Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(3): 1523-1527

Received: 27-03-2018
Accepted: 28-04-2018

MS Kala
Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry Sardar Krushi Nagar Dantiwada Agricultural University, Dantiwada, Gujarat, India

LN Sankhala
Assistant Professor, Department of Pharmacology and Toxicology, College of Veterinary and Animal Science Bikaner, Rajasthan, India

Lakshmi Kant
Department of Pharmacology and Toxicology, College of Veterinary and Animal Science Bikaner, Rajasthan, India

Lalit Kumar
Department of Pharmacology and Toxicology, College of Veterinary and Animal Science Bikaner, Rajasthan, India

SR Puniya
Veterinary Officer, Govt of Rajasthan, Rajasthan, India

NM Shah
Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry Sardar Krushi Nagar Dantiwada Agricultural University, Dantiwada, Gujarat, Indi

Correspondence
LN Sankhala
Assistant Professor, Department of Pharmacology and Toxicology, College of Veterinary and Animal Science Bikaner, Rajasthan, India

Isolation, identification and molecular detection of *Brucella abortus* from bovines of North Gujarat

MS Kala, LN Sankhala, Lakshmi Kant, Lalit Kumar, SR Puniya and NM Shah

Abstract
Brucellosis is a widespread and economically important infectious disease of animals and humans caused by members of the genus *Brucella*. The present study was undertaken for isolation and identification of *Brucella* organisms in vaginal discharge, placental cotyledons and aborted faetal contents and detection of *Brucella* organisms by PCR based method of bovines of North Gujarat. Out of 107 clinical samples of bovines, *Brucella* could be recovered, using *Brucella* agar medium, from five samples. Out of these, two were from cattle while three were from buffaloes. All the five isolates were identified as *Brucella* organisms by cultural, morphological and biochemical characteristics and further confirmed by PCR using three different genus specific primer pairs viz. B4/F5, JPF/JPR and F4/R2.Of the 53 clinical samples tested by direct PCR, using three *Brucella* genus specific primer pairs, 4 were found positive by B4/B5 primer pair, 3 by JPF/JPR primer pair and 3 by F4/R2 primer pair. The B4/B5 primer pair found more suitable than other two as they resulted in highest positive numbers as well as gave all the samples positive that were positive by other two primer pairs.

Keywords: Abortion, brucellosis, genus specific primer, reproductive failure

1. Introduction
Brucellosis is an important disease of livestock and wild animals widely prevalent in most of the developing countries. The disease causes a variety of reproductive disorders, viz., infertility, retained placenta, abortions, endometritis, etc. and resulting in heavy economic losses due to interrupted lactation and also due to loss of calves, wool, meat and milk production which are the main impediments to profitability. The disease has a significant health hazard in contact human beings.
Isolation of the causative agent is most accepted tool for confirmatory diagnosis of brucellosis [10]. It has the advantage of detecting the organisms directly, but it is time consuming since it takes about 10 days or longer for proper identification of the causative agents and it is less efficient in diagnosis of chronic infection. Besides, the culture materials must be handled carefully, as the organisms are class III pathogens [2]. For these reasons, identification based on the genetic characterization using molecular technique *viz*., PCR is much preferred and numerous PCR based assays have been developed for the rapid identification of *Brucella*.

2. Materials and Methods
In the present study, A total of 107 samples comprising of deep vaginal swabs from cows (23) and buffaloes (31) (having history of abortion at last trimester of gestation), placental cotyledons from cows (21) and buffaloes (27) and aborted faetus organs and stomach contents were also collected from cattle (2) and buffaloes (3).

2.1 Isolation and Identification of *Brucella*
For isolation and identification of *Brucella* organisms from deep vaginal swabs and aborted fetal materials the standard procedures described by Alton *et al.* (1988) [2] were followed. Isolated colonies presumed to be of *Brucella* were further confirmed by PCR using various genus specific primer pairs.

