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Research Article
Epidemiological and Molecular Characterization of *Brucella* Species in Cattle

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Abstract

Background: Bovine brucellosis is a severe threat to livestock and mankind as it is a food-borne and occupational zoonosis. Rapid transmission, high morbidity and mortality are the main features of zoonotic diseases, leading to great personal and economic losses within a short period of time. Therefore, this study aimed for prompt identification and characterization of *Brucella* species in livestock to control the spread of infection and epidemiological data for the planning of disease control strategies is required. **Methodology:** Five hundred milk and blood samples were collected from cattle from different regions of Karnataka. All milk and blood samples were examined by Milk Ring Test (MRT) and Rose Bengal Test (RBT) to detect *Brucella* antibodies, polymerase chain reaction to detect *Brucella* specific DNA. Low-stringency Single Specific Primer Polymerase Chain Reaction (LSSP-PCR) gene signatures and Single-Strand Chain Polymorphism Polymerase Chain Reaction (SSCP-PCR) were used to study polymorphic variations of *Brucella* species. **Results:** Amongst a total of 500 blood and 500 milk samples, 4.6% prevalence of brucellosis was found in Karnataka. The PCR assay was affirmative with the Rose Bengal Test (RBT) (4.6%) and Milk Ring Test (MRT) which yielded (3.4%) lower prevalence. The positive samples were confirmed as *Brucella abortus* by Bruce-ladder multiplex PCR. The LSSP-PCR, SSCP-PCR gene signatures showed high genetic similarity and intraspecific similarity which are reproducible. **Conclusion:** Symptoms of brucellosis are not pathognomonic, diagnosis rely mostly on the laboratory tests. Hence, the SSCP-PCR, LSSP-PCR gene signatures can be used as an alternative for detection of brucellosis, screening a large number of clinical samples and identify epidemiological diversity. It also minimizes the drawback of cross-reactivity and only suspected mutants can be sequenced.

Key words: Brucellosis, infected cattle, PCR, PCR- SSCP, PCR-LSSP

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Brucellosis is a highly infectious zoonotic and re-emerging disease for animals and humans. It poses health risk and is a neglected pathogen in developing countries including India[1,2]. Infections cause significant economic losses by comparatively lowering milk production in livestock, abortion, public health and international trade implications and weak off-springs[3]. The real rate of brucellosis is estimated to be 10 to 25 times more than annual reports[4]. The human brucellosis incidence is directly correlated to the level of animal brucellosis in specific regions. The routes of infection are multiple: Food-borne, occupational or recreational, linked to travel and even bioterrorism[5]. *Brucella* is non-motile, small, gram negative, non-spore forming and strictly aerobic cocco-bacilli. It is positive for catalase and oxidase tests and shows variable results in urease tests[6]. *Brucella* genus shows little variation genetically, eleven *Brucella* species have been recognized, they are genetically similar although each one has different host preference[7]. *Brucella* easily transmitted to humans via aerosols and this makes the bacteria attractive for defence researchers[8,9]. The main source of naturally acquired brucellosis in humans is always found to be from animal and very few cases of human to human transmission have been reported by Godfroid *et al.*[5]. Unpasteurized dairy foods, raw meat and carcasses are sources of infection for workers in the meat-packing industry and general population. Veterinarians may acquire brucellosis from assisting births in infected livestock, as well as through close contact and accidental inoculation[10]. The *Brucella* spp. has ability to successfully survive and replicate within different host cells, which explains their pathogenicity. Extensive multiplication of *Brucella* species in trophoblasts of placenta is associated with abortion in their preferential hosts and its persistence in macrophages leads to chronic infections in both natural animal hosts and humans[11,12]. An efficient and proper understanding of epidemiology is required to make proper control strategies as there is a growing concern that the disease may further flare up due to the intensive dairy farming.

