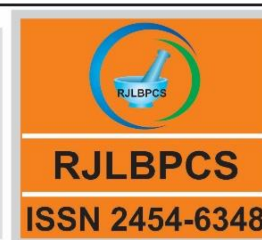

Original Research Article **DOI: 10.26479/2019.0504.14**

EPIDEMIOLOGICAL STUDIES OF BOVINE BRUCELLOSIS IN RURAL VILLAGES OF MANDYA DISTRICT KARNATAKA, INDIA

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ABSTRACT: Bovine Brucellosis is one of the devastating zoonotic diseases that affects the multiple livestock species and causes great economic losses. Therefore, appropriate detection and characterization of brucella species in farm animals to control the spread of infection, and obtain epidemiological data for planning disease control strategies are required. The present study was conducted to estimate the bovine brucellosis prevalence and possible risk factors associated with it in different villages of Mandya District, Karnataka. Blood and Milk samples collected from 210 animals of different villages of Mandya District Karnataka, were examined by the Rose bengal test (RBT) and Milk ring test(MRT). The overall seropositivity was 4.3 % for RBT and 3.3 % for MRT. The seropositive samples were further validated by the polymerase chain reaction (PCR) and differentiation by Bruce ladder PCR. The prevalence of bovine brucellosis by Polymerase chain reaction was found to be 4.3 %. Bruce ladder polymerase chain reaction showed that the isolated samples are Brucella abortus. This study indicated an urgent need of policy for prevention and control of brucellosis in dairy animals.

KEYWORDS: Bovine; Brucellosis; Seroprevalence; polymerase chain reaction.

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1. INTRODUCTION

Brucellosis is an infectious bacterial zoonosis that is caused by different species of brucella. Brucella species has their preferred natural host that serve has a reservoir and it can be transmitted

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by direct contact or indirect contact with the infected animals with brucella most often the it get transmitted via ingestion and also via venereal routes [1]. Brucellae belong to subdivision α–2 of proteobacteria. They are Gram-negative, coccobacilli, aerobic, facultative intracellular and partially acid fast. They are positive to oxidase, catalase, nitrate reductase and urease test [2]. The brucellosis infected cow usually aborts only once after which the cow develops immunity and animal remains infected. At succeeding calving the infected cow excrete huge amount of brucella bacterium in the fetal fluids [3]. Surveillance and vaccination are the two effective approaches to control the disease [4]. Brucellosis inspection may be adopted in many ways like slaughter surveillance, on farm surveillance, livestock market surveillance, enhanced passive surveillance etc [5]. Dairy products prepared from unpasteurized milk such as soft cheeses, yoghurts, and ice creams may contain high concentration of the bacteria and consumption of these is an important cause of brucellosis. It is the commonest mode of transmission in case of B. melitensis and B. abortus infections in general population, raw meat and carcasses are the also sources of infection for workers in meat industry and other population. From assisting the birth of infected cows the veterinarians may acquire brucellosis also by direct contact and accidental inoculation [6]. Brucellosis in livestock seems to be connected primarily with poor farm hygiene, movement of animals, use of local cattle yards and fairs for trading, the practice of returning non-lactating animals to villages for seasonal maintenance, and the use of semen from infected bulls of unknown health status for artificial insemination. As a result, there is a severe human suffering from the disease [7]. Still, epidemiological data on brucellosis prevalence is frequently incomplete. This is partially explained by the lack of proper laboratory diagnostic techniques, under-reporting, poor co-operation and exchange of information between veterinary and health care. The milk ring test and rose Bengal test are widely used for the Seroprevalence screening of bovine brucellosis in developing countries, where other tests are laborious and time consuming to perform large scale screening and it requires special equipment and expertise [8]. Brucellosis in humans is rarely moral, but can lead to severe debilitation and disability however it has been reported that 2% of untreated patients die by brucellosis infection [9]. The disease has the tendency towards the chronocity and persistence by becoming a granulomatus disease capable of affecting any organ system [10]. The appropriate time and accurate diagnosis of human brucellosis continues to challenge clinicians as the symptoms of brucellosis is pathognomonic The presence of brucellosis in wild animals, with great potential to transfer to domestic animals and followed by to humans is another epidemiological issue [11]. The aim of the present study was to identify the epidemiology of brucellosis in rural villages of Mandya district, Karnataka, India, through specific gene and differentiation of brucella species by Bruce ladder speciation PCR, For rapid detection of *B. abortus* in naturally infected Blood and Milk samples collected from the suspected animals; to quickly quarantine the infected animals and to take up the preventive measures.

