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RESEARCH ARTICLE

16S rRNA gene for ribotyping *Brucella*: A monospecific genus

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Abstract : The aim of the study was to assess the usefulness of 16S rRNA gene in identification of *Brucella* spp. and phylogenetic study. For identification purposes, a 1477-bp fragment of 16S rRNA gene of 16 isolates and 3 control reference strains was amplified and 3 isolates with *B. abortus* S19 sequenced. The obtained sequences were submitted to GenBank for species identification. Sequence analysis of a 1477-bp 16S rRNA fragment allows the identification of *Brucella* spp. However, for discrimination of closely related species sequencing of the entire 16S rRNA gene, additional sequencing of the 23S rRNA gene or sequencing of the 16S-23S rRNA intergenic spacer is essential.

Key words : *Brucella*, bovines, 16S rRNA gene PCR, sequencing

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INTRODUCTION

Brucellosis is an economically important zoonotic disease affecting various species of livestock that results from infection with gram-negative, facultative, intracellular bacteria (Gee *et al.*, 2004) classified under the genus *Brucella*, of family *Brucellaceae*, Order *Rhizobiales* Class *Alphaproteobacteria*. Within within the α-2 sub-group of the *Proteobacteriaceae*, these gram negative bacteria creates a genetically coherent taxon which are related more closely to *Ochrobactrum* and distantly to *Rhizobium* and *Agrobacterium* (Nimri and Batchoun, 2011). There are six nomen species in this genus classified based up on different principal hosts: *B. abortus* (cattle), *B. melitensis* (sheep, goats), *B. ovis* (sheep), *B. canis* (dogs), *B. suis* (swine, reindeer) and *B. neotomae* (desert wood rat) (Corbel and Brinley-Morgan, 1984; Unver, *et al.*, 2006; Tiller *et al.*, 2010). Recently, unique types of *Brucella* infecting sea mammals were described, these probably representing the several nomen species. *Brucellae* are facultative intracellular pathogens that infect a wide variety of animal species and humans. Bovine brucellosis caused primarily by *Brucella abortus* and occasionally by *B. melitensis* (Bricker and Halling, 1994).

For the identification of *Brucella*, mainly traditional methods like bacteriological and immunological detection methods are being used in all host species. These tests are time-consuming and often give false-positive results (Herman and Ridder, 1992; Navarro *et al.*, 2002). The antigenic similarity of the members of genus *Brucella* with a number of gram negative bacteria *viz.*, *Yersinia enterocolitica* O: 9, *Francisella tularensis*, *Vibrio cholerae* O:1, *E. coli* O:157, *Salmonella* O:30, makes the diagnostic value of serological tests questionable (Debeaumont *et al.*, 2005).

The members of genus *Brucella* show a great degree of phenotypic similarity and a high DNA–DNA relatedness. A battery of phenotypic and genotypic tests thus is required for species delineation and characterization of novel *Brucella* spp. (Corbel, 1997). The sequence similarity between *Brucella* spp. exceeds 90% (Foster *et al.*, 2009; Verger *et al.*, 1985). The molecular techniques developed in the last two decades are a quick and cost-effective approach towards identification of species and their differentiation to subspecies and strain level. Besides being rapid and specific these techniques are advantageous in non-requirement of handling of virulent

organisms thereby reducing the safety concerns (Seleem *et al.,* 2010).

For instance while detecting *Brucella* spp. the targets utilized for such assays include *Brucella* BCSP31 gene (Baily *et al.,* 1992) and 16S-23S rRNA operon (Romero *et al.,* 1995), While implementing brucellosis eradication program and for epidemiological trace back, several strategies have been explored for identification of *Brucella* spp., subspecies or strain level as AMOS-PCR based on IS711 (Bricker and Halling, 1994), PCR-RFLP of omp2 locus (Leal-Klevezas *et al.,* 1995). Several methods have been employed for molecular characterization of *Brucella* spp. and their discrimination into different molecular types *viz.,* PCR (Fekete *et al.,* 1992), PCR-RFLP analysis (Vizcaino *et al.,* 1996; Cloeckaert *et al.,* 2000), DNA-RFLP or SDS-PAGE (Monreal *et al.,* 2003), multilocus enzyme electrophoresis – MLEE (Benjamin *et al.,* 2001). Considering the above facts the present investigation was planned with a view to undertake molecular characterization of *Brucella* spp. recovered from bovines and to genetically characterize *Brucella* isolates by 16S rRNA gene PCR and sequencing.

