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RESEARCH ARTICLE Indian Journal of Animal Research

Molecular Characterization and PCR-based Detection of *Brucella abortus* Clinical Isolates from Cattle in Maharashtra, India

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10.18805/IJAR.B-5610

ABSTRACT

Background: Brucellosis is an important zoonotic disease in several parts of the world including India. The current study was undertaken for molecular characterization and PCR-based detection of *Brucella* species of cattle from Maharashtra, India.

Methods: A total of 220 clinical samples (vaginal swabs and placental tissues) were collected from cattle of various organized and unorganized farms from different regions of Maharashtra. These samples were processed for isolation of *Brucella* organisms and further characterized by PCR and sequencing.

Result: A total of 10 *Brucella* isolates were recovered from 220 samples (5.54%). BCSP 31 and 16S rRNA PCR showed an amplicon of 223 and 1412 bp in 10 isolates respectively. Species identification was performed using IS711 PCR, conventional methodology, Bruce-ladder PCR and AMOS PCR. During the study period, a total of ten isolates from vaginal swab and placental tissues of cattle were identified as *Brucella abortus*. The phylogenetic analysis detected the close relationship of the organism with that of isolate from Italy. Correct and timely diagnosis of brucellosis is important for control of disease. The molecular methods used in the current study aided to speed up the diagnosis of the disease.

Key words: AMOS PCR, BCSP 31, Bruce-ladder, *Brucella abortus,* Cattle, IS711 PCR.

INTRODUCTION

Brucella spp. are Gram negative facultative intracellular bacteria that are causative agents of brucellosis. Brucellosis is a major infectious disease of livestock and a re-emerging zoonotic disease in several developing countries including India (Shome *et al.,* 2017). Brucellosis is an important cause of veterinary morbidity and mortality and responsible for economic losses in developing countries (Franco *et al.,* 2007). Infection in cattle is mainly caused by *Brucella abortus* and the economic losses associated with bovine brucellosis are primarily related to various reproductive problems, perinatal mortality and chronic or diffuse interstitial mastitis (Corbel *et al.,* 2006; OIE, 2009). Brucellosis prevalence among livestock animals varies widely across India, differing from region to region and farm to farm (Jaismon *et al.,* 2023). Current laboratory diagnostic tests for brucellosis include the serological tests and cultural examination. However, these serological tests are not very sensitive or specific to be used for diagnosis. PCR- based assays proved to be more accurate and sensitive compared to traditional bacteriological methods used for the identification of Brucella species at the biovar level and can identify low amounts of Brucella DNA (Ullah *et al.,* 2024). Improved laboratory diagnosis is possible due to developments in culture and serological methods and the availability of advanced molecular detection and typing methods. These molecular methods could serve as important alternatives to culture methods for the confirmation of the disease and may be a valuable epidemiological tools to trace sources of infection. Samples collected in this study were taken through various PCR assays to identify *B. abortus at genus* and species level. PCR-positive samples were subjected to sequencing in order to confirm the presence of *B. abortus* and to determine the relationships between the various stains. It can be concluded that this work is useful for expanding knowledge related to molecular characteristics of brucellosis in cattle of Maharashtra, India.

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How to cite this article: Hedau, M., Singh, J., Singh, N., Kaore, M., Chaudhari, S.P., Thorat, V. and Kurkure, N.V. (2025). Molecular Characterization and PCR-based Detection of *Brucella abortus* Clinical Isolates from Cattle in Maharashtra, India. *Indian Journal of Animal Research*. 1-7. doi: 10.18805/IJAR.B-5610.

Submitted: 23-04-2025 **Accepted:** 19-08-2025 **Online:** 18-11-2025

MATERIALS AND METHODS

Prior to the start of collection of samples, experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and Institutional Bio-safety Committee (IBSC) of Nagpur Veterinary College, Nagpur, India.

Reference strain

Reference strain included *B. abortus* S99 (VTCCBAA465) obtained from National Centre for Veterinary Type Cultures (NCVTC), Hisar, Haryana. *B. abortus* S99 and *E. coli strain* were used as positive and negative controls, respectively.

Collection of sample

The farms having history of abortion were selected for the present study. A total of 220 samples (vaginal swabs and placental tissues) were collected from cattle of various organized and unorganized farms from different regions of Maharashtra state for the detection and isolation of *Brucella spp*.