2.1.1 Isolation

Isolation of *Brucella* organisms from the vaginal swab and from aborted material from cattle and buffaloes was carried out by Transport swab from Himedia (Transport swab w/Amines medium w/o charcoal in polystyrene tube). Each swab collected from an animal was separately streaked on *Brucella* agar medium (BAM) plates in duplicates. One plate was incubated aerobically in incubator at 37ºC (without CO_2), and the other incubated at 37ºC aerobically in an atmosphere of 5% CO_2 in CO_2 incubator (Binder, Germany) for minimum 15 days. The plates were observed at every 24 h for the growth. The suspected colonies of *Brucella* were picked up and transferred to another BAM plates and incubated under 5% CO_2 tension to obtain a pure culture.The isolates were further inoculated on Blood Agar (BA) and MacConky Agar (MA) for their cultural characteristics.

2.1.2 Morphological and Staining Characters of Isolates

The isolates suspected for *Brucella* were subjected to Gram staining and Modified Ziehl-Neelsen (MZN) staining for confirming the purity of cultures and morphological characters.

2.1.3 Rapid Slide Agglutination Test:

One drop (0.03 ml) of known *Brucella* positive serum (I.V.R.I., Izatnagar) was taken on a glass slide by micropipette. A loopful culture from single suspected colony was mixed thoroughly with the spreader and then the slide was rotated for four min. The result was read immediately. Definite clumping/agglutination was considered as positive reaction, where as no clumping/agglutination was considered as negative. The purpose of conducting the test was to confirm suspected *Brucella* isolates serologically.

2.1.4 Acriflavine Test

One drop of acriflavine solution (1:1000 diluted in distilled water) was placed on a glass slide. A loopful of culture was mixed thoroughly and observed for agglutination. If the suspension remains uniform, the colony was considered s smooth (negative for test), while if clumps were observed, the colony was considered as rough (positive for test). The purpose was to differentiate smooth and rough colony producing *Brucella* isolates

2.1.5 Biochemical Characterization of Isolates by Various Biochemical Tests

The biochemical tests employed in the identification of gram negative organisms were those recommended by Barrow and Feltham, (2003) [3] and Alton *et al.* (1988) [2]

2.1.6 Molecular Detection of *Brucella*

2.1.6.1 Confirmation of *Brucella* Isolates by Using Colony PCR

PCR was used for confirmation of the *Brucella* isolates. The template DNA from colony was prepared according to Wilson (1987) [16] with minor modifications.

2.1.6.2 Nucleic Acid (DNA) Extraction from Colony

Suspected colonies from BAM plates were streaked on BAM slants. Slants were incubated at 37°C for 4 to 5 days at 5% CO_2 tension. Bacterial colonies were picked and suspended in 100 µl mili Q water. The samples were boiled for 15 min, cell debris were removed by centrifugation for 5 min at 8500 rpm and 3 µl of the supernatant was used as a template.In the present study three primer pairs were used for the detection of *Brucella* DNA from the isolated colony on *Brucella* Agar medium and direct clinical samples. The ability of all three primers pairs were compared. These three primer pairs amplified three different fragments *viz.*, (i) B4/B5 primer amplified a 223 bp product of *bcsp*31 region gene which encode 31 kDa immunogenic cell surface protein (ii) F4/R2 primer amplified a product of 905 bp of the 16S rRNA gene of *B. abortus* (iii) JPF/JPR primer amplified a product of 193 bp for gene sequence encoding outer membrane protein (*omp*2). All the 3 sets of primers used in the present study were genus specific to *Brucella* organisms

3. Results and Discussions

During the present study, attempt was made for isolation of *Brucella* organisms from 107 samples from cases of abortion and R.O.P in cows (46) and buffaloes (61) with history of abortion during last trimester of gestation. Of these, 4.35%, and 4.92% samples of cow and buffaloes yielded isolates presumably of *Brucella* organisms and overall prevalence of *Brucella* infection as detected by cultural isolation was 4.67 percent (5/107) as depicted in Table 1.

Table 1: Isolation of *Brucella* from vaginal discharges, placental cotyledons and aborted faetal contents.

Animal	No. of samples						Total	
	Vaginal swabs		Placental cotyledons		Aborted fetus			
	Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
Cows	25	1(4.00)	19	1(5.26)	2	-	46	2(4.35)
Buffaloes	38	1(2.63)	20	1(5.00)	3	1(33.33)	61	3(4.92)
Total	63	2(3.70)	39	2(5.66)	5		107	5(4.67)

Figures in parentheses indicate percentage

The present findings supported by Ghodasara (2008) [7] reported the overall isolation rate of *Brucella* from the vaginal swabs, placental cotyledons and other reproductive disorder in cattle, buffaloes, bitches and goats in Gujarat 4.03%. Kanani (2007) [9] recovered 8 (7.92%) *Brucella* isolates from the 101 bull semen samples processed. The overall isolation rate of *Brucella* from the milk, vaginal swabs and bull semen was 6.2%, Chatterjee *et al.* (1995) [5].