RESEARCH METHODOLOGY

In the present investigation, a total of 3 reference strains and all 16 isolates recovered from clinical cases of abortions in bovines, identified as *Brucella* spp. on the basis of morphology, staining reactions in grams and MZN staining, cultural characters and biochemical properties were selected for PCR and PCR products from 3 *Brucella* isolates and *B. abortus* S19 were subjected to nucleotide sequencing.

DNA purification, DNA amplification and DNA sequencing:

For isolation of DNA from bacterial cultures the protocol described by Romero *et al*. (1995) with some modifications. DNA extracts were stored frozen (-20°C) until PCR was performed. 16S rDNA analysis was performed from DNA extraction as described below.

PCR samples were prepared in a total volume of 25 µl containing 300 ng gDNA, 10 pmol of each primer, 25mM of $MgCl_2$, 10 mM of dNTP mix, 2.5 µl of 10X PCR buffer without $MgCl_2$, 1 unit *Taq* DNA Polymerase and 17.75 µl of nuclease free water to make 25 µl. The oligonucleotides used for amplification correspond to the 5' end 16S F (5'-AGA GTT TGA TCM TGG CTC AG-3') and the 3' end 16S R (5'-AAG GAG GTG WTC CAR CC-3') [22]. PCR conditions were 95°C for 3 min; 30 cycles each of 94°C for 1 min, 58°C for 90 s, and 72°C for 90 s; and a final step at 72°C for 6 min. Prior to sequencing, the amplified products were analysed on 1·5% agarose gels. The amplified products purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany).

Sequencing was carried out using ABI 3130 (4 capillary) electrophoresis instrument with an automated DNA sequencer ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).

Sequence data analysis :

The obtained forward and reverse sequences of each sample were assembled using Chromas lite software, version 2.1. The software was used to assemble each forward and reverse sequence into a consensus sequence and then compared with the different sequences.

Nucleotide sequence accession numbers :

A total of 3 *Brucella* 16S rRNA gene sequences have been deposited in GenBank under accession no. KC790986 for *Brucella abortus* S19; KC790984 for *Brucella* spp. AHM900; KJ873040 for *Brucella* spp. KOL79; KJ914912 *B.abortus* 544 and KC790985 for *Brucella* spp. PUN101.

RESULTS AND DISCUSSION

On species level 16S rRNA gene (1477) amplification by PCR allowed correct identification *Brucella* spp. (Fig. 1) and PCR products were subjected to nucleotide sequencing.

16S rRNA gene sequencing :

The results of PCR sequencing of the PCR products obtained from 16S rRNA PCR revealed that the sizes of amplified fragments generated from all the strains matched with previously published sequences of *Brucella* 16S rRNA gene.

Fig. 1 : 16SrRNA gene profiles of PCR amplified products on 1 per cent agarose gel electrophoresis displaying: Lane M, molecular size markers; Lane 1, *Brucella abortus* S19; lane 2, *B. Melitensis* Rev 1, lane 3, AHM900; Lane 4, PUN101.