Processing of specimens by conventional and molecular methods

The vaginal swabs and placental tissues were processed for isolation of *Brucella* by conventional methods. The isolates suspected of *Brucella* were screened by biochemical tests for identification (OIE, 2009). The isolates recovered were further subjected to PCR. The placental tissue samples were also used for direct DNA extraction and further characterized by PCR assays. The extraction of genomic DNA of *B. abortus* from the bacterial cultures, *Brucella* reference strain and placental tissue was carried out by using the HiPurA® Multi-Sample DNA Purification Kit (HiMedia) as per the manufacturer's protocol. The extracted DNA was quantified spectrophotometrically using nanodrop spectrophotometer (Eppendorf).

BCSP 31, 16S rRNA, IS711, AMOS and Bruce-ladder PCR assays

The BCSP 31 and 16S rRNA PCR was carried out on isolates and clinical samples to detect *Brucella* at genus level with primers mentioned in Table 1 (Baily *et al.,* 1992; Singh *et al.,* 2013). The genus was re-confirmed using conventional methodologies and protocols (OIE, 2009). Brucella species identification was performed using IS711 PCR and AMOS-PCR by using specific primers of *B. abortus, B. melitensis, B. ovis and B. suis* which are listed in Table 1 (Bricker and Halling, 1994; Sonekar *et al.,* 2018). Species identification and differentiation of *B. abortus* was also performed using the Bruce-ladder multiplex PCR protocol using a set of eight primer pairs mentioned in

Table 1: Primer sequences used in the present study.

PCR assay	Gene	Primers sequence 5′-3′	Size (bp)	Reference
Genus specific	*BCSP31*	F-CGCGCTTGCCTTTCAGGTCTG	223	Baily *et al.,* (1992)
		R-TGGCTCGGTTGCCAATATCAA		
	16S rRNA	F-AGAGTTTGATCCTGGCTCAG	1412	Singh *et al.,* (2013)
		R-ACGGCTACCTTGTTACGACTT		
Species specific	*IS711*	F-ACGAACGGAATTTTTCCAATCCC	498	Bricker *et al.,* (1994)
		R-TGCCGATCACTTAAGGGCCTTCAT		
AMOS PCR	*IS711*	R-GCCGATCACTTAAGGGCCTTCAT		
	B. abortus	F-GACGAACGGAATTTTTCCAATCCC	498	Sonekar *et al.,* (2018)
	B. melitensis	F-AAATCGCGTCCTTGCTGGTCTGA	731	
	B. ovis	F-CGGGTTCTGGCACAATCGTCG	976	
	B. suis	F-GCGCGGTTTTCTGAAGGTTCAGG	285	
Bruce-ladder PCR	*wboA*	F-ATCCTATTG CCCCGATAAGG	1682	Garcia-Yoldi *et al.,* (2006)
		R-GCTTCGCATTTTCACTGTAGC		
	bp26	F-GCGCATTCTTCGGTTATGAA	450	
		R-CGCAGGCGAAAACAGCTATAA		
	omp31	F-TTTACACAGGCAATCCAGCA	1071	
		R-GCGTCCAGTTGTTGTTGATG		
	pda	F-ACGCAGACGACCTTCGGTAT	794	
		R-TTTATCCATCGCCCTGTCAC		
	eryC	F-GCCGCTATTATGTGGACTGG	587	
		R-AATGACTTCACGGTCGTTCG		
	abc	F-GGAACACTACGCCACCTTGT	272	
		R-GATGGAGCAAACGCTGAA G		
	rpsL	F-CAGGCAAACCCTCAGAAGC	218	
		R-GATGTGGTAACGCACACCAA		
	crp	F-CGCAGACAGTGACCATCAAA	152	
		R-GTATTCAGCCCCCGTTACCT		

Table 1 (Garcia-Yoldi *et al.,* 2006)**.** The BCSP31, 16S rRNA, IS711, AMOS and Bruce-ladder PCR were set in a final volume of 25 µl as mentioned in Table 2. PCR reactions were carried out in thermal cycler (Eppendorf, vapo.protect) as per cycling conditions mentioned in Table 3. The amplified BCSP31, 16S rRNA, IS711, AMOS and Bruce-ladder PCR products were visualized in ethidium bromide-stained 1.5% agarose gel under Automatic Computerized Gel Documentation and Analysis System (Gel Doc, Syngene). A molecular weight marker with 100 bp and 1kb increments (Himedia) were used as a DNA standard.

