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ISOLATION AND IDENTIFICATION OF *BRUCELLA* SPECIES FROM SUSPECTED BIOLOGICAL SAMPLES OF HUMAN PATIENTS AND INFECTED ANIMALS BY CONVENTIONAL ISOLATION METHOD FOLLOWED BY PCR ASSAY

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ABSTRACT

Brucellosis is an infectious zoonotic disease of domestic and wild animals. It has a serious implication on human health as well as on the economic development in a developing country like India. Rapid identification and treatment of the disease in human and animal is important for the prevention of spreading the disease. Isolation with a conventional bacteriological method for the identification of the disease stands gold standard, but is time consuming and misleading sometimes. Isolation and identification by conventional PCR assay have been done for screening of *Brucella abortus*, *Brucella melitensis* and *Brucella suis* from suspected samples collected from hospitals and local animal farms. In conclusion, six *B. melitensis*, four *B. abortus*, and two *B. suis* isolate has been identified out of 250 animal biological samples and three *B. melitensis* and two *B. abortus* isolates were identified out of seven suspected human blood samples.

KEY WORDS: ***Brucella*, Isolation, Biochemical test, PCR**

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INTRODUCTION

As a zoonotic disease, Brucellosis continues to be an increasing challenge worldwide. In agriculture based countries like India, major groups of human population are with the close association of animals. As a disease, it causes multi-organ infections that may present with a broad spectrum of clinical manifestations, including arthritis, sacroiliitis, spondylitis, encephalitis, endocarditis, epididymoorchitis and abortion [1]. The disease is caused by species of *Brucella*, and the pathogen demonstrates host specificity in infection [2]. *Brucella* spp. is Gram-negative, aerobic, non-motile and nonspore-forming coccobacilli. The genus includes a total of 10 species, namely *B. abortus, B. melitensis, B. suis, B. canis*, *B. ovis, B. pinnipedialis, B. microti, B. ceti, B. neotomae* and *B. inopinata*. Among these species, *B. abortus* (cattle)*, B. melitensis* (humans, goats and sheep)*, B. suis* (pigs)*, B. canis* (dogs)*, B. ovis* (sheep) and *B. neotomae* (wood rats) are involved in brucellosis [3]. However, *B. neotomae* is of a poor importance in terms of human health and economy [4]. The disease gets transmitted by ingestion of undercooked meat, unpasteurized milk and raw milk products, inhalation of aerosols containing the pathogen and accidental inoculation of the contaminated samples in the cuts and wounds [5]. The veterinarians, laboratory workers and farmers are at the higher risk of getting infection [6]. Isolation of the bacterium from biological samples of the infected hosts stands as the “gold standard” for confirmation of the infection [7]. However, concomitant DNA-based identification such as polymerase chain reaction is promising, confirmative and definitive in clinical diagnosis of the disease. Presently, in India diagnosis of brucellosis mainly depends on serological test like Standard Agglutination Test (SAT), Rose Bengal Test (RBT) and seroprevelance of anti-*Brucella* antibodies [8]. However, many of these tests are time consuming, hazardous, laborious and give rise to false positive and false negative results. Additionally, the tests are very sensitive even for vaccine strains and infections include sero-negative cases as well [9].The bacteriological diagnosis of *Brucella* spp can also be done by Gram’s staining, microscopic observation of organisms, and staining of vaginal swabs of animals, however, they are always misleading as they look like Gram positive and in most of the cases they are presumed to be coryneform bacilli or *Micrococcus* spp [10]. In microscopic view, it is really difficult to distinguish *Brucella* spp. from each other as they are highly similar morphologically. There are several different strategies developed in the past decades for diagnosis and clinical differentiation of *Brucella* spp. Among this, AMOS (*B. abortus*, *B. melitensis*, *B. ovis*, *B. suis*) PCR is most convincing and used in this study for identification and differentiation of *B. abortus*, *B. melitensis* and *B. suis*. As we have mentioned before, isolation followed by PCR assay is the most reliable, rapid and unique method for the identification of different *Brucella* spp. Blood samples of human and vaginal swabs, aborted samples, pus and milk of infected or dead animals is the best way for isolation of *B. melitensis*, *B. abortus* and *B. suis*. The spleen and lymph samples of the dead animals during post mortem examination of animals are also best sites for the isolation of *Brucella* organisms. In the present study, an attempt was made to screen suspected biological samples from human patients and animals for *Brucella* infection by isolation and identification of the *Brucella* species including *B. abortus*, *B. melitensis* and *B. suis* using biochemical and serological tests which were followed by polymerase chain reaction assay for confirmations of the disease.