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2. MATERIALS AND METHODS

2.1 Collection of *Brucella* reference strains

Bacterial reference strains such as *Brucella abortus, Brucella melintensis, Brucella suis* were procured from Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly, Uttar Pradesh, India. They were tested for the purity, biochemical and molecular characteristics before use. Type III Biosafety containment was used to culture the bacteria.

2.2 Study Population

Two hundred ten milk and blood samples from the animals with history of abortion were collected from villages of Mandya district, Karnataka, India. Blood and Milk samples collected using aseptic techniques and were transported to the laboratory at 4 ºC. Tubes were centrifuged at 3000 rpm for 3 min to separate the serum and stored at -20 ºC till further use. Milk samples were aseptically obtained from all four quarters of the animal's mammary gland during their milking time and stored at -20 ºC till further use. Samples are tabulated in table (Table 2).

2.3 Rose Bengal test and Milk ring test

The RBT antigen consisted of standardised *B. abortus* antigen sourced from the IVRI, Izatnagar, India. According to Alton *et al.* [8], Equal volume of test serum and antigen (30 µl) were agitated thoroughly using the stick applicator and plate was rocked for 4min and appearance of the agglutination was observed and recorded. According to the Blythman and Forman [12], Milk ring test was performed by adding 30 µl of *B. abortus* bang ring antigen (IVRI, Izatnagar, India). The milk and antigen mixtures were incubated at 37 °C for 1 hour, together with positive and negative control samples. Experiments were conducted in triplicates and repeated three times.

2.4 Extraction of the DNA and determination of Purity by Nanodrop UV-spectrophotometer

Reference bacterial strains DNA and DNA from infected blood samples were extracted using a commercial purification system with columns (QIAamp Blood Midi; QIAGEN GmbH, Hilden, Germany) following the instruction of the manufacturers. *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 down at the absorbance ratio of 260/280 OD.

2.5 Species specific Polymerase chain reaction

PCR assay was carried out for gene *bscp31,* which is conserved in all *Brucella* species, using the specific primers *BSCP31* F and *BSCP31* R according to the protocol of Baily *et al.*[13] Briefly, PCR was performed in a 0.2 ml reaction tube; 50 µl reaction mixture containing 5 µl template DNA, 3 µl forward primer- (5-TGGCTCGGTTGCCAATATCAA-3) and 3 µl reverse primer-(5-CGCGCTTGCCTTTCAGGTCTG-3), 25 µl of Dream Taq green master mix (Thermo Fischer Scientific, India). The PCR was performed in a master gradient thermal cycler (LABNET, NJ,

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USA). The PCR amplification conditions: initial denaturation at 95 °C for 5 min; 30 cycles of denaturation for 30 sec at 94 °C, annealing for 1 min at 54 °C, extension for 45 sec at 72 °C and final extension at 3 min at 72 °C, Following DNA amplification, the products were run on 1.5% agarose gels in the presence of ethidium bromide. Amplified products were visualized under a UV transilluminator. The gel profile was documented in Geldoc 1000 System-PC (Biorad, USA) (Fig 1).