Sequence and phylogenetic analysis

PCR products IS711 of three *Brucella* isolates were submitted for sequencing to Eurofins Genomics India Pvt. Ltd, Banglore (India). The sequences were blast using blast search of NCBI for identity of sequences. Both the sequences were aligned and consensus sequence was prepared with Bioedit (www.mbio.ncsu.edu/BioEdit/bioedit.html). The alignment was carried out with Clustal W of MEGA 7.0 and the phylogenetic tree was constructed based on *Brucella abortus* sequences by neighbor joining method in MEGA 7.0 (Kumar *et al.,* 2016).

RESULTS AND DISCUSSION

In the present study, molecular characterization of *Brucella abortus* clinical isolates from cattle in Maharashtra, India was carried out.

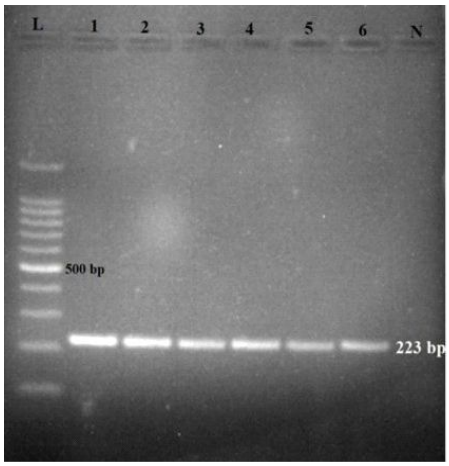
Laboratory findings

A total of 10 *Brucella* isolates were recovered from 220 clinical samples with 4.54% isolation rate. Seven isolates were derived from vaginal swabs and three isolates were obtained from placental tissue. All the isolates exhibited morphology and staining characteristics typical of *Brucella* spp., *i.e.* they were Gram- negative coccobacilli, acid-fast in MZN staining. The Colonies were round, glistening, smooth and mucoid on BAM (Fig 1); non-lactose fermenting on MacConkey agar and non-haemolytic on blood agar. All cultures on Brucella agar medium were typical isolates of Brucella in morphology, colonial appearance and characteristics of growth. The isolates recovered were further confirmed as members of *Brucella* spp. employing different biochemical tests. All isolates produced oxidase, indole, urease, H2S and reduced nitrate.

PCR analysis

All the 10 isolates and the reference strain *B. abortus S99* showed BCSP31 gene-specific amplicon of 223 bp and 16S rRNA specific amplicon of 1412 bp confirming their identity as members of genus *Brucella* (Fig 2 and 3). In IS711 PCR, *B. abortus* S99 and 10 clinical isolates generated a product of 498 bp, confirming the isolates as *B. abortus* (Fig 4). All of the 10 Brucella isolates were identified as *B.*

Fig 1: *Brucella abortus* on Brucella specific agar.



Lane L: DNA ladder 100bp; Lane 1: B. abortus S99 as positive control; Lane 2-6: Field samples; Lane N: Negative control.

Fig 2: Identification of field isolates of *Brucella* spp. by BCSP 31 PCR.

Table 2: PCR reaction mixture for BCSP 31, 16s rRNA, IS711, AMOS and Bruce-ladder PCR.

Ingredients	BCSP 31, IS711 and 16s rRNA PCR	AMOS PCR	Bruce-ladder PCR
2 × Go taq green master mix (Promega)	12.5 µl	12.5 µl	12.5 µl
Forward primer mix (10 pM)	1.0 µl	2.0 µl	4.0 µl
Reverse primer mix (10 pM)	1.0 µl	0.5 µl	4.0 µl
Nuclease free water	8.0 µl	7.0 µl	2.0 µl
DNA	2.5 µl	3.0 µl	2.5 µl
Total	25 µl	25 µl	25 µl

Table 3: Cycling conditions for BCSP 31, IS711, Bruce-ladder PCR, AMOS PCR and 16s rRNA PCR.