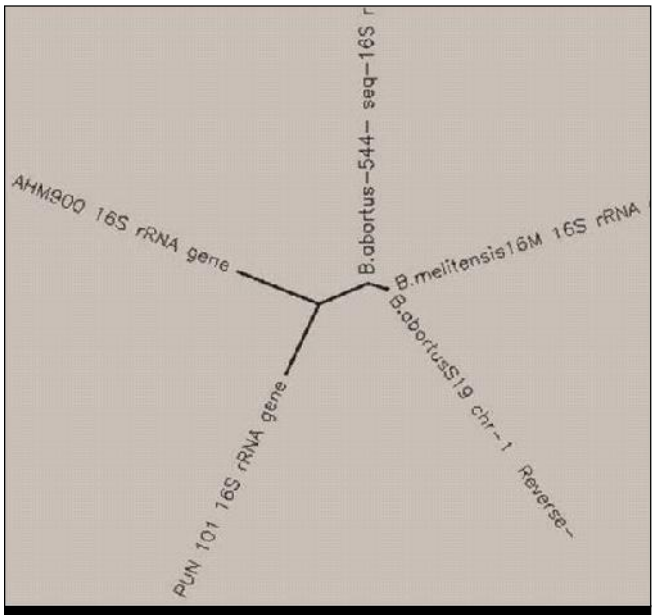

Fig. 2 : Unrooted Phylogenetic reconstruction of 16S rRNA gene sequences *Brucella* species in the study

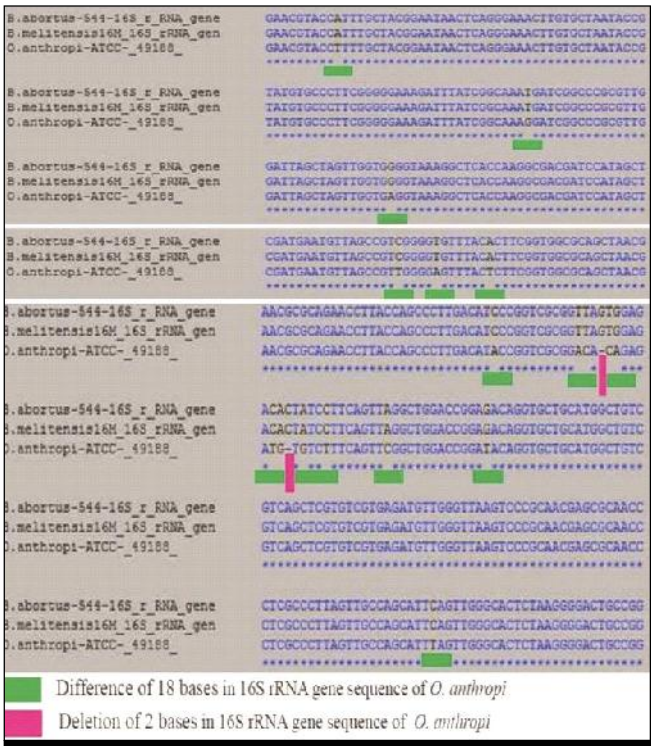

Fig. 3 : Multiple sequence alignment of *O. anthropi* and *Brucella* spp.

The comparison of the *Brucella* 16S rRNA gene sequences with the sequences of bacteria phylogenetically close to *Brucella*, that of *O. anthropi* 58 strain ATCC 49188 with 98% similarity (Fig. 3) with 18 bp difference and two deletions in the sequence of *O. anthropi* (Fig. 3).

Among the sequences of 16S rRNA gene in *Brucella* isolates, *Brucella* spp. PUN101 reveled 98% sequence similarity with 16S rRNA gene sequences of *B. abortus* 544 and 97% similarity with 16S rRNA gene sequences of *B. abortus* S19 and *B. melitensis* 16M. There was one insertion and 4 deletions in the sequence of this isolate as compared to consensus sequence. The *Brucella* spp. AHM900 isolate shared 97% similarity with all the *Brucella* spp included in the study with a difference of 23 bp, 4 insertions and 3 deletions when compared to 16S rRNA gene consensus sequence. 2 nucleotide difference, 3 insertion in *Brucella* spp. KOL79 and showed 96% similarity with all the *Brucella* spp. included in the study while there was 95% similarity with other of *Brucella* spp. isolates.