MATERIALS AND METHODS

1. *Bacterial strains and growth conditions*: *Brucella* reference strains used in this study, listed in table 1, were procured from Indian Veterinary Research Institute, Bareilly, India. The strains were cultured on *Brucella* selective agar base and trypticase soy broth and agar (Himedia, India). The cultures were preserved at -80 $^{\circ}$C in presence of 15% of glycerol for further use. All the cultures were handled in Biosafety level III containment.

Table 1
List of reference strains used in this study.

Bacterial Strains	Source
B. abortus 544	IVRI
B. abortus S19	IVRI
B. melitensis 16M	IVRI
B. suis 1330	IVRI

2. Biological samples

Blood samples from seven suspected human patients for *Brucella* infection were collected from NIMHANS, Bengaluru, India. A total of 250 biological samples consisting blood, pus shed, vaginal exudates and fluids during abortion were collected from suspected animals which included, bovine, goats and swine from Veterinary College, Bengaluru, India.

3. Serological testing

Nine milliliters of blood were collected by vein puncture from *Brucella* spp. infected human patients and dispensed in 3 ml aliquots; 3 ml was not treated with anticoagulants and was used to obtain sera to perform the RBPT tests as described by Alton et al [7]. The antigen for the RBPT was procured from Indian Veterinary Research Institute (IVRI), Izatnagar.

4. Microbiological analysis

Three milliliters of the test sample was inoculated in 25 ml of trypticase soy broth and incubated at 37 $^{\circ}$C, 200 rpm for 72 hours. Aliquots were plated onto standard *Brucella* selective agar weekly during a 3-week period and incubated at 37 $^{\circ}$C under aerobic condition without CO_2 supplementation. Suspect *Brucella* colonies were identified by conventional biochemical methods as described elsewhere [11]. To brief, the tests included Gram staining, a modified Ziehl–Neelsen stain, growth characteristics, catalase and oxidase activity, motility test, urease production, nitrate reduction and H_2S production (4 days). The other biochemical test included the growth of cultures on media containing 20 µg/ml basic fuchsin.

5. Whole genomic DNA extraction from isolates and samples

Three millitres of heparinized blood sample and 1 ml of suspect *Brucella* isolates, cultured in TSB were used for DNA extraction. To extract DNA from blood, heparinized sample was centrifuged at 4000 x *g* for 5 minutes and resulting cell pellet was resuspended in 1 ml of erythrocyte lysis solution containing 155 mM NH_4Cl, 10 mM $NaHCO_3$, and 100 mM disodium EDTA (p^H 7.4). The resuspended blood cells were centrifuged at 4000 x *g* for 5 minutes repeatedly with fresh erythrocyte lysis solution until the leukocyte pellets lose the reddish colour. Leukocyte pellet was further resuspended in 400 µl of lysis solution (2% Triton X 100, 1% sodium dodecyl sulphate, 100 mM NaCl, 10 mM Tris-HCl, p^H 8.0) and 10 µl of proteinase K (10 mg/ml); incubated at 50 $^{\circ}$C for 30 minutes. The culture pellet of suspect *Brucella* isolate was resuspended in 400 µl of lysis solution containing 10 mM Tris HCl, 1 mM EDTA, 1% sodium dodecyl sulphate, p^H 8.0 and 10 µl of proteinase K (10 mg/ml); incubated at 50 $^{\circ}$C for 30 minutes. Resultant leukocyte and *Brucella* culture lysates were subjected to traditional phenol – chloroform treatment followed by DNA precipitation using 95% ethanol and 5 M NaCl. Followed by DNA precipitation, DNA was pelleted by centrifugation at 12000 x *g* for 15 minutes and washed with 70% ethanol, air dried and resuspended in 100 µl of 10 mM Tris-HCl. DNA concentration was determined spectrophotometrically using Nanodrop 2000 (Thermo Scientific, Bengaluru, India); diluted to a concentration of 50 ng/µl and aliquots were stored at -20 $^{\circ}$C for further use.