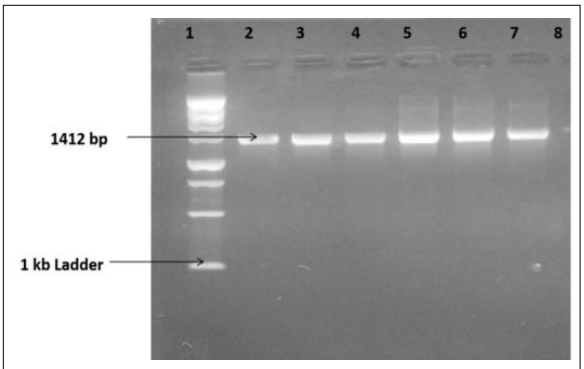
		BCSP 31 PCR	IS711 PCR		Bruce-ladder PCR		AMOS-PCR		16s rRNA PCR	
Step I	Initial denaturation	95°C for 3 min.	95°C for 2 min.	1 cycle	95°C for 7 min.	1 cycle	95°C for 2 mins	1 cycle	95°C for 5 min.	1 cycle
Step II	Denaturation	95°C for 45 sec.	95°C for 1.15 min	}35 cycles	95°C for 35 sec	}30 cycles	95°C for 1.15 mins	}30 cycles	95°C for 45 sec.	}35 cycles
	Annealing	60°C for 45 sec.	55.5°C for 2 min		64°C for 45 sec		55.5°C for 2 mins		57°C for 1 min	
	Extension	72°C for 2 min.	72°C for 2 min		72°C for 3 min		72°C for 2 min.		72°C for 1 min.	
Step III	Final extension	72°C for 10 min	72°C for 5 min	1 cycle	72°C for 6 min	1 cycle	72°C for 10 min	1 cycle	72°C for 10 min	1 cycle
Step IV	Hold	4°C	4°C		4°C		4°C		4°C	

abortus using the AMOS-PCR method (Fig 5). The Bruce-ladder PCR identified all the ten isolates as *B. abortus* by amplifying genes *crp, bp26, eryC, pda* and *wboA* of 152 bp, 450 bp, 587 bp, 794 bp and 1682 bp respectively (Fig 6).

Molecular characterization of *Brucella abortus* by nucleotide sequencing

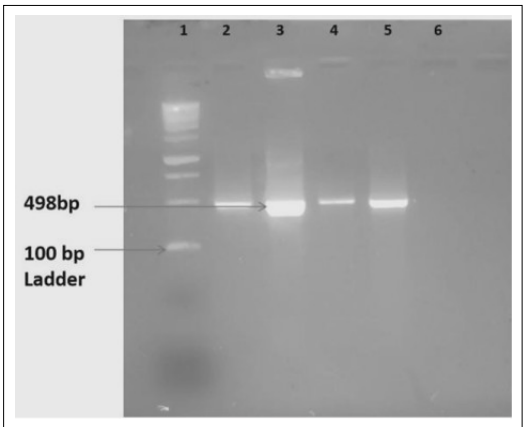
Three isolates were subjected to sequencing of 498 bp amplicon of IS711 gene. It was observed that amplicons of the IS711 gene of the three isolates of *B. abortus* had 99 to 100% homology with the available sequences of *B. abortus* in NCBI. Results of phylogenetic analysis indicated that *B. abortus* isolates *i.e.* NGP1, NGP2 and NGP3, showed similarity with isolates from Italy (Fig 7).

Brucellosis is considered one of the world's most serious zoonotic diseases which causes enormous economic losses in both developed and developing countries. The disease has disseminated worldwide, despite massive attempts to eradicate it in many countries (Elrashedy *et al.,* 2022). Several workers in India have attempted isolation of *Brucella* spp. with varying rates of



Lane 1: 1 kb DNA ladder; Lane 2: *B. abortus* S99, 3 to 7: field samples, 8: Negative control.

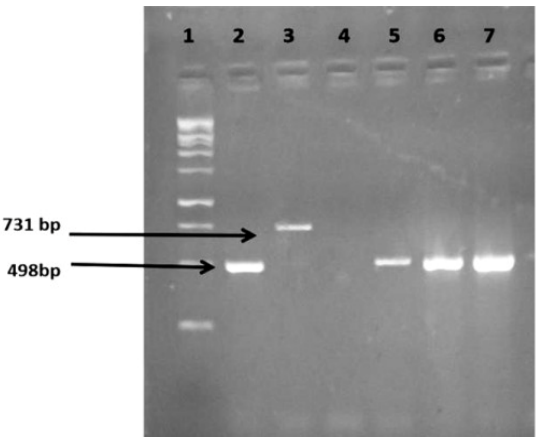
Fig 3: Identification of field isolates of *Brucella* spp. by 16S rRNA PCR.



