Isolation, identification, and molecular characterization of *Brucella melitensis* from aborted sheep fetuses in Kars, Turkey

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SUMMARY

Brucellosis is an enzootic and economically important zoonotic infection in Turkey and the causative agent of ovine brucellosis in this region has not been genetically characterized in detail. This study aimed to isolate and identify *Brucella melitensis* from aborted sheep foetuses in Kars, Turkey and to characterize one of the *B. melitensis* isolate by 16S rRNA gene sequencing and Polymerase Chain Reaction (PCR)-Restriction Fragment Length Polymorphism (RFLP) of *omp*2a gene. *B. melitensis* was isolated and identified from 14 out of 37 aborted sheep foetuses and the 16S rRNA gene sequence of one of the *B. melitensis* isolate was identical to that of type strain of *B. melitensis* (16M strain). PCR-RFLP analyses of *omp*2a revealed that the current isolate was genetically close to the isolates from different Mediterranean countries, particularly those from France, Spain and Israel, however, different from vaccine (Rev.1) and type (16M) strains. The genetic characterization of *B. melitensis* in this study can establish a base for epidemiological studies, management of outbreaks and control and eradication programs of ovine brucellosis in this region. This is the first report of the 16S rRNA gene sequence of *Brucella* in Turkey.

Keywords : *Brucella melitensis* - sheep - Turkey - PCR-RFLP.

RÉSUMÉ

Isolement, identification, et caractérisation moléculaire de *Brucella melitensis* de foetus issus d'avortement de brebis à Kars en Turquie. Par A. UNVER, H. M. ERDOGAN, H. I. ATABAY, M. SAHIN et O. CELEBI.

La brucellose est une infection bactérienne zoonotique importante sur le plan économique en Turquie et, à ce jour, la bactérie responsable de la brucellose chez les ovins, *Brucella melitensis,* n'a pas été caractérisée génétiquement. La présente étude a visé à isoler et identifier la bactérie à partir de fœtus provenant de brebis ayant avorté dans la Province de Kars en Turquie. Un isolat représentatif a été caractérisé (i) par séquençage d'une région du gène de l'ARN 16S et (ii) par la technique de PCR-RFLP (PCR et polymorphisme de longueur des fragments de restriction) pour le géne *omp*2a. *B. melitensis* a été isolée et identifiée chez 14 sur 37 fœtus soumis à l'analyse. La séquence de la région amplifiée du gène de l'ARN 16S de l'isolat représentatif était identique à celle de la souche de *B. melitensis* de type 16M. L'analyse par PCR-RFLP du gène *omp*2a a montré que l'isolat était génétiquement proche des isolats des différents pays méditerranéens, en particulier ceux de France, d'Espagne et d'Israel, mais différent du vaccin Rev.1 et de la type 16M. Cette première caractérisation génétique des souches de *B. melitensis* en Turquie permet donc d'établir une base pour les études épidémiologiques sur la brucellose ovine, sur la gestion des mouvements d'animaux et la mise en place de programmes d'éradication dans ce pays.

Mots-clés : *Brucella melitensis* - ovin - Turquie - PCR-RFLP.

Introduction

Brucellosis is an emerging zoonosis and an economically important infectious disease of livestock with worldwide distribution [14]. The disease is manifested by abortion and infertility and caused by *Brucella* spp. which are Gram-negative, facultative intracellular bacteria. Six species in the genus of *Brucella* are currently recognized on the basis of the phenotypic characteristics, antigenic properties and host distribution [13]. *Brucella melitensis* is the main etiological agent of brucellosis in small ruminants and it can be responsible for bovine brucellosis in some areas [14, 23]. It is the most important and pathogenic *Brucella* sp. for humans causing clinically apparent human brucellosis. The disease is usually contracted by direct contact of the contaminated fetal tissues or consumption of contaminated unpasteurized milk or milk products. Brucellosis is geographically distributed in Mediterranean region, Middle East, parts of Africa, Western Asia and Latin America [8, 14, 20, 23]. *B. melitensis* is enzootic in different regions of Turkey and is the main cause of abortion in sheep [1, 2, 8, 9, 15, 20, 22-24].

