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Isolation & characterization of *Brucella melitensis* isolated from patients suspected for human brucellosis in India

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***Background & objectives*: Brucellosis is endemic in the southern part of India. A combination of biochemical, serological and molecular methods is required for identification and biotyping of *Brucella*. The present study describes the isolation and biochemical, molecular characterization of *Brucella melitensis* from patients suspected for human brucellosis.**

***Methods*: The blood samples were collected from febrile patients suspected to have brucellosis. A total of 18 isolates were obtained from 102 blood samples subjected to culture. The characterization of these 18 isolates was done by growth on *Brucella* specific medium, biochemical reactions, CO_2 requirement, H_2S production, agglutination with A and M mono-specific antiserum, dye sensitivity to basic fuchsin and thionin. Further, molecular characterization of the isolates was done by amplification of *B. melitensis* species specific IS*711* repetitive DNA fragment and 16S (rRNA) sequence analysis. PCR-restriction fragment length polymorphism (RFLP) analysis of *omp2* locus and IS*711* gene was also done for molecular characterization.**

***Results*: All 102 suspected samples were subjected to bacteria isolation and of these, 18 isolates could be recovered on blood culture. The biochemical, PCR and PCR-RFLP and 16s rRNA sequencing revealed that all isolates were of *B. melitensis* and matched exactly with reference strain *B. melitensis* 16M.**

***Interpretation & conclusions*: The present study showed an overall isolation rate of 17.64 per cent for *B. melitensis*. There is a need to establish facilities for isolation and characterization of *Brucella* species for effective clinical management of the disease among patients as well as surveillance and control of infection in domestic animals. Further studies are needed from different geographical areas of the country with different level of endemicity to plan and execute control strategies against human brucellosis.**

Key words Brucellosis - *Brucella melitensis* - PCR - PCR-RFLP - sequencing

Brucellosis, a zoonosis caused by the genus *Brucella* is responsible for considerable human morbidity and causes enormous economic losses due to abortion and infertility in livestock[1]. Brucellosis in humans occurs as an acute, sub-acute or chronic illness which develops from direct or indirect contact with animals or by consumption of unpasteurized milk, milk products or meat of infected animals. Six classical

species of *Brucella* that differ in their host preference are *B. abortus* which preferentially infects cattle; *B. melitensis* infects sheep and goats; *B. suis* infects pigs; *B. canis* the dog; *B. ovis*, sheep and goats; and *B. neotomae* infects the desert wood rat[2]. Later on, new species isolated from marine mammals namely *B. ceti* and *B. pinnipedialis* have been described[3]. Two more species namely, *B. microti* (common vole; seals)[4-6] and *B. inopinata* from a human breast implant infection were also isolated and characterized as a new member of genus *Brucella*[7]. A novel *Brucella* strain originating from wild native rodent species in North Queensland, Australia, and a *Brucella* isolate from two cases of stillbirth in non-human primates have also been described but not yet included in the genus *Brucella*[2,8].

The low infection dose and easy transmission to humans via aerosols make *Brucella* species a potential biological warfare agent[9,10]. In the absence of reliable, reproducible and validated molecular tests for confirmation, culture isolation is still considered as gold standard for brucellosis. A combination of biochemical, serological and molecular methods is needed for reliable identification and biotyping of *Brucella*. Biochemical tests like CO_2 requirement, H_2S production, dye sensitivity, urease, oxidase, catalase tests are also used for identification of *Brucella* species[11]. In addition, biotyping method based on agglutination with mono-specific A and M antiserum is also helpful in characterization of isolates[11]. Several serological tests like serum tube agglutination test (STAT), Rose Bengal plate test (RBPT), 2-mercaptoethanol (2ME), complement fixation test (CFT) and enzyme linked immunosorbent assay (ELISA) are used for the diagnosis of human brucellosis[12]. The major disadvantages with these serological tests include the presence of background antibodies from endemic areas and no differentiation of acute cases from previous exposure. Numerous PCR based assays have been developed and evaluated ranging from identification of the genus *Brucella* (Genus-Specific PCR assay) to single unique genetic locus that is highly conserved in *Brucella* species (*e.g.* 43 kDa *omp*, *BCSP31*, IS*6501*/*711* or 16S rRNA genes)[13-15]. In the present study an attempt was made to characterize the isolates of *Brucella* obtained from patients suspected for brucellosis using biochemical, serological and molecular methods.