Lane 1: 100 bp DNA Ladder; 2: S99; 3 to 5: Field samples; 6: Negative control.

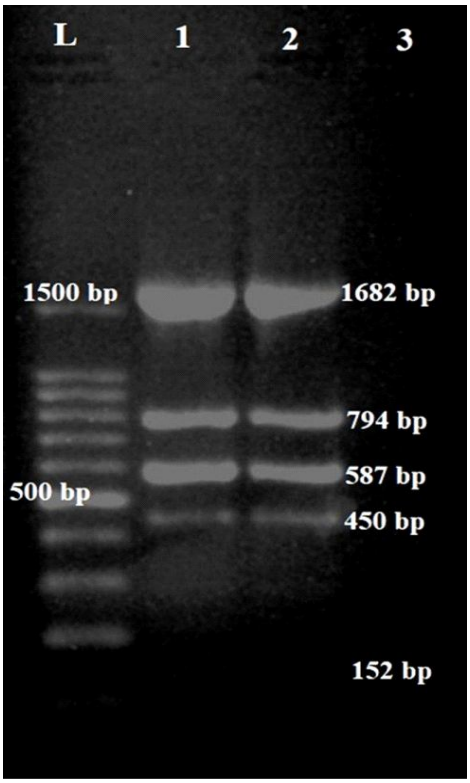
Fig 4: Identification of *Brucella abortus* by IS711 PCR assay.

isolation. Recovery of 30% isolates from abomasal contents of aborted foetuses, 16% isolates from foetal tissues and 8.42% from vaginal swabs have been reported earlier (Thorat and Bannalikar, 2022). Isolation of *B. abortus in* 8.92% cases of bovine while *B. abortus* from vaginal swabs in 15% cases have also been reported by various workers (Jeyaprakash *et al.,* 1999; Shrimali *et al.,* 2017). In the present study, isolation rate was relatively low (4.54%) than the previous reports. However, isolation rate of 4.4% for bovine brucellosis appear to similar as reported by Mittal *et al.,* (2018) might be due the slow growth and fastidious nature of the *Brucella spp* (Seleem *et al.,* 2010; Patel *et al.,* 2017). While India, prevalence of brucellosis in animals is ranging from low to moderate, however, the isolation rate of *Brucella* spp. from clinical samples of animals tends to be low. While many animals may test positive for antibodies to Brucella (seropositive), the actual bacteria are not frequently isolated from them which can be attributed to factors like the stage of infection, the type of sample tested *etc.* Isolation data and clinical pictures indicated that typically isolates were obtained from vaginal swabs of animals with a history of late pregnancy abortion (7-9 months). In the present study, only animals with a history of abortion were sampled to increase the probability of detecting Brucella-infected animals. The presence of *Brucella* in vaginal swabs of cattle has been recorded in other studies (Thorat and Bannalikar, 2022; Efrem *et al.,* 2024). Hence, the presence of *Brucella* in the vagina signifies that the Veterinarians and farmers managing difficult calvings and retained placenta cases in cows should use personal protective equipment (Mengele *et al.,* 2024). Furthermore, a large number of animals (n=210) with a recent history of abortion were negative on cultural examination. In these cases, other factors and infectious agents can induce abortion at different stages of the gestation period and abortion was not necessarily caused by Brucella infection (Efrem *et al.,* 2024). All the 10 isolates obtained in the present investigation were initially confirmed by the cultural, morphological and biochemical tests as *Brucella* species (Koneman *et al.,* 1997). The BCSP31 and 16S rRNA PCR assay detected all the isolates as Brucella at genus level. Similar efficacy of the BCSP31 primers in detecting *Brucella* organism at the genus level was previously recorded (Kaur *et al.,* 2018; Thorat and Bannalikar, 2022). In the present study, 16S rRNA gene amplification was used as a rapid confirmatory identification tool for *Brucella* genus (Singh *et al.,* 2013). The results of IS711 assay recorded in the present investigation agree with the findings of earlier researchers (Awwad *et al.,* 2015; Thorat and Bannalikar, 2022). They identified *B. abortus* by IS711 PCR assay and found it to be effective. Molecular approaches for identification of *Brucella* species have proved to be powerful tools to confirm the disease and to establish the genetic relationship among field isolates (Allen *et al.,* 1998). The reduced risk of laboratory-acquired infections, diagnosis within short time and accessibility are among the great advantages of these methodologies (Scholz and Vergnaud, 2013). Phylogenetically, *B. abortus* isolates in the current study showed similarity with isolates from Italy. Similar phylogenetic analysis findings have been reported (Barua *et al.,* 2016; Ahmed *et al.,* 2017; Thenamutha *et al.,* 2017). The current study has identified that *B. abortus is* circulating in the cattle population in Maharashtra, India. Therefore, molecular characterization of *Brucella* species is becoming increasingly important to understand the epidemiological aspect (Oliveira *et al.,* 2017).