The reports on brucellosis in Turkey are mainly diagnosed based on the isolation of the organism followed by identification with set of phenotypic tests, detection of *Brucella* DNA by Polymerase Chain Reaction (PCR) and serological tests [1, 2, 8, 9, 15, 20, 22-24]. However, *B. melitensis* strains isolated or detected in Turkey have not been genetically characterized in detail. The characterization of the causative agent of an infectious disease has a pivotal role in epidemiological studies, management of the outbreaks and implementing control and preventive measures. PCR-Restriction Fragment Length Polymorphism (RFLP) of Outer Membrane Protein (OMP) genes of *Brucella* has been widely and successfully used for genotyping of several *Brucella* isolates from geographically divergent regions including recent isolates from marine mammals [7, 10-12, 16].

Since the genomes of *Brucella* spp. are highly homogeneous (>95 % homology at the DNA-DNA pairing) among each other [19, 21, 27, 28] and 16S rRNA genes have very little or no polymorphism among the isolates and biovars,

several researchers proposed that all *Brucella* spp. should be placed in one specie name [17, 19, 27, 28], however, this has not generally been accepted and systematized yet. Therefore, the old nomenclature was used in this article.

The current study was conducted to isolate and identify *B. melitensis* from aborted sheep foetuses in Kars region, Turkey and to genetically characterize a *B. melitensis* isolate by 16S rRNA gene sequencing and PCR-RFLP of *omp*2a gene.

Materials and methods

FOETUS SAMPLES

Aborted sheep foetuses were collected during the lambing seasons from 2002 to 2004. Thirty seven aborted foetus samples were investigated for the presence of *Brucella* sp. by cultural methods and PCR. The samples were collected from different flocks in Kars center, Yalcinlar, Oguzlu, and Has Ciftlik in Kars province, Turkey. Aborted sheep foetuses were collected during the visits to the farms after the report of an ovine abortion cases or the samples were directly submitted to the laboratory of the Microbiology Department, Faculty of Veterinary Medicine, Kafkas Univesity, Kars.

BACTERIOLOGY

Samples from lung and stomach contents of the aborted foetuses were inoculated onto Brucella agar plate (Oxoid, Hampshire, England). After incubation of plates at 37°C for 2-3 days, the observed colonies were investigated and identified as *Brucella* spp. by morphologic, cultural and biochemical properties such as oxidase, H_2S production, urease and CO_2 requirement.

DNA ISOLATION

After 3 days of incubation, 4-5 colonies identified as *Brucella* spp. were taken with a loop and suspended in 1 ml of phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4 [pH 7.2]). After centrifugation, the bacterial pellet was resuspended with 100 µl of PBS and the cells were lysed with the addition of 400 µl of NETS buffer (10mM NaCl, 1 mM EDTA, 10 mM Tris-Hcl [pH7.6], 0.5%SDS). After addition of 50 µg of Proteinase K (MBI Fermantes, St. Leon-Rot, Germany), the mixture was incubated at 65°C for 20 min. An equal volume of phenol:choloroform:isoamyl alcohol (25:24:1) was added and vortexed. The aqueous layer was carefully removed to a new tube and 0.1 volume of the 3M sodium acetate and 2-3 volumes of cold absolute ethanol were added. The mixture was incubated at -20°C for at least 2 hours. After centrifugation at 13,000 rpm for 10 min, supernatant was discarded and the pellet was washed with 70 % of ethanol. After final centrifugation, the pellet was air dried and resuspended with 40 µl of nuclease-free distilled deionized sterile water.

IDENTIFICATION BY PCR

The PCR was used to confirm identification of isolated *Brucella* spp. from aborted fetal samples as described by Bricker and Halling [4] with a modification by utilizing primers, *B. melitensis* and IS711, specific to IS711 gene of *B. melitensis* (Table I). Briefly, the amplification was carried out in a 50-µl reaction mixture including PCR buffer, 1.5 mM $MgCl_2$, 20 pmol of primer pairs, 0.2 mM each of dNTP mixture, 1.5 U of *Taq* DNA polymerase and 5 µl of DNA, with 3 min of denaturation at 95(C followed by 35 cycles each consisting of 1 min of denaturation at 95°C, 1.5 min of annealing at 56°C and 1.5 min of extension at 72°C. The final extension was allowed to continue for 7 min. DNA isolated from Rev.1 strain of *B. melitensis* and PCR reaction mixtures without addition of template were used as positive and negative controls, respectively. PCR products were electrophoresed in 1.5% agarose gels and visualized with ethidium bromide (EtBr).