Brucellosis diagnosis is mainly based on detection of *Brucella* lipopolysaccharide (LPS) specific antibodies in serum and milk samples based on serological tests. Sero-diagnosis does not differentiate between an acute and chronic infection[11]. Gram negative bacteria that have antigenic similarities with *Brucella* LPS and that can lead to false results by cross reactivity with Yersinia enterocolitica O:9, Escherichia coli and some *Salmonella* spp[13]. Isolation of *Brucella* is required for confirmatory diagnosis of disease. Classically, detection and identification of *Brucella* spp. have been based on cultural and phenotypic characters. Although undoubtedly providing valuable information, bio-typing was and remains; a highly specialised and laborious approach and require expertise. Reagents were ideally used under secured biosafety containment. 16S rRNA based PCR method amplify DNA fragment common to all *Brucella* spp. but cross-reacts with members of genus *Ochrobactrum*[14]. The sensitive and specific PCR-based methods are not well established. Consequently, a great deal of work is required for verification, validation and establishment of standard positive controls before any of these methods may be used in routine laboratory testing for brucellosis[15]. Bruce-ladder has been recommended by the OIE (Office International des Epizooties) as a rapid and simple one-step molecular test for speciation of *Brucella* spp.[16].

The LSSP-PCR is extremely rapid and simple PCR-based technique that permits the detection of single or multiple mutations in gene sized DNA fragments. Purified DNA fragments are subjected to PCR using single specific oligo-nucleotide primer with large amounts of *Taq* DNA polymerase at very low annealing temperature. Under these conditions, the primer binds specifically to its complementary region and non-specifically to multiple sites within the DNA fragments in a sequence-dependent manner, producing a heterogeneous set of reaction products that produce a unique gene signature profile. In this method even single base pair mutation can drastically vary the banding pattern, producing different signatures that are diagnostic of the specific alterations[17].

The SSCP technique was initially developed for detection of point mutations in human DNA[18,19]. It has been also used as a complementary technique with species-specific PCR in the development of genetic markers for rapid identification of three *Phytophthora* species pat hogenic on potato[20,21]. Using species specific primers to carry out SSCP-PCR and its genetic signatures shows the validation of the *Brucella* spp. in the clinical samples, where we can rule out the cross contamination problem additionally it is a robust, rapid and inexpensive detection method.

The aim of this study was to develop a new diagnostic technique for rapid, sensitive, accurate and efficient diagnosis of brucellosis. In this study the epidemiology of brucellosis, identification through specific gene, Bruce ladder multiplex PCR and genetic signatures of SSCP-PCR and LSSP-PCR technique for the rapid identification of the *Brucella* spp. and for the detection of *B. abortus* in naturally infected bovine blood and milk samples and take preventive measures.

MATERIALS AND METHODS

***Brucella* reference strains:** *Brucella* strains -*Brucella* S19, *Brucella melitensis*, *Brucella* suis were procured from Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, Uttar Pradesh, India. *Brucella* S99 was obtained from Indian Council of Agricultural Research-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Bangalore, India. They were tested for the purity, biochemical and molecular characteristics before use. Type III Biosafety containment was used to culture the bacteria.

Study population: Five hundred milk and blood samples from the animals with history of abortion were collected from different regions of Karnataka, India. Blood was aspirated from jugular veins in plain vacutainer tubes without anticoagulant using aseptic techniques. The samples were kept on ice at about 4°C and transported immediately to the laboratory. Tubes were centrifuged at 3000 rpm for 3 min to separate the serum and stored at -20°C till further use. Milk samples were aseptically obtained from all 4 quarters of the animal's mammary gland during their routine milking time and stored at -20°C till further use. The blood and milk samples collected from different regions of Karnataka are shown Table 1.

Milk ring test and rose bengal test: The serum samples collected were tested with Rose Bengal Test (RBT) as described by Alton *et al.*[22]. The appearance of agglutination was recorded. Milk Ring Test (MRT) was performed according to Blythman and Forman[23], positive samples were differentiated. Experiments were conducted in triplicates and repeated three times.

DNA extraction and determination of purity and yield of DNA samples: The DNA from all blood samples and bacterial strains was extracted using a commercial purification system with columns (QIAamp Blood Midi; QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. *Brucella* cultures were grown overnight in *Brucella* selective broth at 37°C and DNA was extracted with the QIAamp DNA mini Kit (Qiagen, Germany) after inactivation for 2 h at 80°C. The purity and concentration of the genomic DNA extracted from samples was estimated by Nanodrop spectrophotometer (Thermoscientific, USA). The concentration of DNA was noted at the absorbance ratio of 260/280 OD. The DNA extracted from these samples was used as the template for PCR, SSCP-PCR and LSSP-PCR.

