Original Article

Molecular typing of *Brucella* species isolates from Human and livestock bloods in Isfahan province

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Abstract

Background: Human brucellosis is caused by infection with certain species of the genus *Brucella* and is characterized by bacterial persistence and inflammation of many host tissues. Handling all live Brucella involves risk of laboratory infection and very strict biosafety rules must be observed. In order to avoid these disadvantages, method based on the PCR-RFLP shows excellent typeability, reproducibility, stability, and epidemiological concordance. The *omp2* locus contains two gene copies (named *omp2a* and *omp2b*) coding for porin proteins and has been found particularly useful for molecular typing and identification of *Brucella* at the species, biovar, or strain level. This study is designed to evaluate the molecular epidemiology of *Brucella spp* from human and livestock in Isfahan province, central region of Iran in order to use the findings in efficient disease prevention programs.

Materials and Methods: One hundred ninety blood samples were collected from human and cattle with active brucellosis and 40 aborted ewes fetuses were collected and genotyped using PCR-RFLP technique, DNA polymorphisms such as the restriction patterns of the PCR-amplified omp2a and omp2b genes.

Results: The molecular characterization performed to assess the species and the biovar of the *Brucella* strains. Analysis of the 230 isolates examined in this study generated three unique RFLP profiles. One of the profiles was the most common being present in 134/180.

Conclusion: Our findings confirm abundance of *B. melitensis*, particularly biovar 1 in human and sheep are identical but *B. abortus* biovar 3 as the etiological agent of cattle brucellosis most frequently isolated in the Isfahan area.

Key Words: Brucella, brucellosis, molecular typing, omp2, PCR-RFLP

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INTRODUCTION

Brucellosis, a typical zoonotic disease, systematic infection transmitted from animals (wild or domestic) to human, remains a worldwide veterinary and medical problem contributing to health and economic situation of affected regions.[1] The source of naturally acquired brucellosis in humans is almost always to be found in the animal reservoirs although very few cases of human to human transmission have also been reported.[2,3]

Brucellosis is present throughout the five continents and it is still an uncontrolled serious public health problem in many developing countries.[4,5] It is endemic in sheep and goats in most countries of the

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Mediterranean.[6] Distinction between the *Brucella* species and biovars in the past was mainly based on phenotypic characteristics.[7] Traditional methods of identifying *Brucella* to the species level is time consuming, subjective, and can pose a hazard to laboratory personnel in the absence of suitable biocontainment facilities.[8,9]

The *omp2* locus contains two gene copies (named *omp2a* and *omp2b*) coding for porin proteins and has been found particularly useful for molecular typing and identification of *Brucella* at the species, biovar, or strain level.[10]

Today, with advancement of molecular techniques, several molecular typing methods to find DNA polymorphism are used, which are capable to identify *Brucella* biovars and species.[11,12] polymerase chain reaction-fragment length polymorphism (PCR-RFLP) is a common approach for typing of *Brucella* spp., providing a good tool for taxonomic, epidemiological, evolutionary and diagnostic studies.[10-12] The method has especially been utilized in studies of various outer membrane protein (*omp*) genes.[13] Several Brucella *omp* genes were considered as potential targets for typing schemes, however, the *Omp2* locus [*Omp2a and Omp2b*) shows greatest polymorphism among the *Brucellae* spp.[14,15] No data, however, are available about the molecular epidemiological characterization of *Brucella* isolates from humans and livestock bloods. In this study, the genetic profiles of *B. melitensis* and *B. abortus* isolates from humans and animals were analyzed and compared by PCR-RFLP of *omp2a* and *omp2b* genes; a specific marker was detected grouping the human and animals *Brucella* spp.[15] PCR-RFLP of the *omp2* locus has good reproducibility and has been useful for differentiation of *Brucella* species, even though it is somewhat limited by the lack of natural sequence differences at the biovar level.[16] This study was designed to evaluate the molecular epidemiology of *Brucella spp* from human and livestock in the Isfahan area and applying the findings to planning for disease control in this province.

MATERIALS AND METHODS

We collected 150 human blood samples from outpatients with active brucellosis. These patients were seen in health centers in Isfahan province during March 2009 to April 2010. STA tests for all the patients were positive (titers of; 100 IU/mL) at the beginning of the disease. We also collected bovine blood samples from 40 cattle. The blood collection from these cattle was done to determine bovine seropositivity . 5 ml blood was taken from patients and bovine blood samples were transferred to laboratory and were kept at –20°C.