In contrast to the present study very high rate of recovery was obtained by Pal and Jain (1985) [13] who isolated *B. abortus* from 9 (20.93%) out of 43 clinical samples from abortion in buffaloes. Similarly, Das *et al.* (1990) [6] reported the presence of *Brucella* organisms in 38.18% in cows and 14.28% of buffaloes from cases of abortion. Aldomy *et al.* (1992) [1] obtained 34 isolates (16.5%) of *Brucella melitensis* from aborted, still birth or week full term animals and vaginal swabs from aborted sheep, goats and cows. Jeyaprakash *et al.* (1999) [8] obtained 10 (15.62%) isolates from milk samples from cows having symptoms of abortion and retained placenta. Patel (2007) [17] obtained 4 (7.56%) isolates of *Brucella* out of 53 milk samples processed from cattle and buffaloes having a history of abortion.

Keid *et al.* (2007) [11] examined 144 female dogs having reproductive problems. Of these 49 (34.02%) were blood

culture positive, 6 (4.1%) were vaginal swab culture positive for *Brucella* organisms.

A isolate of *Brucella abortus* was isolated from vaginal swabs (4%) and one from placental cotyledons (5.25%) of cows. While in buffaloes out of 3 isolates one from vaginal swabs (2.63%), one from (5%) placental cotyledons in buffaloes another one from (33.33%) from aborted faetus stomach contents. Overall isolation rate from vaginal discharge, placental cotyledons and aborted faetus contents was 4.67 percents in cattle and buffaloes.

In agreement with the present study, Ocholi *et al.* (2005) [12] reported recovery of 25 (3.01%) isolates of *Brucella* from the aborted fetuses, hygroma fluids, milk and vaginal swabs obtained from abortion cases of various animals.

In contrast to present study higher percentage of recovery rate were obtained by Verma *et al.* (2000) [15] who reported 2 (28.57%), 1(6.67%), 1(1.08%) and 4 (14.29%) isolates of various *Brucella* species from 7 aborted cows, 15 aborted does, 93 cases of endometritis among does and from 28 aborted ewes, respectively. Jeyaprakash *et al.* (1999) [8] reported 15.62% milk samples, culturally positive from cows having symptoms of abortion and retained placenta. Whereas, Chahota *et al.* (2003) [4] obtained 100% recovery rates of *B. abortus* from aborted morbid materials from all cows tested. Kaur *et al.* (2006) [10] recovered 17 (27.86%) isolates of *B. abortus* out of 61 samples processed from aborted cattle and buffaloes.

3.1 Idenification

3.1.1 Morphological and Staining Character of *Brucella* Isolates

The isolates presumed to be of *Brucella* were small gram's negative, coccobacillary rods on gram's staining while, on MZN staining they appeared red coloured as described by Alton *et al.* (1988) [2]. Similarly, Pal and Jain (1985) [13] revealed a gram negative coccobaciilary organism on gram's staining. Kanani (2007) [9] and Ghodasara (2008) [7] observed red coloured coccobacilli *Brucella* organisms on MZN staining.

3.1.2 Rapid Slide Agglutination Tests

In this study, all the isolates were tested for agglutination by known positive anti *Brucella* serum. All the isolates from cows (C1, C2) and buffaloes (B1, B2, B3) exhibited clear agglutination with known positive anti *Brucella abortus* serum describe by Alton *et al*. (1988) [2].

3.1.3 Acriflavine Test

In the present study, in presence of (1:1000) acriflavine, all the isolates from the cows (C1, C2) and buffaloes (B1, B2, B3) were identified as smooth *Brucella* species as they did not form clumps, as described by Alton *et al.* (1988) [2]. Similarly, Kanani (2007) [9] and Patel (2007) [17] reported smooth *Brucella* species isolated from the semen of bull (Bovine) and milk of bovines, respectively

3.1.4 Biochemical Characterization of *Brucella* Isolates

In the present study, all the five isolates were positive for oxidase and catalase reaction. The isolates from cows (C1, C2) and buffaloes (B1, B2, B3) failed to induce urea hydrolysis, no motility and no H_2S production was observed on Motility Sulphide Medium and the isolates were negative for indole production and VP test. All the isolates were found to reduce nitrate.