Analyses of 16S rRNA gene have been extensively used for molecular detection and taxonomic analyses of many different bacterial species (Gee *et al.,* 2004; Weisburg *et al.,* 1994; Moreno *et al.,* 2002). The technique has been widely exploited for demonstration of phylogenetic relationships between bacteria at intra- and inter-generic levels. The recent reports suggests sequencing is a reliable tool for rapid genus level identification of *Brucella* spp as sequences 16S rRNA gene *Brucella* spp. and strains are significantly conserved (Amann *et al.,* 1990; Amann *et al.,* 1995).

Considering the potential of this region in characterization of *Brucella* isolates, we determined the nucleotide sequences of 16S rRNA genes of reference *Brucella* strains and field isolates. Initially, the amplification of 16S rRNA gene was done using published primer sequences (Weisburg *et al.,* 1991). All the reference strains and field isolates generated amplification products of 1477 bp these are findings are similar with the observations of Weisburg *et al*. (1991).

A BLAST query on GenBank indicates that the 16S rRNA genes sequences of *B. abortus* S19 (GenBank accession number KC790986), *B. abortus* 544 and *B. melitensis* 16M were a 100% match. The nucleotide sequences in the present investigation revealed that the sequences were a 100% match to the 16S rRNA gene consensus sequences of respective species. These observations are in agreement with the findings of Gee *et al.* (2004), who determined the sequences of 16S rRNA gene in *B. abortus* U.S. strain 19, *B. melitensis* strain ATCC 23456, *B. neotomae* strain ATCC 23459, and *B. canis* strain ATCC 23365 and found that all the four sequences were a 100% match to the *Brucella* consensus sequence. It is well documented that the members of genus *Brucella* including its different species and biovars share a high nucleotide sequence similarity and the six species are so closely related that a monospecies genus has been suggested (Verger *et al.,* 1985). This, hypothesis has been confirmed by analysis of 16S rRNA gene sequence (Moreno *et al.,* 1990). This fact has been used to propose that the genus *Brucella* contains only a single species *B. melitensis* and the remaining

species considered its biovars (Halling, 1997).

Analysis of 16S rRNA sequences of field isolates revealed that there was a great deal of similarity between the sequences of the 3 *Brucella* isolates investigated. The observations pertaining to similarity of 16s rRNA sequences observed during present investigation are in consistent with the previously published data describing the conserved nature of the 16S rRNA sequence of *Brucella* spp. and strains (Gee *et al.,* 2004; Unver *et al.,* 2006).

The 16S rRNA gene sequences of *B. abortus* 544 and *B. abortus* S19 showed 98% similarity with that of *Ochrobactrum anthropi* (ATCC 49188). The results observed in the present study are in agreement with those of Gee *et al*. (2004) who recorded 98.8% similarity between 16S rRNA gene sequences of *Brucella* and *O. anthropi* (5D). They observed difference in 16 bp, 1 insertion and 1 deletion in the sequence of *O. anthropi*. This variation could be due to the difference in the strains used for comparison.

The *O. anthropi* is known to be phylogenetically close to genus *Brucella* and several studies have shown a close relationship between these organisms (Fig. 2). Herman and Ridder (1992) who developed PCR assay targeting 16S rRNA gene for identification of *Brucella* spp. found cross-reactivity of *O. anthropi* with the primers designed for amplification of 16S rRNA gene of *Brucella*. They suggested that *O. anthropi* and *Brucella* spp. were most closely related and formed one rRNA branch together with the genera *Phyllobacterium*. Similar observation indicating close phylogenetic relationship between *O. anthropi* and *Brucella* was recorded by Romero *et al.* (1995). They designed the primers based up on the 16S rRNA sequence of *B. abortus* and evaluated them on the representative strains *Brucella* spp. The primers did not show any amplification in 10 phylogenetically related strains, 5 Gram negative bacteria showing serological cross-reactions with *Brucella*, however, *Ochrobactrum anthropi* biotype D yielded amplification, suggesting a closer relationship between these organisms. The close relationship of *O. anthropi* with *Brucella* is not only confined to the 16s rRNA gene but is also evident with the other regions of the DNA e.g. 31 kDa *Brucella* protein gene, genes encoding heat shock proteins *viz.*, DnaJ, DnaK, HtrA and GroEL (Da Costa *et al.*, 1996).