6. Primers and gene targets

The list of primers used in the present study is tabulated in table 2. A total of four pairs of reported diagnostic primers was used; one each for detection of *Brucella* genus [12], *B. abortus*, *B. melitensis* and *B. suis* [13]. 16S rDNA and IS711 were targeted as genus and species specific region respectively for the PCR assay. The oligonucleotide primers were synthesized at Eurofins Genomics Pvt. Limited, Bengaluru, India.

Table 2
List of oligonucleotides and their amplicon size (in base pair).

Primer	Primer sequence (5′–3′)	Amplicon Size (bp)
B. abortus (F)	GACGAACGGAATTTTTCCAATCCC	498
B. abortus (R)	TGCCGATCACTTAAGGGCCTTCAT	
B. melitensis (F)	AAATCGCGTCCTTGCTGGTCTGA	731
B. melitensis (R)	TGCCGATCACTTAAGGGCCTTCAT	
B. suis (F)	GCGCGGTTTTCTGAAGGTTCAGC	285
B. suis (R)	TGCCGATCACTTAAGGGCCTTCAT	
Ba148-167 (F)	TGCTAATACCGTATGTGCTT	800
Ba928-948 (R)	TAACCGCGACCGGGATGTCAA	

[F] forward, [R] reverse

7. Monoplex PCRs

For genus specific PCR assay, the 25 µl reaction mixture consisting of 100 ng of test DNA, 1x PCR buffer, 2.0 mM $MgCl_2$, 250 µM of the dNTPs (Fermentas, India), 0.6 µM of Ba148-167F and Ba928-948R each and 1 U of Taq DNA polymerase (Sigma, India) was used. The PCR reaction condition included initial denaturation at 94 °C for 4 minutes, followed by 35 cycles of denaturation at 94 °C, annealing at 50 °C and extension at 72 °C for 1 minute each and a final extension step of 8 minutes at 72 °C. For *B. abortus*, *B. melitensis* and *B. suis* specific PCR assay, each reaction included 100 ng of test DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 250 µM of dNTPs (Fermentas, India), 0.2 µM of Ba148-167F and Ba928-948R each and 1.25 U of Taq DNA polymerase (Sigma, India). The PCR reaction condition included an initial denaturation at 94 °C for 4 minutes, followed by 35 cycles of denaturation at 94 °C for 1.15 minutes, annealing at 55.5 °C for 2 minutes and extension at 72 °C for 2 minutes and a final extension step of 5 minutes at 72 °C. Appropriate positive (standard strains of *Brucella*) and negative (double distilled water) controls were including in each PCR assay. Approximately 5 µl of each PCR product was subjected to agarose gel electrophoresis and visualized using UV transilluminator.