SEQUENCE ANALYSIS OF THE 16S rRNA GENE

One of the *B. melitensis* isolate was used to amplify and sequence nearly entire 16S rRNA genes for further genetic analyses. 16S rRNA genes from this sample was amplified with primer pairs 16SrRNF1 - 16SrRNR1 and 16SrRNF2 - 16SrRNR2 (Table I), designed to amplify 839- and 699 bp-fragments, respectively, overlap each other. PCR was carried

Primer names	Sequences (5'- 3')	Target gene	Purpose
B. melitensis	AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA	IS711	Identification
IS711	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT	IS711	Identification
16SrRNAF1	AGA-GTT-TGA-TCC-TGG-CTC-AG	16S rRNA	Sequencing
16SrRNAR1	AAT-CTT-GCG-ACC-GTA-GTC-CC	16S rRNA	Sequencing
16SrRNAF2	ACG-ATG-AGT-TGC-TAG-CCG-TC	16S rRNA	Sequencing
16SrRNAR2	ACG-GCT-ACC-TTG-TTA-CGA-CTT	16S rRNA	Sequencing
2aA	GGC-TAT-TCA-AAA-TTC-TGG-CG	*omp*2a	PCR-RFLP
2aB	ATC-GAT-TCT-CAC-GCT-TTC-GT	*omp*2a	PCR-RFLP

TABLE I. — PCR primers used in this study.

out in a 50-µl reaction mixture including PCR buffer, 1.5 mM $MgCl_2$, 20 pmol of primer pairs, 0.2 mM each of dNTP mixture, 1.5 U of *Taq* DNA polymerase and 5 µl of DNA template, with 4 min of denaturation at 94°C followed by 35 cycles each consisting of 1 min of denaturation at 94°C, 1 min of annealing at 58°C and 1 min of extension at 72°C. The final extension was allowed to continue for 7 min. PCR products were electrophoresed in 1.5% agarose gels and visualized with EtBr. Two microliters of amplicons observed at the expected sizes of 839 and 699 bp on agarose gel electrophoresis were cloned into the PCRII vector of a TA cloning kit (Invitrogen Co., San Diegio, CA, USA) as described by the manufacturer. Recombinant plasmids were purified using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) and the inserts were sequenced by a dideoxy chain termination method with the universal synthetic primers M13 and T7 in the Plant- Microbe Genomics Facility at the Ohio State University, Columbus, OH, USA. The sequences of two clones from the sample were determined on both DNA strands. The alignment of DNA sequences and the comparisons with other sequences were performed by using the DNASTAR program (DNASTAR Inc., Madison, WI, USA).

PCR-RFLP ANALYSES OF A *BRUCELLA* ISOLATE

For genotyping of one of the *B. melitensis* isolate, *omp*2a gene was amplified by PCR. The primers (Table I) and amplification conditions were identical with that of described by Cloeckaert et al. [11]. Five µl of isolated DNA was used as template for *omp*2a amplification and 10 µl of PCR product was digested with 1 U of *Pst*I restriction enzyme (Invitrogen Co., San Diegio, CA, USA) which can differentiate the 2 different restriction profiles derived from *B. melitensis* strains demonstrated by Cloeckaert et al. [11]. Restriction digestion was performed with buffer and incubation temperature as described by the manufacturer. The digested PCR product was electrophoresed in 1.5% agarose gels and visualized with EtBr.

NUCLEOTIDE SEQUENCE ACCESSION NUMBER

The 16S rRNA gene sequence of *B. melitensis* reported here has been assigned the GenBank accession number AY922323.

Results

B. melitensis was isolated from 14 out of 37 (38%) aborted sheep fetus samples examined in this study. The organisms were isolated from both lung and stomach content in all *B. melitensis*-positive samples. The identification was performed by biochemical tests and PCR utilizing primers specific to IS711 gene of *B. melitensis. B. melitensis*-specific DNA fragments with 731 bp were amplified from all isolates and no DNA was observed in negative control samples (data not shown). One of the *B. melitensis* isolate was used for genetic characterization. For this purpose, nearly complete (1,407 bp) 16S rRNA gene of this isolate was sequenced. The obtained 16S rRNA gene was aligned and compared with other GenBank-accessible 16S rRNA gene sequences of *B. melitensis* and other *Brucellae* spp. The 16S rRNA sequence obtained in the current study was identical to that of the recent sequence of type strain of *B. melitensis* (ATCC 23456,