2.6 Bruce ladder multiplex PCR

PCR was performed in 25µl reaction volume with slight modification containing 1µl 0.4 µM of each primer (eight primer sets cocktail represented in Table 1) [23] 10 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.4 mM dNTPs, 60–100 ng of template DNA, and Dream TaqGreen master mix(Thermo Fischer Scientific, India). The samples were subjected to amplification (35 s at 95 °C, 45 s at 63 °C, 3 min at 72 °C) 35 cycles with initial denaturation for 7 min at 95 °C and final extension for 6 min at 72 °C in a MultiGene thermocycler (Labnet International, Inc. USA). The products were corroborated by 1.5 % agarose gel electrophoresis and image was documented using Geldoc 1000 System-PC (Biorad, USA) (FIG 2).

Table 1: Oligonucleotides used for the Bruce ladder speciation PCR

Primer designation	Sequences 5'→3'	Amplicon size (bp)	References
BMEI0998 F BMEI0997 R	ATC CTA TTG CCC CGA TAA GG GCT TCG CAT TTT CAC TGT AGC	1, 682	Garcia *et al.*[14]
BMEI0535 F BMEI0536 R	GCG CAT TCT TCG GTT ATG AA CGC AGG CGA AAA CAG CTA TAA	450 (1,320)	
BMEII0843 F BMEII0844 R	TTT ACA CAG GCA ATC CAG CA GCG TCC AGT TGT TGT TGA TG	1,071	
BMEI1436 F BMEI1435 R	ACG CAG ACG ACC TTC GGT AT TTT ATC CAT CGC CCT GTC AC	794	
BMEII0428 F BMEII0428 R	GCC GCT ATT ATG TGG ACT GG AAT GAC TTC ACG GTC GTT CG	587	
BR0953 F BR0953 R	GGA ACA CTA CGC CAC CTT GT GAT GGA GCA AAC GCT GAA G	272	
BMEI0752 F BMEI0752 R	CAG GCA AAC CCT CAG AAG C GAT GTG GTA ACG CAC ACC AA	218	
BMEII0987 F BMEII0987 R	CGC AGA CAG TGA CCA TCA AA GTA TTC AGC CCC CGT TAC CT	152	

2.7 Statistical analysis

Sample size estimates were based on data pertaining to prevalence based on milk testing and risk factors were analyzed on IBM SPSS statistical software version 20.0 using chi square test.

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3. RESULTS AND DISCUSSION

Bovine brucellosis is widespread zoonotic disease in India and appears to be increasing in recent times due to increased farming and trade of animals causing complications for the veterinarians that are confronted, not only with the cattle and the risk is associated with public health implication in developing countries like India is the major concern. In our study, for the first time, a large population based brucellosis survey covering diverse cattle populations in villages of Mandya district Karnataka, India is reported. Though several brucellosis eradication programs have been launched in many countries, but the success of these programs still has not reached the desired level. Samples collected from different places and results of bovine brucellosis infection are tabulated in table 2.

Table 2: Screening of milk and blood samples by Rose Bengal Test and Milk Ring Test collected from villages of Mandya District, Karnataka

Sl. No.	Total no. of samples			MRT positive	RBT positive
	Regions	Milk	Blood		
1	Kennalu	57	57	2	3
2	Alpahalli	33	33	1	1
3	Chikkade	23	23	2	2
4	Chinakuralli	19	19	0	0
5	Hiremarali	09	09	0	0
6	Banangadi	27	27	1	2
7	Manchanahalli	24	24	1	1
8	Basthihalli	18	18	0	0