Robert *et al.* (1982) [18] typed isolates of *Brucella abortus* were typed based on various biochemical tests. Of these, isolates were found positive for CO_2 requirement, nitrate reduction and negative for urea hydrolysis, but in contrast to the present study, were negative for catalase and were H_2S producer. Pal and Jain (1985) [13] reported biochemical character of *B. abortus*, as catalase and oxidase positive, but were H_2S producer. Poole *et al.* (1972) [19] biotyped isolates obtained from human (female) by various biochemical tests were CO_2 dependent and positive for Nitrate reduction, but in contrast to the present study, were H_2S producer.

Rhyan *et al.* (1994) [14] carried out various biochemical tests for the identification of *B. abortus*, reported catalase and oxidase activity, CO_2 dependent, but in contrast to present study, observed urease hydrolysis and H_2S producer among the *B. abortus* isolates. Variability among the *Brucella* isolates was also described by Alton *et al.* (1988) [2].

3.1.5 Molecular Detection of *Brucella* from Culture and Clinical Samples

3.1.5.1 Confirmation of *Brucella* by Genus specific primer pairs from culture

Colony PCR for reference *Brucella* strain-19 and the *Brucella* isolates were carried out using three different *Brucella* genus specific primer pairs (i) B4/B5 primer pair amplified a 223 bp region of the sequence encoding a 31 kDa immunogenic *bcsp*31 (ii) F4/R2 primer pair amplified a 905 bp region of the sequence 16S rRNA of *B. abortus* (iii) JPF/JPR primer pair amplified a 193 bp region of the sequence encoding an outer membrane protein (*omp*2)

In present study 46 clinical samples were taken from cattle for cultural isolation. Only 2 (4.35%) were positive for culture while 61 clinical samples were taken from buffaloes for cultural isolation. Only 3 (4.92%) were positive further confirm by colony PCR. In the present study overall 4.67% samples (5 out of 107) were isolated on *Brucella* Agar medium found positive and all five were confirmed by colony PCR.

The desired product of 223 bp using B4/B5 primer pair (Plate 1) was amplified in all the 6 isolate including the reference strain. However, isolates B3 could not produce a desired product of 193 bp using primer pair JPF/JPR (Plate 2) and isolates C1 could not produce a desired product of 905 bp using primer pair F4/R2 (Plate 3) even after repeated trials. The results of amplification using various sets of primers are depicted in Table 2.

Table 2: Confirmation of *Brucella* isolates from culture by PCR

Sr. No.	Isolates	Primer pairs		
		B4/B5	JPF/JPR	F4/R2
1.	C1	+	+	-
2.	C2	+	+	+
3.	B1	+	+	+
4.	B2	+	+	+
5.	B3	+	-	+
6.	Reference strain *B. abortus* strain-19	+	+	+

C = Cattle, B = Buffaloes

+ = Amplified desired product - = Do not amplified desired product

For positive samples amplified product sizes for different primers were: B4/B5 - 223bp, JPF/JPR - 193bp and F4/R2 - 905bp