Species specific PCR assay: The PCR assay was carried out for gene *bcsp31* encoding an immunogenic outer membrane protein of 31 kDa of *B. abortus*, which is conserved in all *Brucella* spp. using the specific primers *BSCP31* F and *BSCP31* R according to the protocol of Baily *et al.*[24]. Amplified PCR products corroborated through 1% agarose gel and the gel profile documented in Geldoc 1000 System-PC (Biorad, USA) (Fig. S1).

Bruce ladder multiplex PCR: The PCR was performed in 25 µL reaction volume containing 1 µL 0.4 µM of each primer (eight primer sets cocktail represented in Supplementary Table S1)[25]. The products were analyzed by electrophoresis through ethidium bromide stained 1.5% agarose gel and image was documented in Geldoc 1000 System-PC (Biorad, USA).

SSCP-PCR analysis: Ten microliter of individual eluted (50 ng μL^{-1}) PCR products were mixed with 25 µL of denaturing buffer (95% formamide, 20 mM EDTA and 0.05% bromophenol blue). The mixtures were heated at 96°C for 10 min and immediately chilled with ice[21]. Thirty five microliter

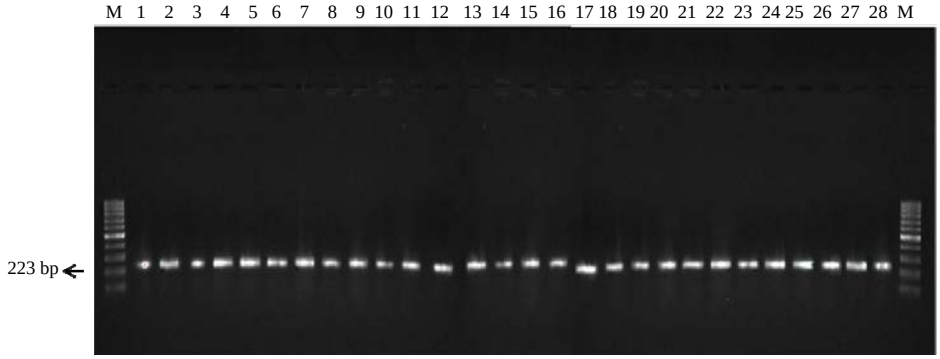

Fig. S1: Amplified PCR products of different *Brucella* spp.
Agarose gel electrophoresis of PCR products. Lane M: Marker, Lane 1: *Brucella abortus,* Lane 2: *Brucella melitensis* , Lane 3: *Brucella suis,* Lane 4-28: Isolated *Brucella* spp.

Table 1: Screening of anti-*Brucella* antibodies and *Brucella* specific DNA in milk and blood samples from different regions of Karnataka

	Total no. of samples				PCR assay
Regions	Milk	Blood	Milk ring test positive	Rose bengal test positive	Blood
Mysore	170	170	6	9	9
Chamarjanagara	60	60	3	4	4
Mandya	90	90	3	3	3
Maddur	90	90	3	3	3
Hassan	90	90	2	4	4

Table S1: List of primers used in the present study

Primer designation	Sequences (5′→3′)	Amplicon size (bp)	References
BSCP31 F	TGGCTCGGTTGCCAATATCAA	223	
BSCP31 R	CGCGCTTGCCTTTCAGGTCTG		Baily *et al.*[24]
BMEI0998 F	ATC CTA TTG CCC CGA TAA GG	1, 682	Garcia-Yoldi *et al.*[25]
BMEI0997 R	GCT TCG CAT TTT CAC TGT AGC		
BMEI0535 F	GCG CAT TCT TCG GTT ATG AA	450 (1,320)	
BMEI0536 R	CGC AGG CGA AAA CAG CTA TAA		
BMEII0843 F	TTT ACA CAG GCA ATC CAG CA	1,071	
BMEII0844 R	GCG TCC AGT TGT TGT TGA TG		
BMEI1436 F	ACG CAG ACG ACC TTC GGT AT	794	
BMEI1435 R	TTT ATC CAT CGC CCT GTC AC		
BMEII0428 F	GCC GCT ATT ATG TGG ACT GG	587	
BMEII0428 R	AAT GAC TTC ACG GTC GTT CG		
BR0953 F	GGA ACA CTA CGC CAC CTT GT	272	
BR0953 R	GAT GGA GCA AAC GCT GAA G		
BMEI0752 F	CAG GCA AAC CCT CAG AAG C	218	
BMEI0752 R	GAT GTG GTA ACG CAC ACC AA		
BMEII0987 F	CGC AGA CAG TGA CCA TCA AA	152	
BMEII0987 R	GTA TTC AGC CCC CGT TAC CT		

of reaction mixture was loaded and electrophoresed in pre-chilled 1X TBE buffer at 200 V for 2 h at room temperature. Gels were silver stained and documented by Geldoc 1000 System-PC (Biorad, USA).