The true presence of bovine brucellosis infection is unknown in most of the developing countries which includes India. Nine out of 210 serum samples were positive by Rose Bengal test (RBT), 7 out of 210 milk samples were positive by Milk ring test (MRT). Resulting in an apparent prevalence of 4.3 % Rose Bengal test (RBT) and 3.3 % Milk ring test (MRT) similar to the studies of Islam *et al*. [15] in Bangladesh. Animals that show negative milk ring test result do not mean that they are not infected with *Brucella* species. Several possible reasons have been taken into account to explain inconsistency of *Brucella* detection. The *Brucella* could be located only in the lymph nodes and did not yet reach the milk at the sampling time. The stage of the infection may influence the antibody level and the number of the bacteria [8]. Some serological diagnostic techniques lack sensitivity to differentiate antibodies produced after the vaccination of the animal and from those produced after the infection of brucella [16]. Corbel, [4], reported that Brucellosis infection is underreported globally because of its unclear clinical symptoms and it is difficult for diagnosis in laboratory due to lack of understanding by the medical professionals. The implementation of “test-and-slaughter” policy for infected animals is hindered by lack of wealth to

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compensate affected owners. Some agrarians agree to slaughter their infected animals, but, due to the high level of errors in serology methods, the culling of all sero-positive animals in the group leads to high economic losses. Some errors may occur due to lack of reliability on serology hence molecular detection methods are in need for the confirmation of the brucellosis infection. [17]. *Brucella* genus specific primers targeting *bscp31* gene synthesized primers showed the apparent prevalence of 4.3 % of brucella infection (Fig 1).

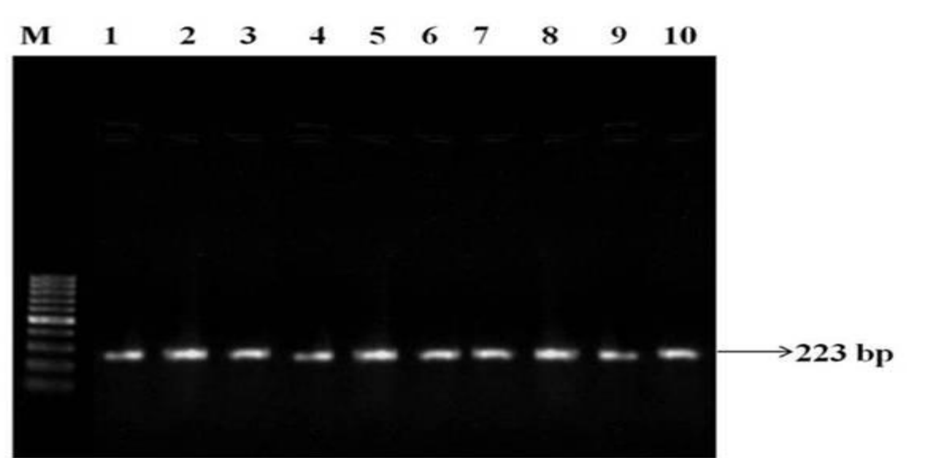

Fig 1. Amplified PCR products of different *Brucella* spp. showing amplication of amplicons size 223bp. PCR products Lane M- Marker, lane1-10; lane 1- *Brucella abortus,* lane 2- *Brucella melitensis* ,lane 3- *Brucella suis,* 4-10 isolated *Brucella* spp.

According to OIE the molecular technique PCR assays tend to be simple and robust but it can be used only for the detection of brucella species and when the differentiation of the brucella species is not relevant, such as diagnosis of human brucellosis or contamination of food products [4]. Though various gene targets are reported for PCR assay, in our study 31kDa *Brucella* surface cell protein was used as the target for the detection of the brucella infection. Differentiation of the detected brucella positive samples were subjected to the Bruce ladder speciation PCR and samples subjected resulted as *Brucella abortus* (Figure 2).