Material & Methods

This study was conducted in the Microbiology Division, Defence Research and Development Establishment (DRDE), Gwalior, on the isolates collected at the department of Microbiology, BLDEU's Shri B.M. Patil Medical College, Bijapur, Karnataka, India. A total of 496 febrile patients from endemic region of Bijapur were selected consecutively during the period 2010-2012. Of these, 102 high risk patients (5-55 yr) were identified based on the history given by them that included animal exposure, raw milk ingestion and clinical symptoms such as fever, headache, nausea, vomiting, backache, joint pains. Blood samples (15 ml) were collected aseptically from these 102 patients. Five ml of blood in duplicate was immediately used for blood culture and the remaining was saved for separation of serum for serological testing of brucellosis. All serological tests and isolation were performed at Shri B.M. Patil Medical College, Bijapur, Karnataka, and serum samples and culture were sent to DRDE for further biochemical and molecular confirmation of isolates.

Serological tests with patients' serum samples: The serum sample collected was subjected to conventional serological tests (STAT, 2ME and RBPT) to check for the presence of anti-*Brucella* antibodies. In STAT, patient serum was serial diluted from 1:10 to 1:1280 with 0.5 per cent phenol saline, *Brucella* antigen (Division of Biological products, IVRI, Izatnagar, India) was added and incubated at 37°C for 24 h. The maximum dilution that exhibited 50 per cent agglutination was considered as the end point of serum activity and recorded as the titre of antibodies present in the individual against *Brucella.* RBPT and 2ME tests were done as per the protocol described earlier[16-18].

Isolation of bacterial culture: The isolation of bacteria from patient's blood was performed using conventional Castaneda culture technique[19]. Five ml of blood was inoculated aseptically into 50 ml broth phase of Castaneda biphasic medium consisting of brain heart infusion (BHI) agar and broth (Hi-media, Mumbai) in duplicate. The contents were mixed well and tapped daily to allow the blood-broth mixture to flow over the agar slant. The bottles were then incubated in upright position in incubator at 37°C, one with and another without CO_2 atmosphere and examined daily for growth[19]. If no colonies were observed, the bottles were tilted and re-incubated and the cycle was repeated for a minimum of 30 days. If still no growth was observed after 30 days, the samples were considered negative and discarded.

Microbiological and biochemical identification of isolates: All isolates were grown on *Brucella* selective

medium (Hi-media, Mumbai) and studied for colony morphology, culture characteristics like smooth or rough colony by examination under inverted microscope. The cultures were subjected to Gram staining. The isolates were characterized by biochemical tests such as oxidase, catalase, urease, CO_2 requirement, H_2S production, methyl red, indole and sensitivity to thionin, basic fuchsin and safranin dyes[20]. *B. melitensis* biotype I expresses M antigen[21]; therefore, all isolates were also reacted with monospecific A and M agglutinating antiserum (Veterinary laboratories agency, Addlestone, UK) for culture identification.

The standard bacterial strains of *Brucella* species used in this study for comparison were *B. melitensis* 16M and *B. abortus* S19. Both these species were maintained in 30 per cent glycerol stocks at -80°C as well as in liquid nitrogen.

DNA isolation: DNA of all the isolates and standard strains were isolated using conventional phenol: chloroform: isoamylalcohol method[22]. Purity and the concentration of DNA were checked spectrophotometrically at an absorbance 260/280 nm and visualized under UV on 0.8 per cent agarose gel electrophoresis.

Molecular characterization

PCR amplification of 16S rRNA and sequence analysis: PCR amplification of 16S rRNA gene of *Brucella* species was done in two fragments using the primers as described earlier[23]. The PCR reactions were standardized in 25 µl reaction mixture of 200 µM each dNTPs, 1.5 mM of $MgCl_2$, 10 pmol of each primers, 1 U of Taq polymerase and 50 ng of DNA in 1x PCR buffer. All the PCR reagents used in the study were procured from MBI, Fermentas, USA. The PCR cycling conditions were standardized as initial denaturation of 95°C for 4 min followed by 30 cycles of denaturation at 95°C for 50 sec, annealing at 58°C for 50 sec and extension of 72°C for 1 min and then final extension at 72°C for 10 min. The PCR products were run on 1.2 per cent agarose gel, stained with ethidium bromide and visualized under gel documentation system (Alpha innotech, USA). The PCR products were purified using QIAGEN gel extraction kit, USA and the purified PCR products were sequenced from both ends using forward and reverse primers in separate reaction by dideoxy chain termination method[24] in ABI 3730 sequencer (Applied biosystem, USA). The alignment of DNA sequences was performed by using software DNA star 9.0 version (*www.dnastar.com*) along with the reference sequence of *B. melitensis* 16M (GenBank accession no. DQ845343). The BLAST analysis of the aligned sequences was also done to compare with other reported sequences of *Brucella* species[25]. The 16S rRNA sequence of 18 isolates were submitted to GenBank and assigned accession numbers JF939172 to JF939189.