Because diagnostic methods targeting rRNA sequences have been shown to be very reliable for different kinds of samples, we apply 16S rRNA of *Brucella* spp. in the PCR. We have sequenced the 16S rRNA genes of *Brucella* spp. represent the species commonly encountered in bovine infections.

In the present study the *Brucella* 16S rRNA gene sequences revealed 98% similarity with *O. anthropi* with 18 bp difference and 2 deletions in the *O. anthropi* sequence which could be of potential value in differentiating 16S rRNA gene of the *Brucella* from that of *O. anthropi*. This observation could be of potential value in differentiating 16S rRNA sequence of the *Brucella* from that of *O. anthropi*. Thus in conclusion, 16S rRNA gene sequence analysis is a valuable tool for rapid identification of phylogenitically related bacteria.

LITERATURE CITED

Amann, R.I., Krumholz, L. and Stahl, D.A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.,* **172** (2) : 762–770.

Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.,* **59** (1) : 143–169.

Baily, G.C., Kraahn, J.B., Drasar, B.S. and Stokeer, N.G. (1992). Detection of *Brucella* melitensis and *Brucella* abortus by DNA amplification. *J. Trop. Med. Hyg.,* **95** (4) : 271-275.

Benjamin G., Merino, A.L., Rogel, M.A. and Romero, E.M. (2001). Limited genetic diversity of *Brucella* spp. *J. Clinical Microbiol.,* **39** (1) : 235–240.

Bricker, B.J. and Halling, S.M. (1994). Differentiation of *Brucella abortus* bv. 1, 2 and 4, *Brucella melitensis*, *Brucella ovis* and *Brucella suis* bv. 1 by PCR. *J. Clin. Microbiol*., **32** (11) : 2660-2666.

Cloeckaert, A., Grayon, M. and Gripinet, M.O. (2000). An IS711 Element Downstream of the bp26 Gene Is a Specific Marker of *Brucella* spp. Isolated from Marine Mammals. *Clin. Diagnost. Lab. Immunol.,* **7** (5) : 835-839.

Corbel, M.J. (1997). Brucellosis: an overview. *Emerg. Infect. Dis*., **3** (2) : 213–221.

Corbel, M.J. and Brinley-Morgan, W.J. (1984). Genus *Brucella* Meyer and Shaw (1920). 173AL. In: Krieg, N. R.; Holt, J. G. (Eds.), *Bergey's Mannual of Systematic Bacteriology*.

Da Costa, M., Guillou, J.P., Garin-Bastuji, B., Thiebaud, M. and Dubray, G. (1996). Specificity of six gene sequences for the detection of the genus *Brucella* by DNA amplification. *J. Appl. Bacteriol*., **81** (3) : 267-275.

Debeaumont, C., Falconnet, P.A. and Maurin, M. (2005). Real-time PCR for detection of *Brucella* spp. DNA in human serum samples. *Eur. J. Clin. Microbiol. Infect. Dis.,* **24** (12) : 842–845.

Fekete, A., Bantle, J.A. and Halling, S.M. (1992). Detection of *Brucella* by polymerase chain reaction in bovine fetal and maternal tissues. *J. Vet. Diagn. Invest*., **4** (1) : 79-83.

Foster, J.T., Beckstrom-Sternberg, S.M., Pearson, T., Beckstrom-Sternberg, J.S., Chain, P.S., Roberto, F.F., Hnath, J., Brettin, T. and Keim, P. (2009). Whole-genome-based phylogeny and divergence of the genus. *Brucella. J. Bacteriol*., **191** (8) : 2864–2870.