LSSP-PCR analysis: The LSSP-PCR was carried out in 20 µL reaction mixture containing 3 µL of purified amplified DNA template (50 ng) DreamTaq green master mix (contain 0.25 mM each dNTP, 2 mM $MgCl_2$ and Taq DNA polymerase) and 50 pmol of primer *BSCP31* F (5′-TGGCTCGGTTGCCAA TATCAA-3′) and *BSCP31* R (5′-CGCGCTTGCCTTTCAGGTCTG-3′) primers in 0.2 mL reaction tubes after denaturation step at 95°C for 10 min, program consisted of 35 cycles of 94°C for 1 min and 30°C for 1 min[17]. The same experimental condition were repeated with respect to MultiGene thermocycler (Labnet International, Inc. USA). Five microliter of LSSP-PCR products were analysed by electrophoresis on 1.5% agarose gel and results were documented by Geldoc (Biorad, USA).

Statistical analysis: Sample size estimates were based on power analyses conducted using Win Episcope 2.0. Overall prevalence estimates were generated as an average of the individual cohort prevalence estimates and as an overall individual animal prevalence.

RESULTS AND DISCUSSION

Collection and screening for brucellosis: Bovine brucellosis is one of the most re-emerging infectious diseases causing difficulties for veterinary authorities that are confronted, not only with animal but also with major public health implications. Here in, for the first time, a large population based brucellosis survey covering diverse cattle populations in different regions of Karnataka, India is reported. Sampling distribution and results of brucellosis assay are presented in Table 1. Using Rose Bengal Test (RBT); 4.6% and Milk Ring Test (MRT); 3.4% prevalence was observed. According to Corbel[26] globally brucellosis is under-reported because of its unclear clinical flu like symptoms, difficult in laboratory diagnosis and lack of understanding by medical professionals. In the present study 3.4% of brucellosis positive for MRT test showed concordant matches to study of Islam *et al.*[27] in Bangladesh. Since MRT is a presumptive test, negative results did not mean animals are not infected with *Brucella* spp. Several possible reasons have been taken into account to explain the relatively inconsistent performance of *Brucella* detection. The *Brucella* could be located only in the lymph nodes and not yet reached the milk at the sampling time. The infection stage also influences the antibody level and the number of the

bacteria[22]. Some serological tests lack sensitivity and it is impossible to differentiate antibodies produced after vaccination from those produced after infection[28]. Gram negative bacteria that have antigenic similarities with *Brucella* LPS can lead to false results by cross reactivity with *Yersinia enterocolitica* O:9, *Escherichia* coli and some *Salmonella* spp.[13].

PCR analysis: *Brucella* genus specific primers (synthesized by the Sigma Aldrich, India) targeting *bscp31* gene showed the apparent prevalence of 4.6%. The homology of the sequence were analyzed using BLAST analysis considering the query coverage and percentage identity of *Brucella abortus*, in which primers *BSCP31* F and *BSCP31* R showed 100% homology to *B. abortus*. The gene sequenced were deposited to GenBank database with the accession number KX389302,KX389303, KX389304, KX389305, KX424943, KX424944, KX424945, KX424946 and KX424947. The PCR assays tend to be simple and robust and can be utilized when differentiation of the species is not relevant, such as diagnosis of human brucellosis or contamination of food products[29]. Though several targets are used for the PCR assay, in this study 31 kDa *Brucella* cell surface protein was employed. Even, though this assay may be considered as specific, in some protocols cross reactivity has been observed with closely related genus *Ochrabactrum*[30,31]. The gene sequences obtained from the study were compared with the sequences from the NCBI containing *B. abortus*, *B. melitensis*, *B. pinnipedalis* and *B. ceti* formed a clade and *Brucella* species, *B. canis, B. microti* and *B. ovis*. In the maximum likelihood analysis *Orchobactrum* species was taken as outgroups in the analysis and observed two clades with the strong bootstrap supports >80%. The variation within our isolates and NCBI sequences were very low and are similar. Between the clades, variation can be observed with our isolate clade as longer branch length (Fig. 1). Since there was high genetic similarity and no variation, we sequenced only few isolates and deposited them in the GenBank.