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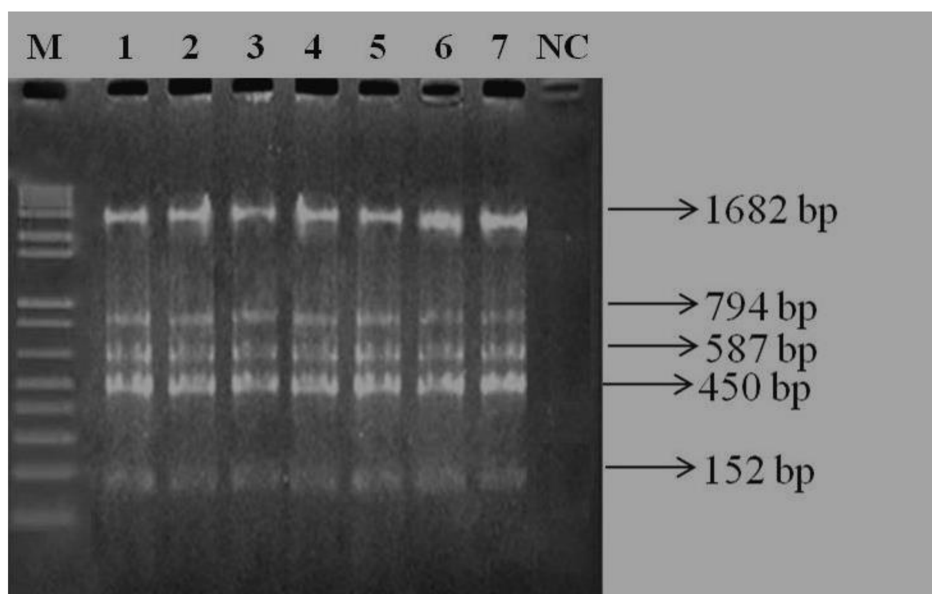

Fig 2. Differentiation of all *Brucella* species by Bruce ladder speciation PCR. Lane M-Marker, Lane 1-7 infected isolates were identified as *Brucella abortus.*

Bruce ladder multiplex PCR results highlighted the application and novelty of PCR in discrimination of *Brucella* spp. Ignacio *et al*. [18] in his studies reported the application of Bruce-ladder PCR in speciation of *Brucella* species. Bruce-ladder multiplex PCR results were concordant to previous reports of Ignacio *et al.* [18] and Mohandoss *et al*. [19]. Therefore, to dispose of a rapid, accurate and highly sensitive assay as PCR and Bruce ladder speciation PCR gives a reliable tool to be used by authorities, in order to implement control measures and spread of the disease among human and animal population [20]. The treatment relapse of brucellosis by raising antimicrobial resistance is another concern for the control of the brucellosis spread [21]. Bruce ladder pcr is in accordance with the studies of Mathew et al. [22], Ali et al. [23]. Importantly there is ~ 94 % genetic similarity amongst the members of the genus [24], although specific genomic islands have been identified [25]; whole genomic sequence of brucella spp. including B. melitensis [26] B. suis [27], two strains of B. abortus [28, 29] and B. ovis [30]. From the present study the brucellosis prevalence was reported and prevention measures were taken for the control of the disease. Due to the pathognomonic nature of brucellosis, disease is of emerging zoonotic importance and diagnosis is of high priority for its prevention. So that further the vaccination strategies for animal can be planned effectively only after obtaining the epidemiological data in particular region. To reduce the prevalence of human infections in endemic areas it is tremendously important to carry out awareness campaigns to emphasize to farmers to use only boiled milk for all purposes, because most cases occur after consumption of unpasteurized dairy product.

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4. CONCLUSION

Bovine brucellosis prevalence in villages of Mandya District, Karnataka, India was screened and 4.3 % Seroprevalence of bovine brucellosis was identified by RBT and 3.3 % by MRT and it was confirmed by molecular characterization 4.3% prevalence was observed by PCR and Bruce ladder PCR, In conclusion these results also signify significant public health implications following great economic losses to poor people particularly in the rural areas of Karnataka. As vaccination for the humans are unavailable the control of human brucellosis is possible only through the mass vaccination of animals in the infected regions, testing the animals, slaughtering, vaccination, sanitation and control of movement of the infected animals. In India the slaughtering of cattle is prohibited due to religious and ethical issue. Knowledge of the brucellosis infection distribution, spread of the infection and screening of brucellosis in different livestock and wildlife species is important to effectively implement the control strategies.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest in the article.

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