Gee, J.E., De, B.K., Levett, P.N., Whitney, A.M., Novak, R.T. and Popovic, T. (2004). Use of 16S rRNA Gene Sequencing for Rapid Confirmatory Identification of *Brucella* Isolates. *J. Clin. Microbiol*., **42** (8) : 3649–3654.

Halling, S.M. (1997). The organism: Genetics. The 50th Anniversary Meeting of *Brucella* Research Conference. 19-25.

Herman, L. and Ridder, H.D. (1992). Identification of *Brucella* spp. by using the polymerase chain reaction. *App. Environ. Microbiol.,* **58** (6) : 2099-2101.

Leal-Klevezas, D.S., Martinez-Vazquez I.O., Lopez-Merino A. and Martinez-Soriano, J.P. (1995). Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. *J. Clin. Microbiol.,* **33** (12) : 3087-3090.

Monreal D., Grillo, M.J., Gonza´lez, D., Marín, C.M., De Miguel, M.J., Lopez-Goni, I., Blasco, J.M., Cloeckaert, A. and Moriyon, I. (2003). Characterization of *Brucella* abortus O-Polysaccharide and Core Lipopolysaccharide Mutants and Demonstration that a Complete Core Is Required for Rough Vaccines To Be Efficient against *Brucella* abortus and *Brucella* ovis in the Mouse Model. *Infect. Immun.,* **71** (6) : 3261–3271.

Moreno, E., Cloeckaert, A. and Moriyon, I. (2002). *Brucella* evolution and taxonomy. *Vet. Microbiol.,* **90** (1-4) : 209-227.

Moreno, E., Stackebrandt, E., Dorsch, M., Wolters, J., Busch, M. and Mayer, H. (1990). *Brucella* abortus 16S rRNA and lipid A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class Proteobacteria. *J. Bacteriol.,* **172** (7) : 3569–3576.

Navarro, E., Escribano, J., Fernandez, J.A. and Solera, J. (2002). Comparison of three different PCR methods for detection of *Brucella* spp. in human blood samples. *FEMS Immun. Med. Microbiol*., **34** (2) : 147-151.

Nimri, L. and Batchoun, R. (2011). Genetic Homogeneity of Clinical Isolates of *Brucella Melitensis*: A Single Ribotype. *Webmed Central Microbiol*., **2**: WMC001508.

Romero, C., Gamezo, C., Pardo, M. and Lopez-Goni, I. (1995). Specific Detection of *Brucella* DNA by PCR. *J. Clin. Microbiol*., **33** (6) : 615-617.

Seleem, M.N., Boyle, S.M. and Sriranganathan, N. (2010). Brucellosis: A re-emerging zoonosis. *Vet. Microbiol.,* **140** (3-4) : 392–398.

Tiller, R.V., Gee, J.E., Lonsway, D.R., Gribble, S., Bell, S.C., Jennison, A.V., Bates, J., Coulter, C., Hoffmaster, A.R. and De, B.K. (2010). Identification of an unusual *Brucella* strain (BO2) from a lung biopsy in a 52 year-old patient with chronic destructive pneumonia. *BMC Microbiol.,* **10**: 23.

Unver, A., Erdogan, H.M., Atabay, H.I., Sahin, M. and Celebi, O. (2006). Isolation, identification, and molecular characterization of *Brucella* melitensis from aborted sheep fetuses in Kars, Turkey. *Revue Méd. Vét.*, **157** (1) : 42-46.

Verger, J.M., Grimont, F., Grimont, P.D.A. and Grayon, M. (1985). *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *Internat. J. Syst. Bacteriol.*, **35** (3) : 292-295.

Vizcaino, N., Cloeckaert, A., Zygmut, M.S. and Dubray, G. (1996). Cloning, Nucleotide Sequence, and Expression of the *Brucella melitensis omp31* Gene Coding for an Immunogenic Major Outer Membrane Protein. *Infect. Immun*., **64** (9) : 3744–3751.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991). 16S Ribosomal DNA Amplification for Phylogenetic Study. *J. Bacteriol.,* **173** (2) : 697-703.