Bruce ladder multiplex PCR: All the positive isolates subjected for Bruce ladder speciation PCR confirmed that, all isolated cultures are *B. abortus* when compared with reference strains; *Brucella* S19, *Brucella melitensis*, *Brucella suis* (Fig. 2). Bruce ladder multiplex PCR was applied for all positive isolates and obtained data highlighted the application and novelty of PCR in discrimination of *Brucella* spp. Lopez-Goni *et al.*[32] successfully demonstrated the application of Bruce-ladder PCR in speciation of *Brucella* species. Bruce-ladder multiplex PCR results showed 100% concordant match with the previous reports of Lopez-Goni *et al.*[32] and Nagalingam *et al.*[33].

SSCP PCR and LSSP PCR assay: In the SSCP-PCR species specific *bscp31* gene was used to identify and discriminate the *B. abortus* at species level for the first time. The SSCP-PCR finger print exhibited similar banding patterns and there was no variation in the banding patterns with that of reference strains; *B. abortus*, *B. melitensis* and *B. suis* (Fig. 3). Also SSCP-PCR did not show any genetic profile variations at intraspecies level. The SSCP analysis may eliminate false diagnoses due to cross contamination[6], which is a serious problem commonly associated with enzyme-linked immunosorbent assay (ELISA) and species-specific detection[34]. LSSP-PCR assay using 5′-TGGCTCGGTTGCCAATATCAA-3′ F primer and 5′-CGCGCTTGCCTTTCAGGTCTG-3′ R primers *Brucella abortus* showed there was no interspecific variability among the positive isolates. *Brucella abortus* showed variable number of banding pattern but it showed similar banding patterns in all the isolated PCR positive samples (Fig. 4, 5). The phylogenetic dendrogram obtained from the LSSP-PCR also exhibited that there was no inter-specific variability among the PCR positive samples.

The SSCP-PCR and LSSP-PCR analysis were carried out for all the samples to find out if there is any genetic diversity. So only the mutated sequences or polymorphic variation samples can be further sequenced, which will be significant in large scale screening of the infected animals. The LSSP-PCR technique for the characterization of *Brucella*, specially designed primers from the sequence of the *BSCP31* protein was used for the first time. Segatto *et al.*[35] showed similar banding pattern obtained for primers C in their study, because of sequence similarity. Significantly, results also showed sequence similarity while using specific primers in LSSP-PCR. Although the LSSP-PCR is used for strain typing techniques, LSSP PCR has also been used for the identification purposes such as detection of *Leptospira* directly from the clinical specimen and for discrimination of the serogroups from different animal reservoir[36]. One of the main advantages of using LSSP-PCR for genetically typing brucellosis from biological samples is the possibility of detecting *Brucella* first by means of a PCR reaction and subsequently identifies them through the comparison of their genetic signatures with known LSSP-PCR profiles. This is clearly exemplified by our results, which showed specific signatures with no variation at intra species level. Thus, LSSP-PCR may be very useful in screening test for genetic identity and establishment of matrilineal relationships, prior to the more definitive study of this region by DNA sequencing. The major limitation of SSCP technology for the analysis of community DNA is the high rate of re-annealing of DNA strands after an initial denaturation during electrophoresis. The SSCP analysis would be ideal tool