RESULTS

Isolation and biochemical characterization

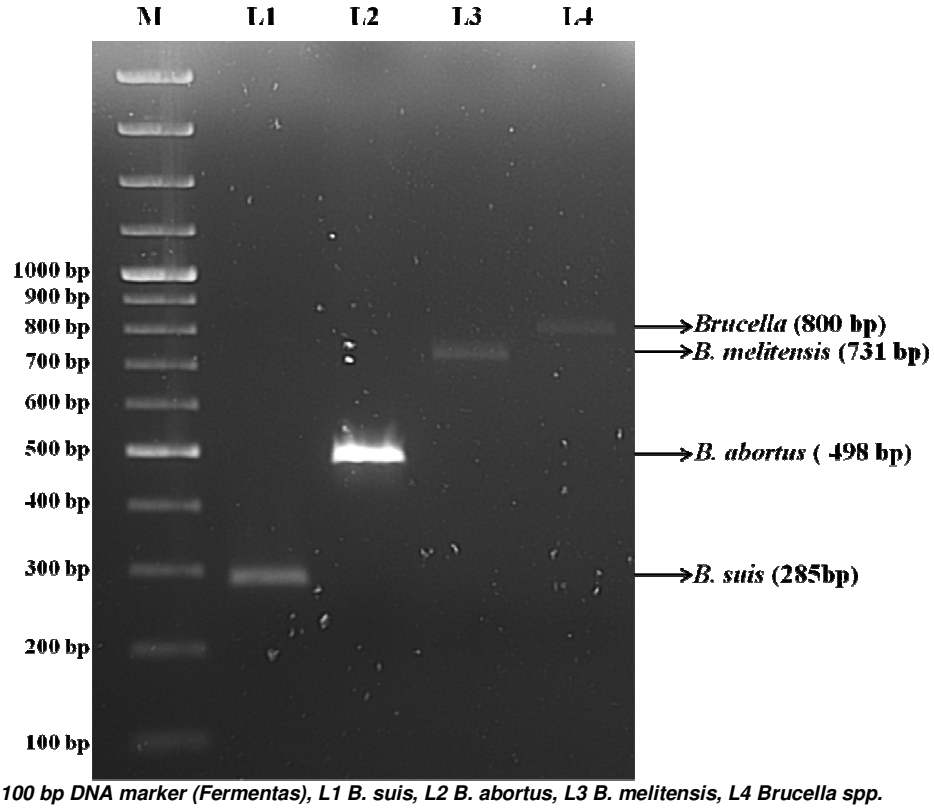
Of the total samples screened, 31 samples which comprised of three human samples and 28 animal samples resulted in the growth of typical circular, raised and convex colonies on *Brucella* selective agar, after five days of incubation at 37 °C without CO_2 environment. The widely used serological test that is Rose Bengal Test was carried out using suspected serum samples to detect brucellosis. The samples which showed agglutination were used further for isolation. The purified cultures were primarily identified microscopically by Gram's staining which revealed that the cells were small coccobacilli. A modified Ziehl–Neelsen staining showed that the cells were stained red against a blue background. Further evaluation of all presumptive isolates were carried out by a few key biochemical tests which included catalase and oxidase activity, urease and H_2S production test. All the cultures were found to be positive for catalase and oxidase test. Nineteen samples were found to be non-motile. Fifteen isolates were found to be positive for urease production on Christensen's Urea Agar within 4h of incubation. Two isolates were urease positive after overnight incubation. Fifteen isolates reduced nitrate to nitrites whereas two isolates did not. Large amount of H_2S production was observed in two samples. One of the sample produced H_2S in trace amounts and no H_2S production was observed in the rest of the samples screened. Fourteen strains isolated were able to grow in the presence of basic fuchsin.

Monoplex PCR/PCR confirmation

Preliminary conventional PCR-based screening of all the 19 presumptive isolates with the genus specific primer [12] resulted in the amplification of an 800 bp region 16S rDNA of *Brucella*. Further PCR analysis of the

19 *Brucella* isolates with *B. abortus, B. melitensis* and *B. suis* specific primer pairs revealed that six isolates were *B. abortus* (498 bp), nine isolates were *B. melitensis* (731 bp) and two isolates were *B. suis* (285 bp) (Table 3) as visualized on 1 % agarose gel upon electrophoresis (Fig 1).