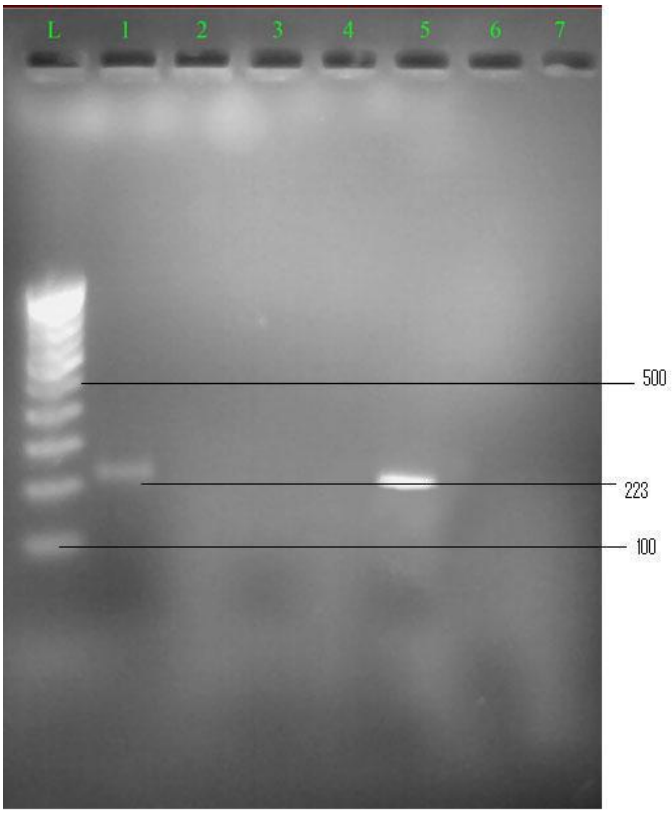

Plate 1: Agarose gel electrophoresis pattern of *Brucella bcsp31* gene 223 bp specific PCR product amplified with primer B4/B5
L: DNA molecular weight ladder 100 bp
1: Positive control
2: Negative control
5: Positive field sample

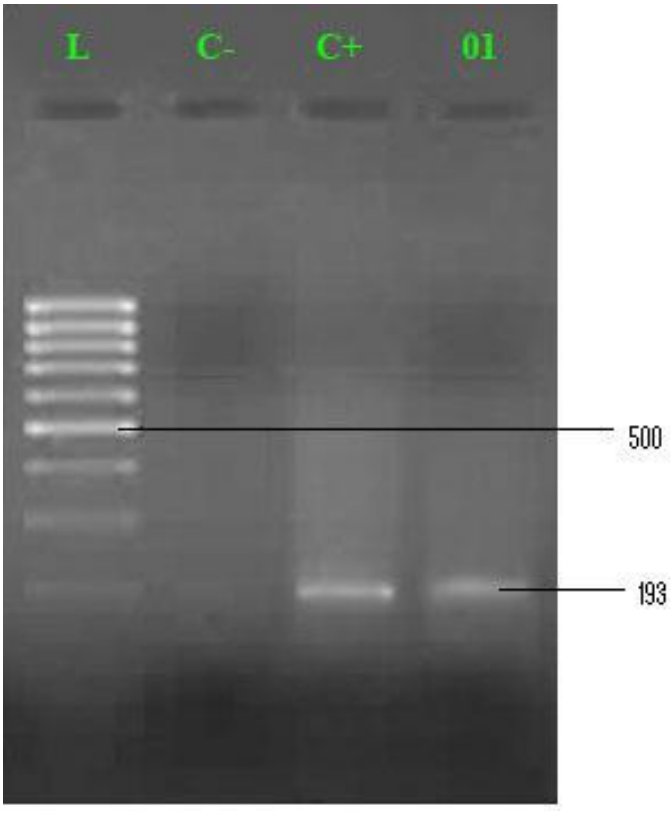

Plate 2: Agarose gel electrophoresis pattern of *Brucella omp2* gene 193 bp specific PCR product amplified with primer JPF/JPR
L: DNA molecular weight ladder 100 bp
C-: Negative control
C+: Positive control
01: Positive field sample