PCR amplification of omp2a and restriction fragment length polymorphism (RFLP) analysis: The 1233 bp *omp2a* gene was amplified and PCR reaction was standardized using the primers and the protocol as described above for 16S rRNA[23]. The *Brucella omp2a* gene was PCR amplified, purified using QIAGEN gel extraction kit and digested with *Hinf* I, *Alu* I, *Ban* I and *Pst* I restriction enzymes. The restriction digestion reaction was standardized in 30 µl reaction mixture of 10 µl of purified *omp2a* PCR product, 3 µl of 10x buffer, and one unit of restriction enzyme (Fast digest, Fermentas). The reaction mixtures was incubated at 37°C for one h and digested products were electrophoresed in 2 per cent agarose gel, stained with ethidium bromide and visualized under gel UV in documentation system (Alpha innotech, USA).

PCR amplification of IS711 gene, sequence and RFLP analysis: The species level confirmation of the isolates was done by PCR amplification of the 733 bp *B. melitensis*-specific DNA fragment of IS*711* repetitive genetic element. The amplification of 733 bp *B. melitensis*-specific DNA fragment was carried out using specific primers[23]. The PCR conditions were optimized as in the case of 16S rRNA except annealing at 68°C. The amplified products were run on 1.2 per cent agarose gel, stained with ethidium bromide and visualized under gel documentation system. Sequencing of IS*711* gene was also performed as per protocol described above. The BLAST analysis of the final sequences obtained for each isolates was done and compared with other reported sequences of *Brucella* species in NCBI database (*www.ncbi.nlm.nih.gov*).

The 733 bp *B. melitensis*-specific IS*711* gene was also used for PCR-RFLP analysis for confirmation of isolates as *B. melitensis*. The IS*711* gene was PCR amplified and purified using QIAGEN gel extraction kit and digested with *Hinf* I and *Tru* I restriction enzymes (Table). The restriction digestion reaction was performed in a 30 µl reaction volume containing 10 µl purified IS*711* PCR product, 3 µl of 10x buffer, and 1U of restriction enzyme (Fast digest, Fermentas). The reaction mixture was incubated at 37°C for 1 h

and the digested products were electrophoresed in 2 per cent agarose gel stained with ethidium bromide and visualized under UV in gel documentation system (Alpha innotech, USA). The sequences of IS*711* gene of all 18 isolates were submitted to GenBank and assigned accession numbers JF939142 to JF939150, JF939153 to JF939157 and JF939161 to JF939169. Phylogenetic analysis of isolates was done based on IS*711* sequence, closest match of the deduced sequences of IS*711* isolates was determined by BLAST search and a phylogenetic tree were constructed by neighbor-joining method[26] using Mega software version 5 with 1000 bootstrap (*www.megasoftware.net*).

Results

Isolation of Brucella and biotyping: Of the 102 samples subjected to blood culture, 18 isolates were obtained with an overall isolation rate of 17.64 per cent. These 18 cultures were initially identified as *Brucella* species based on Gram staining and colony morphology. The colonies grown on *Brucella* selective medium were round, convex, with smooth margin, translucent, honey-coloured, glistening and Gram-negative coccobacilli. These isolates were positive for catalase, oxidase, and urease tests and negative for methyl red, indole, CO_2 requirement and H_2S production. The biochemical tests profile of these 18 isolates was identical and matched with that of standard strain *B. melitensis* 16M. The isolates were positive with *Brucella* anti-M monospecific serum confirming that all 18 isolates belonged to *B. melitensis* biotype 1. The isolates were also able to grow in presence of dyes namely thionin, basic fuchsin and safranin.

Serological characterization: The serum samples of 18 patients who were positive for culture isolation also showed significant titre with STAT (≥160 IU), 2ME (≥80 IU) and were positive by RBPT, confirming the presence of anti-*Brucella* antibody in these patients.