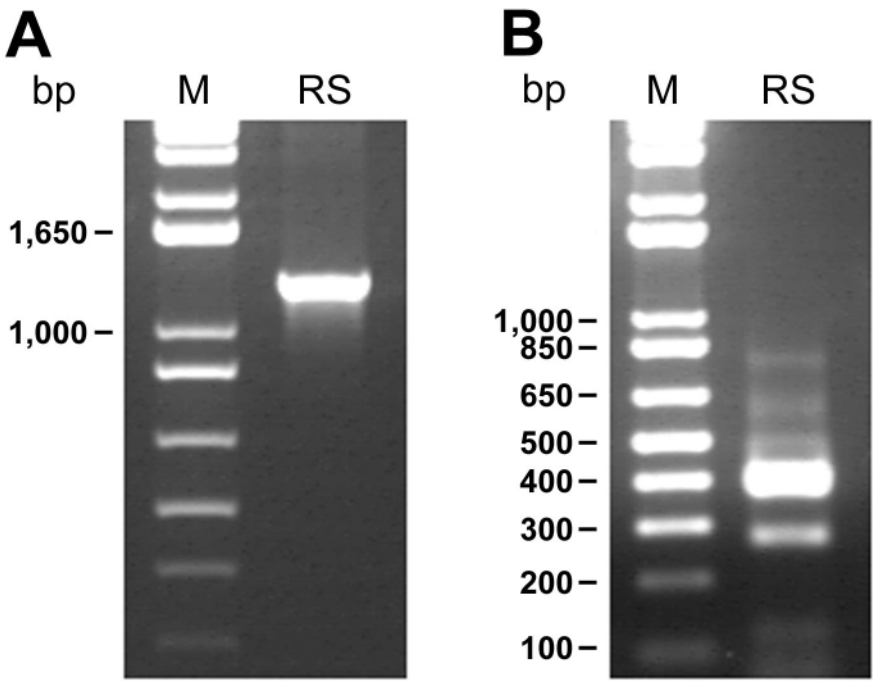

FIGURE 1. — A ; PCR product amplified from *omp*2a gene of one of the *B. melitensis* isolate. B ; Restriction pattern of PCR-amplified *omp*2a gene by *Pst*I. Amplified products were resolved on agarose gels containing EtBr. Lanes M, molecular size markers (1 Kb Plus DNA Ladder, Invitrogen, Carlsbad,CA, USA) ; Lanes RS, sample from one of the *B. melitensis* isolate. Sizes of markers are indicated on the left.

GenBank access no : AY594215) and two bases different than that of previously sequenced same strain (GenBank access no: L26166). Several other GenBank-deposited 16S rRNA sequences of *B. melitensis* biovar/strain *abortus, suis, ovis* and *canis* were also found to be identical to that of obtained in the current study and this current sequence corresponds to the *Brucella* 16S rRNA consensus gene sequences described by Gee et al [17].

For PCR-RFLP analyses, *omp*2a gene was amplified from one of the isolate and nearly 1,233 bp amplified PCR product was observed on agarose gel (Fig.1A).

*Pst*I was chosen for digestion of amplified *omp*2a since it has the only restriction site polymorphic between two groups of *B. melitensis* strains [11]. The restriction digestion of the amplicon by *Pst*I generated 399, 376, 267, 118 and 77 bp DNA fragments (Fig. 1B). Since 1.5% agarose gel was not able to resolve 399- and 376-bp fragments clearly, they looked as forming a broad band together in this figure. This pattern was similar to the pattern P2 described by Cloeckaert et al. [11]. Among several isolates/strains previously analyzed by this method, the pattern P2 obtained in the current study is similar to that of obtained from isolates from several Mediterranean countries, France, Spain and Israel [11].

Discussion

Turkey is an endemic region for brucellosis studied extensively by serological methods and culture isolation of the organism. Recently, detection of *Brucella* DNA from aborted sheep fetuses by PCR was reported [1, 2, 8, 9, 15, 20, 22-24]. However, the reports on the genetic characterization of Brucellae from Turkey are very limited and 16S rRNA gene sequence, the most commonly used target gene for taxonomy in bacteria, has not been analyzed. Since isolation and genotyping of the infectious agents are imperative for control and eradication programs and important for epidemiological studies, we isolated *B. melitensis* from aborted sheep fetuses in Kars district and genetically characterized it by 16S rRNA gene sequencing and PCR-RFLP on *omp*2a gene. This is the first report of 16S rRNA gene sequencing of Brucellae from Turkey and the first PCR-RFLP analyses of sheep-originated *Brucella* spp. in this region.