Lane 1: DNA ladder 1kb; Lane 2: *B. abortus* S99; Lane 3: *B. melitensis* Rev 1; Lane 5-7:Field samples; Lane 4- Negative control.

Fig 5: Identification of *B. abortus* by AMOS PCR.



Lane L: DNA ladder; Lane12: *B. abortus* S99; Lane 2: Field sample; Lane 3- Negative control.

Fig 6: Identification of *B. abortus* by Bruce-ladder PCR.

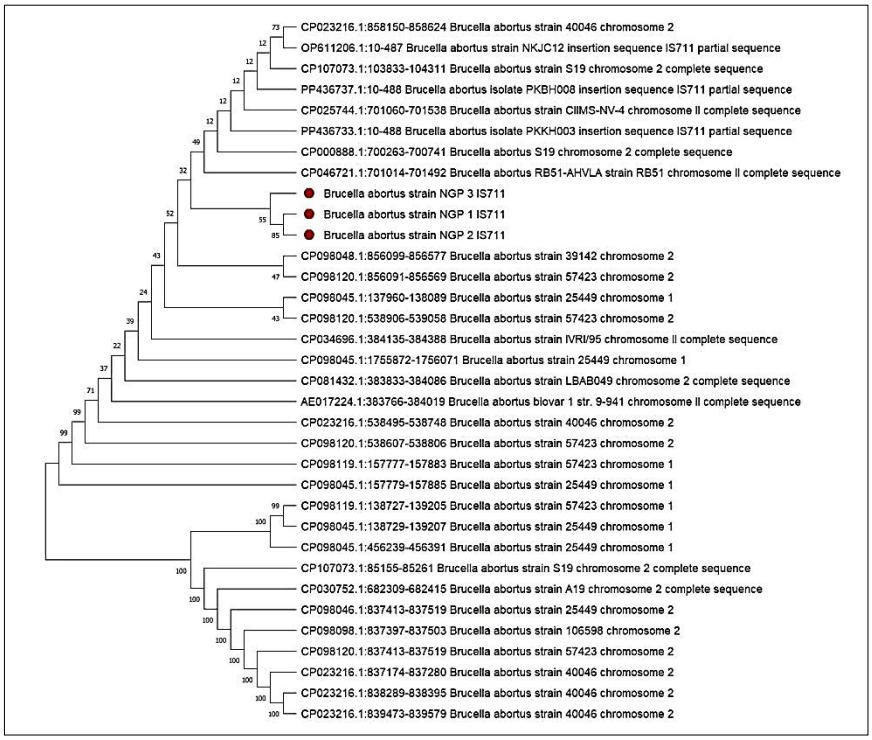


Fig 7: Phylogenetic tree of *Brucella* isolates based on IS711 sequences.

This study also provides insights on the strains of *B. abortus* circulating in India, which certainly help for the better understanding of the epidemiology and control of bovine brucellosis in the country.

CONCLUSION

The current study confirms that *Brucella abortus is* circulating in cattle of Maharashtra, India and is likely to be causing clinical disease. This calls for the control and prevention of this disease else it will not only cause economic loss to animal husbandry but also pose threat to human health. Further studies should be conducted on *Brucella* species circulating in small ruminants and other domestic animals to help in the epidemiology of brucellosis. Further, there is a need to create awareness for safe disposal of aborted materials among animal owners.

ACKNOWLEDGEMENT

The authors are thankful to the funding agency, the Indian Council of Medical Research (ICMR), for supporting this study through a funding. This study was funded by the grant from Indian Council of Medical Research (ICMR), New Delhi; IRIS ID Zon/54/2020/ECD-II.

Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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