Molecular characterization: The PCR results for 16S rRNA amplification showed amplification of 839 bp product with first primer set and 699 bp with second primer set. PCR amplification of O*mp2a* gene amplified 1233 bp products, and 733 bp for *B. melitensis* specific IS*711* gene along with the reference strain of *B. melitensis* 16M. For further confirmation of isolates, PCR-RFLP of *omp2a* locus and IS*711* was performed. *Omp2a* gene was digested with *Pst* I, *Ban* I and *Hinf* I restriction enzymes revealed similar restriction profiles in all isolates and also matched with *B. melitensis* 16M (Table). The restriction profile of all isolates did not match with *B. abortus* S19 reference strain. Similarly, the restriction pattern generated for IS*711* gene when digested with *Hinf* I and *Tru* I restriction enzymes was similar for all isolates and matched with *B. melitensis* 16M reference strain (Table). Sequencing analysis of 16S rRNA and IS*711* gene was done for 18 isolates using dideoxy chain termination and the obtained sequences were aligned and matched with *B. melitensis* 16M reference. The BLAST analysis of deduced sequences of 16S rRNA revealed 100 per cent similarity with *Brucella* species, whereas IS*711* BLAST analysis revealed nearest match with *B. melitensis* species thereby confirming that all the isolates belonged to *B. melitensis* 16M (biotype1).

Phylogenetic analysis: The phylogenetic tree based on 733 bp sequence of IS*711* repetitive genetic element for all isolates was constructed. IS*711* gene sequencing and phylogenetic analysis revealed that all 18 isolates closely matched with *B. melitensis* 16M standard strain and were of clonal origin. One of the isolates from Israel also showed 100 per cent similarity to the isolates of present study in clade 1 (Figure). The isolates of *B. melitensis* from other countries are placed into different clades along with the other species of *Brucella* and showed dissimilarity with the present isolates (Figure).

Table. Restriction fragment length polymorphism analysis (*www.neb.com*)

S.No.	Restriction enzymes	Target gene	No. of restriction sites	Fragments size on agarose gel (bp)
1	*Pst* I	*Omp2a*	3	475, 375, 266, 117
2	*Ban* I	*Omp2a*	6	705, 195, 111, 84, 72, 46, 20
3	*Hinf* I	*Omp2a*	5	499, 259, 255, 193, 20, 7
4	*Alu* I	*Omp2a*	4	657, 275, 211, 78, 10
5	*Hinf* I	IS*711*	3	267, 237, 147, 82
6	*Tru* I	IS*711*	5	271, 213, 125, 87, 24, 13