PCR utilizing different gene targets has recently become the most common way of diagnosis for human and animal brucellosis [4-6, 8, 18, 22]. Even though it is more sensitive, more rapid and less biohazardous than cultural techniques, the isolation of the organism is still accepted as gold standard. The culture isolation followed by the confirmation by PCR in this study is an another approach of diagnosis since PCR confirmation can fasten the identification at species level while remaining isolates can provide material for further biologic, phenotypic and antigenic studies.

The rate of the isolation or detection of *Brucella* spp. from aborted sheep fetuses were found to be 20-31% in Turkey reported by several independent investigators from different regions [8, 15, 20, 22-24]. The isolation followed by PCR detection of the *B. melitensis* from 38% of the aborted sheep fetuses in Kars district also in consistent with the previous seroepidemiologic studies confirming the endemicity of the infection in this region.

Analyses of 16S rRNA gene have been extensively used for molecular detection or taxonomic analyses of many different bacterial species [17, 21, 25, 26, 29]. 16S rRNA gene sequences among *Brucella* species and strains identical or significantly conserved and it was recently reported that 16S rRNA gene sequencing is a reliable tool for rapid genus-level identification of *Brucella* spp. [17]. The *Brucella* 16S rRNA consensus gene sequences described in that study was obtained comparing different *Brucella* spp. from North America with only one exception from New Zealand. The finding of identical sequence from *Brucella* in Turkey with the 16S rRNA consensus gene sequences from divergent geographical locations also confirms that 16S rRNA gene sequences among *Brucella* spp. are extremely conserved.

Several other gene targets were found to be more polymorphic than 16S rRNA gene among isolates to be used for genotyping of Brucellae [4-6, 13, 14, 16, 19, 21, 28]. The *omp*2a and *omp*2b genes encoding 36 kDa OMPs of Brucellae were reported to be highly diverse among *Brucella* spp., biovars and strains [16]. Since then, this method was extensively used to genotype *Brucella* isolates [6, 10-12, 21]. This method, therefore, was carried out to compare one of the *B. melitensis* isolate in the current study with previously characterized isolates/strains. Two *B. melitensis* isolates from Turkey, 63/9 (ATCC 23457 ; BCCN R2) and BCCN 83.35, originated from goat and human, respectively, were analyzed by PCR-RFLP on *omp*2a gene [11]. *B. melitensis* from sheep in the current study is most likely genetically close to these two strains since they all generated pattern P2 by PCR-RFLP on *omp*2a gene. *B. melitensis* described in this study may also be genetically close to isolates originated from different Mediterranean countries, particularly those from France, Spain and Israel for the isolates originated from sheep only since they all showed the similar pattern by PCR-RFLP analyses.

The methods of 16S rRNA gene sequencing and PCR-RFLP of *omp2a* gene were found to be successfully and practically applicable on *B. melitensis* isolate from sheep in this study and these analyses need to be applied on more isolates from different host species from variable regions in Turkey for more detailed molecular characterizations and taxonomic analyses. Sequencing of *omp2a* gene or other genes such as *GroEL* or other *omps* of the *Brucella* isolates in Turkey may also help to further characterize these organisms in this region.

Live-attenuated Rev.1 strain of *B. melitensis* is extensively used as vaccine in sheep in Turkey [21]. PCR-RFLP analyses showed that the *B. melitensis* described here demonstrated restriction profile different from Rev.1 strain suggesting that field strain of *B. melitensis* in Turkey may be genetically different from the vaccine strain of the same organism. Although there are no studies on the capacity or efficiency of this vaccine for protection of brucellosis in this region, the field strains need to be also antigenically characterized and compared with the vaccine strain in order to better evaluate potential of Rev.1 vaccine. This antigenic deter-

mination should be a subject for a further study to establish a base for vaccine developments if necessary.

*Pst*I site polymorphism of its *omp2* gene was previously used to differentiate *B. melitensis* field isolates from that of vaccine (Rev.1) and prototype (16M) strains [3]. Based on the analyses in that study, Rev.1 strain was found to be responsible for some of the brucellosis cases in humans and animals most likely derived from the adverse effects of the vaccination [3]. PCR-RFLP analyses used in the current study may also be applicable on the determination of potential hazards associated with the use of live-attenuated Rev.1 vaccine.

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