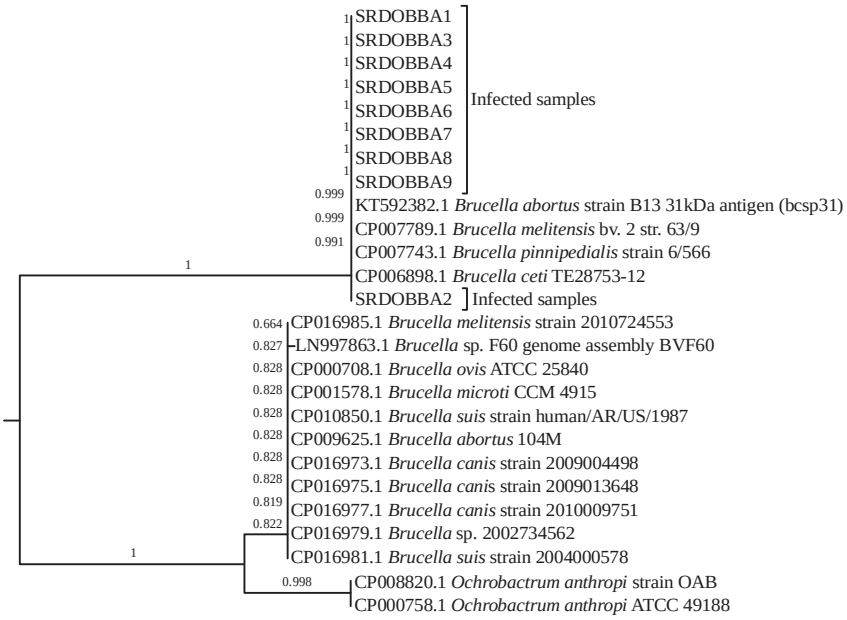

Fig. 1: Phylogenetic tree constructed by maximum likelihood analysis for *Brucella* isolates compared with NCBI database
Between the clades, variation can be observed with our isolate clade as longer branch length

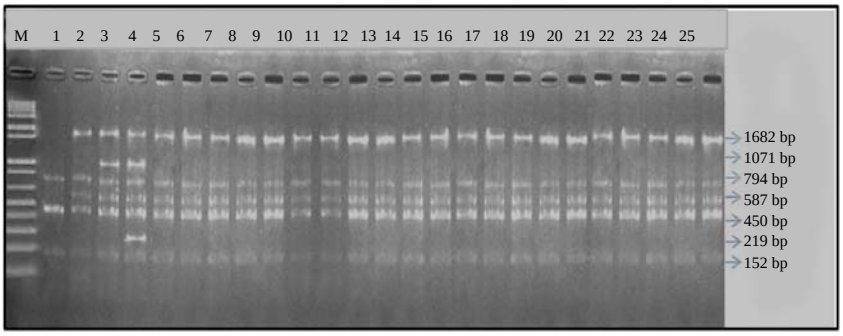

Fig. 2: Differentiation of all *Brucella* species
Lane M: Marker, Lane 1-4: Rreference strains, Lane 1: *Brucella* S19, Lane 2: *Brucella abortus*, Lane 3: *B. melitensis*, Lane 4: *B. suis*, Lane 5-25: *Brucella* spp. isolated from infected animals. The infected isolates were identified as *Brucella abortus*

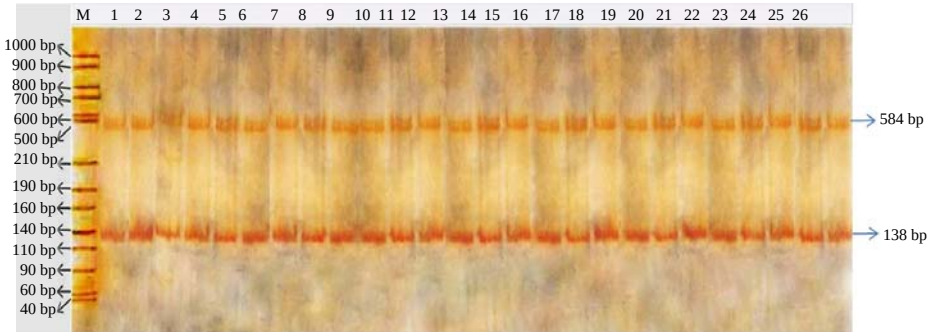

Fig. 3: SSCP-PCR profile of *BSCP31* region in *Brucella* spp.
Lane M: Marker, Lane 1-4: Reference strains, Lane 1: *Brucella* S19, Lane 2: *B. abortus*, Lane 3: *B. melitensis*, Lane 4: *B. suis* Lane 5-26: *B. abortus* isolated from infected animals. Similar banding patterns compared to reference strains