Figure. 1
Agarose gel (1 %) showing amplified monoplex PCR products of isolates.



Lanes: M 100 bp DNA marker (Fermentas), L1 B. suis, L2 B. abortus, L3 B. melitensis, L4 Brucella spp.

Table 3
Correlation between biochemical and PCR results.

Strains	Rose Bengal Test	Catalase, Oxidase and Urease test	H_2S	Nitrate reduction test	Dye tolerance test Basic fuchsin 20µg/ml	PCR
B. abortus	+ve	+ve	1+ve, 5-ve	+ve	+ve	6+ve
B. melitensis	+ve	+ve	9-ve	+ve	+ve	9+ve
B. suis	+ve	+ve	2+ve	-ve	-ve	2+ve

DISCUSSION

Brucellosis, one of the major biological warfare and agroterrorism threats, is endemic in India. Its transmission and epidemiology mainly depends on the host specific *Brucella* infection. Moreover, the epidemiology is also dependant on different *Brucella* biovars. Therefore, rapid detection and identification is important for prevention of spreading, control and treatment strategies. The rapid detection can help in proper vaccination of the livestock, which can save economic loss as well as proper treatment of infected patients. However, the diagnostic tests routinely followed in the referral laboratory include immunoassays such as Coombs Test [14,15], complement fixation test, Milk Ring Test,

Serum Agglutination Test and Rose Bengal Test [16,14,15]. These tests are time consuming (1-4 weeks) which is enough for rapid spreading of the disease. Furthermore, highly skilled personnel are required to perform those above mentioned experiments. Sometimes results are associated with false positive and false negative results of considerable percentage. A procedure of conventional isolation followed by AMOS PCR [13] is promising and rapid as well. It can also differentiate the species successful. Culturing on selective media helps in isolating the target pathogen eliminating the background flora of the source. Though prepared microscopic slides of the organism for identification are easy, it needs a skilled person for observation. However, in most of the cases they appear like Gram positive coccobacilli, which lead to misdiagnosis and treatment. The biochemical method of identification after the isolation of a pathogen has been followed since a very long period. A number of biochemical tests such as dye tolerance test, oxidase test, urease, nitrate reduction test, dye tolerance and H_2S production test are performed routinely at hospital and laboratories. No proficiency is required in the preparation of media, but they are tedious and give ambiguous results. Sugar fermentation tests also give varying results depending on the intra-strain variation. There are easy and convenient methods of identification which include agglutination tests like RBT and STAT [17]. The serological tests such as Rose Bengal Test or other agglutination tests are user friendly and used widely when the sample number is large, but always chance a high degree of unreliability because of false positive results [18]. Numerous immunoassays which are either antigen or antibody based are commercially available, but they cannot differentiate the species. An alternative to these detection systems would be the molecular technique developed which can be used to screen a large number of samples after processing or directly.

Three *B. melitensis* strains isolated from human blood samples and six *B. melitensis* isolates from animals were able to grow in the presence of basic fuchsin. Four *B. abortus* strains were isolated from cattle and two *B. abortus* strains were isolated from suspected human blood samples. Two *B. suis* isolates were found from aborted materials of a pig. All biochemical tests gave positive results when compared with standard reference strains of *B. abortus*, *B. melitensis* and *B. suis*. Our laboratory evaluated the reliability of AMOS PCR on those genomic DNA samples of each isolate. A 100% agreement of the traditional isolation method with AMOS PCR-based identification of the pathogen was observed (Table 3). An attempt was made to screen the field samples directly by AMOS PCR also reciprocated the results (data not shown); inferring the potential of the technique of being the reliable replacement for the traditional method of isolation and laborious biochemical characterization and immunoassays. The method being faster and sensitive plays crucial role in the advancement of molecular diagnostics of Brucellosis.

CONFLICT OF INTEREST

Conflict of interest declared none.

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