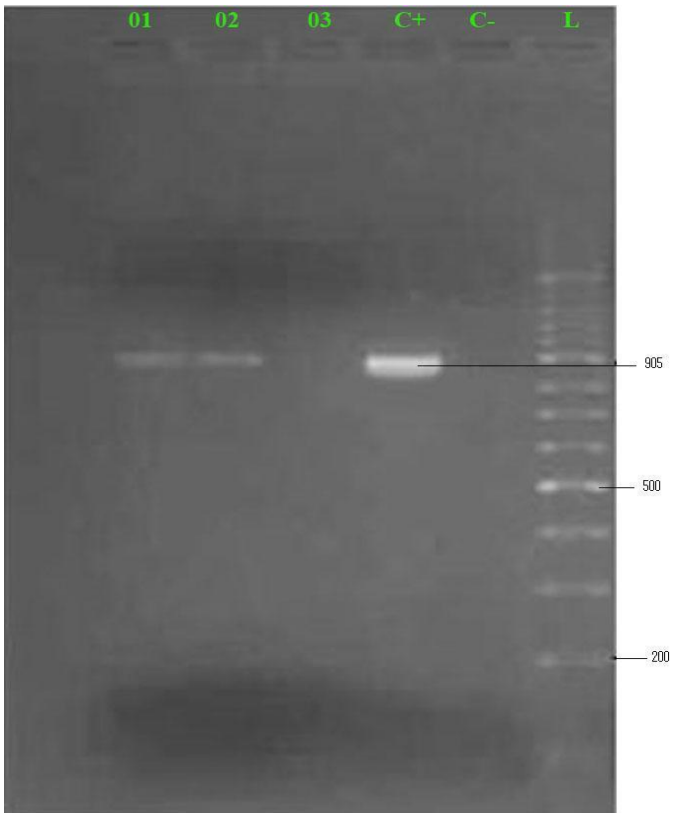

Plate 3: Agarose gel electrophoresis pattern of *Brucella* gene encoding 16S rRNA of *B. abortus* 905 bp specific PCR product amplified with primer F4/R2
L: DNA molecular weight ladder 100 bp
C-: Negative control
C+: Positive control
01 and 02: Positive field sample

3.1.5.2 Direct detection of *Brucella* by Genus specific primer pairs from clinical samples

In present study total 23 samples were taken from cattle for direct detection of brucella from clinical samples by PCR. Out of 23 samples, 2 (8.67%) were positive by clinical samples PCR.Total 30 samples were taken from buffaloes for direct detection of brucella from clinical samples by PCR. Out of 30 samples, 2 (6.66%) were positive by clinical samples PCR.In the present study overall 7.55% samples(4 out of 53) found positive by direct clinical samples PCR. The desired product of 223 bp using B4/B5 primer pair was amplified in all the 4 isolates. The C1 samples could not produce a desired product of 905 bp using primer pair F4/R2 even after repeated trials. The results of amplification using various sets of primers are depicted in Table 3.

Table 3: Direct detection of *Brucella* from clinical samples by PCR

Sr. No.	Clinical samples	Primer pairs		
		B4/B5	JPF/JPR	F4/R2
1.	C1	+	+	-
2.	C2	+	+	+
3.	B1	+	+	+
4.	B2	+	+	+
5.	Reference strain *B. abortus* strain-19	+	+	+

C = Cattle, B = Buffaloes
+ = Amplified desired product - = Do not amplified desired product
For positive samples amplified product sizes for different primers were:
B4/B5 - 223bp, JPF/JPR - 193bp and F4/R2 - 905bp.

Earlier Navarro *et al.* (2002) [20], Kanani (2007) [9] and Patel (2007) [17] used the same three primer pairs for detection of *Brucella* DNA from isolates. In this present study, desired product of 223 bp using B4/B5 primer pair and desired product of 193 bp using primer pair JPF/JPR was amplified in all the 5 isolates and 4 clinical samples, whereas the isolate C1 could not produce desired amplicon of 905 bp using primer pair F4/R2 on repeated trials of the cultured isolate and clinical sample,. Hence, the non-amplification might be due to probable mutation in primer attachments sights particularly at 3' end. Further, in the absence of sequence information of the annealing site of field isolate, no conclusive inference could be drawn about the behaviour of this primer pair (Kanani, 2007) [9]. Navarro *et al.* (2002) [20] observed a slightly different sensitivity of these three primer pairs with the conclusion that difference in sensitivity might be due to sample pretreatment methods and extraction methods of DNA. Patel (2007) [17] also observed the difference in the sensitivity of the same three primer pairs with high sensitivity by B4/B5 primer.

4. Conclusion

Comparatively PCR was found more suitable method for detection *Brucella* in Clinical samples as compared to cultural methods because more numbers of bovines were found positive by this method as well as all culturally positive bovines also found positive in PCR. Among the three different genus specific primer pairs used for amplification by PCR, B4/B5 primer pair detected *Brucella* DNA better than F4/R2 and JPF/JPR primer pairs. The isolation results showed the presence of *B. abortus* in clinical samples which is of public health concern due to its zoonotic importance. There is a need to educate the peoples about prevention and control of brucellosis due to its high zoonotic importance.

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