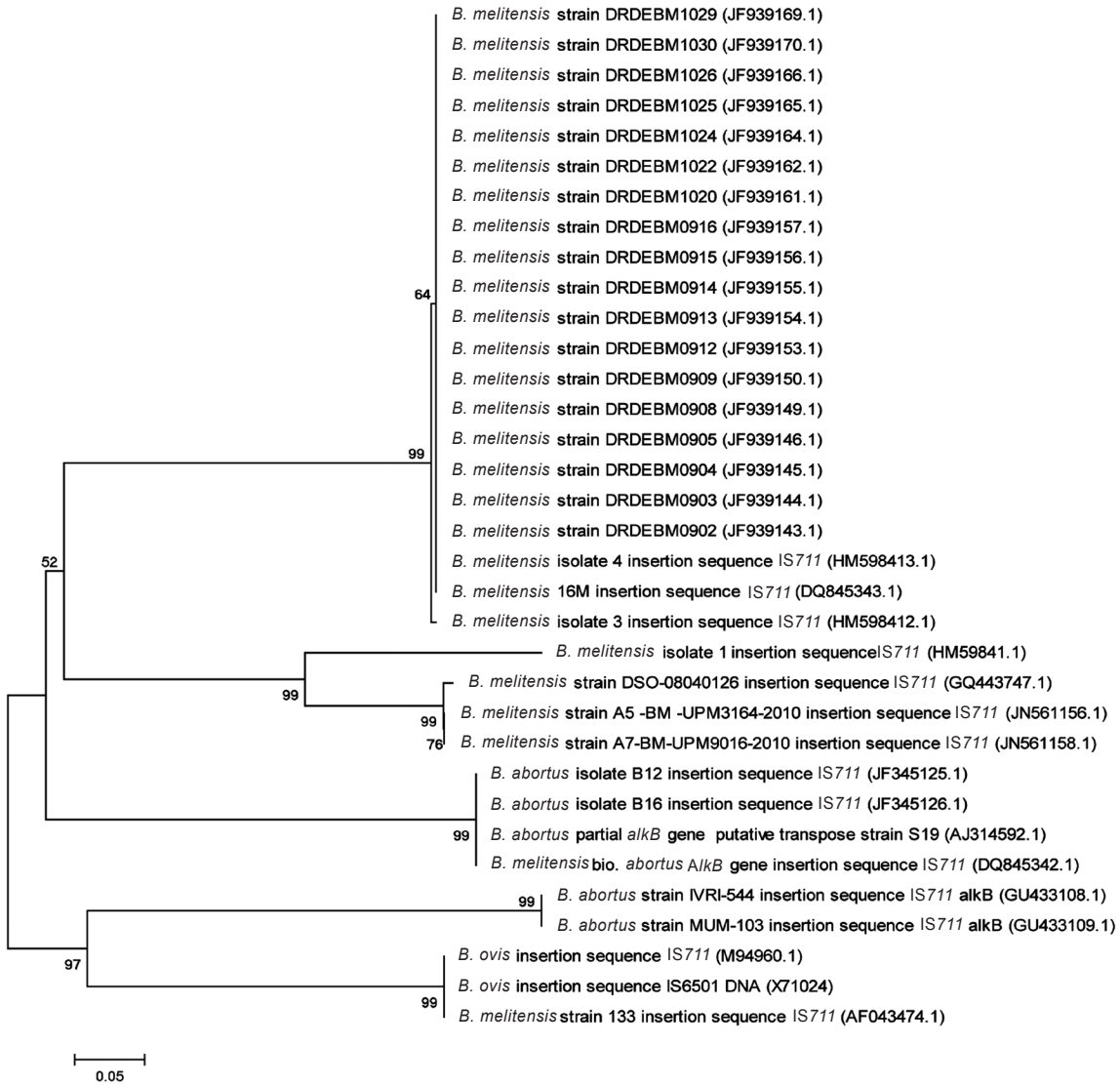

Figure. Neighbor joining tree based on IS*711* gene sequences of *B. melitensis* isolates and related reference strains constructed with 1000 bootstrap using Mega software version 5. The bootstrap values are given at the nodes.

Discussion

Among all the *Brucella* species, *B. melitensis* is mostly responsible for human brucellosis and prevalent in Southeast Asian countries including India[27]. Isolation and genotyping of the bacterial species is essential to know the exact incidence of a disease in a region and also to plan epidemiological studies, control and eradication programmes. The identification of isolates from culture is the gold standard in diagnosis of brucellosis both in humans and animals. Molecular confirmation by PCR utilizing different gene targets has become the most common approach for confirmation of human and animal isolates[28,29]. In this study, we reported the isolation of *B. melitensis* from human patients suspected for human brucellosis and characterized the isolates by conventional biochemical and serological methods. The molecular confirmation was done by PCR, PCR-RFLP and gene sequencing analysis. Sequence analysis of 16S rRNA gene is extensively used for molecular detection or taxonomic analysis of different bacterial species; 16SrRNA gene sequence among *Brucella* species is significantly conserved and it has been reported that 16S rRNA gene sequencing is a reliable tool for rapid genus level identification of *Brucella*[30]. The *omp2a* and *omp2b*

genes encoding 36 kDa outer membrane proteins (OMPs) of *Brucellae* are reported to be highly diverse among *Brucella* spp, biovars and strains[31]. The present study also showed that PCR-RFLP at *omp2a* locus was an important tool in identification and biotyping of *Brucella* species. The amplification of 733bp repetitive genetic element of IS*711* and sequencing are highly suitable and practically applicable for molecular characterization of *B. melitensis* isolates from human patients. The successful isolation protocol followed by the conventional biochemical tests and confirmation by PCR, PCR-RFLP and sequence analysis of 16S rRNA and IS*711* genes followed in this study could be a practical approach for not only disease diagnosis but also as a model for surveillance of human brucellosis in India.

In India, only a few studies[32,33] have addressed the importance of brucellosis as a public health problem. The disease may be overlooked and misdiagnosed because of the difficult diagnosis and lack of experience with the laboratory testing. It has also been estimated that the true incidence of brucellosis may be 25 times higher than the reported incidence due to misdiagnosis and underreporting[31]. Our results indicate that human brucellosis can be a common public health problem in many of the States of India. The disease is normally associated with persons who are in close contact with animals like shepherds and veterinarian and those who have the habit of consumption of raw milk of sheep and goat. More studies from different geographical regions of the country on isolation of this important bacterial species are needed. In addition, facilities and expertise required for isolation and characterization should be established in endemic areas so as to help in prompt diagnosis and treatment as well as to understand the actual incidence of human brucellosis in the country.

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