , (1992)
<i>bbsp31</i> R	TGG CTC GGT TGC CAA TAT CAA		

Nucleotide sequences of *Brucella* species specific primers

Name of the primer	Sequence 5'– 3'	Product length (bp)	Reference
<i>B. abortus</i> F	GAC GAA CGG AAT TTT TCC AAT CCC	498	Bricker and Halling (1994)
<i>B. melitensis</i> F	AAA TCG CGT CCT TGC TGG TCT GA	731	
<i>B. suis</i> F	GCG CGG TTT TCT GAA GGT TCA GG	285	
<i>IS 711</i> R (Common reverse primer)	TGC CGA TCA CTT AAG GGC CTT CAT		

Bacterial isolation, identification and characterization

Culturing of 100 samples collected from 25 aborted cases, yielded five isolates. These five isolates were initially identified as *Brucella* species based on colony morphology, Grams reaction, acriflavine test and agglutination tests. Later confirmed as *B. melitensis* by species specific PCR. The colonies grown on Brucella selective medium were round, convex, with smooth margin, translucent, honey-coloured, glistening and Gram-negative coccobacilli. All the isolates showed homogenous suspension in acriflavine test showing characteristic of smooth form as in smooth reference strains. Further biochemical and antigenic profile of all the five isolates along with *B. abortus* S99, *B. melitensis* 16M and *B. suis* 1330 were listed in Table 2. The biochemical test profile of all the isolates was identical and on par with that of standard reference strain *B. melitensis* 16M. Similar findings were reported by Erdenlig and Sen (2000) and Habtamu *et al.*, (2013) in their isolation studies.

The isolates were further differentiated phenotypically into species and partially to biovars employing CO₂ requirement, H₂S production, and growth on media plates containing thionin and basic fuchsin dyes at three different concentrations. Accordingly, *Brucella* species grown on TSA media containing both thionin and basic fuchsin dyes at a concentration of 20 µg/ml (1 in 50,000) were considered as *Brucella melitensis*.

The isolates agglutinated with *Brucella* anti-M monospecific serum confirming that, all five belonged to *B. melitensis* biotype 1 the most prevalent. There are three biotypes under *B. melitensis*, isolated at different frequencies by several workers. These findings suggest that *B. melitensis* plays an important role in sheep and goat Brucellosis

in Karnataka, India and present study confirms that *B. melitensis* biotype 1 is predominant biotype.

All the field isolates obtained from brucella affected sheep and goat were identified and confirmed to *B. melitensis* based on cultural, biochemical profiles, biotyping techniques and the species specific PCR assay. These results are in accordance with the reports of Paquet *et al.*, (2001), Khosravi *et al.*, (2006) and Vivekananda *et al.*, (2012) as they reported similar biochemical and antigenic properties in different *Brucella* isolates.

Molecular detection of *Brucella* spp. by genus and species specific PCR

Brucella genus specific PCR targeting bcs31 gene yielded 223bp amplicon from all the field isolates and reference strain (Fig. 1).

The targeted sequence codes for an immunogenic protein of 31 kDa outer-membrane immunogenic protein which is conserved across the *Brucella* species (Baily *et al.*, 1992).

Similarly, the *Brucella* species specific PCR target the insertion sequence (IS) element called IS711 with varied copy number yielded 731 bp (Fig. 2) indicating that all five isolates in the present study confirming to *Brucella melitensis*. The AMOS multiplex PCR using four sets of primer resulted in amplification of 498 bp fragment, specific to the *B. abortus* spp., 731 bp for *B. melitensis* and 285 bp for *B. suis* (Bricker and Halling, 1994). *B. abortus* carries six complete and one truncated copy of IS711 element whereas, *B. melitensis* and *B. suis* contain seven complete copies of this element (Ocampo-Sosa and Garcia-Lobo, 2008).

In the present study all the five isolates recovered from *Brucella* affected sheep and

goats were identified and confirmed as *B. melitensis* based on cultural characteristics, biochemical profiles and the species specific PCR. These results were in accordance with that of Paquet *et al.*, (2001), Khosravi *et al.*, (2006), Vivekananda *et al.*, (2012) and Habtamu *et al.*, (2013) as they reported similar amplified products in different *Brucella* isolates.

B. melitensis biotype 1 is the predominant and has a major role in sheep and goat abortions in Karnataka. Extensive studies are need to be carried out by including different geographical areas of the country with varied endemicity to establish the prevalence fo *B. melitensis* infections in sheep and goats. This forms the basis for instituting the effective control strategies to minimise the ovine and caprine brucellosis and intern human brucellosis too.

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