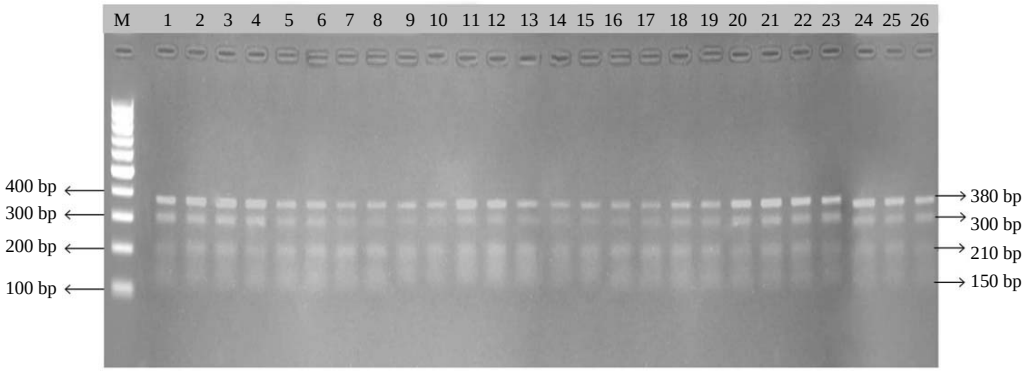

Fig. 4: LSSP signature of *BSCP31* region forward primer of *Brucella* spp. isolated from different regions
Lane M: Marker, Lane 1-4: Reference strains, Lane 1: *Brucella* S19, Lane 2: *B. abortus*, Lane 3: *B. melitensis* Lane 4: *B. suis*, Lane 5-26: Isolates of *B. abortus* for forward primer. Similar banding patterns were observed compared to reference strains

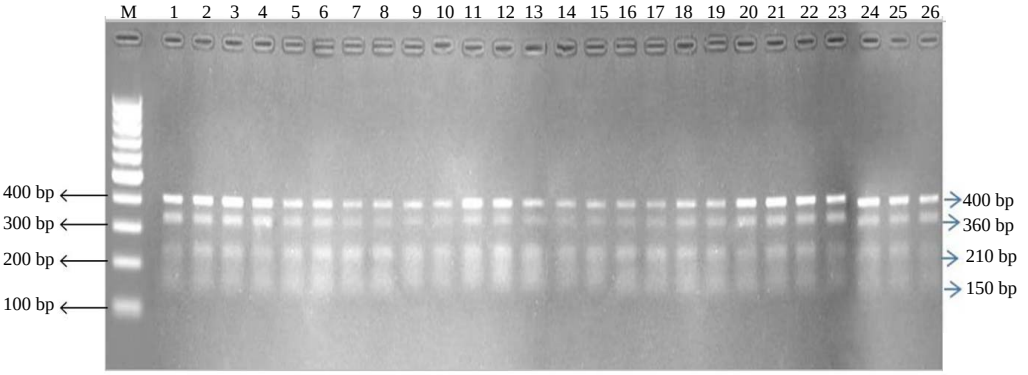

Fig. 5: LSSP signature of *BSCP31* region reverse primer of *Brucella* spp. isolated from different region
Lane M: Marker, Lane 1-4: Reference strains, Lane 1: *Brucella* S19, Lane 2: *B. abortus*, Lane 3: *B. melitensis*, Lane 4: *B. suis*, Lane 5-26: Indicates isolates of *B. abortus* for reverse primer. Similar banding patterns were observed compared to reference strains

to identify clones with DNA products identical to those originally extracted from the amplified community DNA. The LSSP PCR presents almost unlimited molecular application where rapid and sensitive detection of mutation and sequence variation is important. Due to low cost, sensitivity, specificity, simplicity of execution and high reproducible genetic profile, the use of SSCP-PCR, LSSP-PCR technique could be extended to other pathogenic bacteria identification and can be considered as a valuable microbiological tool to study genetic diversity of quarantine pathogens along with their epidemiology in developing countries.

CONCLUSION

Bovine brucellosis prevalence in Karnataka was screened and 4.6% prevalence of bovine brucellosis was identified. The SSCP-PCR and LSSP-PCR gene signatures were developed for rapid detection of bovine brucellosis which helps in the rapid detection even when the sample number is larger and genetic diversity of *Brucella* species was found to be highly similar. In conclusion these findings also portend significant public health implications following great economic losses to poor people particularly in the rural areas. This study also connected with the background of nationwide epidemiological surveillance, required to quantify extent of economic loss on farm animal and there control strategies.

SIGNIFICANCE STATEMENT

This study discovered 4.6% prevalence of bovine brucellosis in Karnataka, India. The SSCP-PCR analysis would be ideal tool to identify clones with DNA products identical to those originally extracted from the amplified community DNA and LSSP-PCR in this study presents rapid and sensitive detection of mutation and sequence variation. From the present study SSCP-PCR and LSSP-PCR gene signatures developed can be used for rapid detection of pathogens and quickly quarantine before the spread of infection.

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