Extraction of genomic DNA

DNA was extracted from the blood specimens using a commercial purification system (Wizard Genomic DNA Purification Kit, Promega, Madison, WI) according to the manufacturer's instructions for DNA purification from blood. Final pellets were resuspended in 50 µL of TE (10 mMTris, 1 mM EDTA, pH 7.2).[12,14]

Bacteriological examinations

Samples from abomasum content and liver of the ewes' aborted fetuses were inoculated onto blood agar plates containing 7% defibrinated sheep blood with and without *Brucella* supplement (Oxoid, SR083A). The cultures were incubated at 37°C under an atmosphere with 10% CO2. *Brucella* were identified by morphological, cultural and biochemical characteristics such as oxidase, urease, CO2 requirement, H2S production, growth in the presence of thionin and basic fuchsin (20 µg/ml). The strains were bio typed by agglutination with mono specific A and M antiserum.

DNA preparation

DNA preparation for *Brucella* genomic DNA extraction, *Brucella* sample strains were cultured for 24 h at 37°C on tryptic soy agar-yeast extract slopes and harvested, in 3 ml of sterile distilled water, by centrifugation at 2,000 × g for 10 min. The pellet was suspended in 567 µl of TE/sodium buffer (50 mM Tris, 50 mM EDTA, and 100 mM NaCl [pH 8.0]). Then, 30 µl of 10% (wt/vol) sodium dodecyl sulfate (SDS) solution and 3 µl of 2% (wt/vol) proteinase K solution were added, and the mixture was kept at 37°C for 1 h. The lysed cell suspension was extracted twice with phenol-chloroform, and nucleic acids were precipitated by gently mixing the aqueous phase with 2 volumes of cold ethanol. The precipitate was dissolved in 100 µl of TE (10 mM Tris, 1 mM EDTA [pH 8.0]).

The amount of DNA was measured by electrophoresis of an aliquot of each sample through 0.8% agarose gels and was compared with standard DNA solutions [6]. The extracted DNA was then used for PCR amplification of part of omp2a gene.

PCR amplification

The *Brucella omp2a* gene was used as target DNA for PCR amplification.[13] Specific oligonucleotides (*omp2a*F 5'-CCTTCAGCCAAATCAGAATG-3', *omp2a*R 5'-GGTCAGCATAAAAAGCAAGC-3') were used for amplification of this gene locus.[3,17]

Amplification conditions

The reaction mixture contained 5 µL of 10x PCR buffer,

10 pMol of the primer mix, 0.2 μMol of dNTP mix, 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase, 100 pg of sample DNA in a volume of 50 μL. No template negative controls were routinely processed to monitor contamination with *Brucella* DNA and positive controls were included to monitor amplification success. Amplifications were initiated by denaturing the sample for 3 min at 95°C, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. After the last cycle, samples were incubated for an additional 5 min at 72°C before storage at 4°C.[12]

DNA digestion

Five microliters of PCR-amplified *omp2a* gene was digested by the restriction enzyme *PstI* (Promega Corporation, Madison, WI), at 37°C for 3 h in a 20 μL reaction volume using manufacturer's recommended buffer. The mixtures were then electrophoresed on a 2% agarose gel containing DNA green viewers.[13]

Statistical analysis

Statistical analysis was conducted to determine how many samples were positive for *Brucell spp* biovar, as well as those positive for two *Brucella spp*. Perspective analyses were performed and data rounded numerical values (percentage) were documented.

RESULTS

The molecular characterization performed to assess the species and the biovar of the *Brucella* strains. Analysis of the 230 isolated examined in this study generated three unique profiles. One of the profiles was the most common being present in 134/180.[14,15,18] This profile was predominantly associated with 74% of the isolate but was also less frequently seen in cattle [Table 1].

According to our findings, since 62% of human and sheep isolates were identified as *Brucella melitensis* biovar 1 [Figure 1 pattern 3], 35% *B. melitensis* biovar 1,2 and *B. abortus* biovar 3,5,6 [Figure and Table 1 pattern 1], 5% *B. abortus* biovar 1,2,4 and *B. melitensis* biovar 1,2,3 [Figure and Table 1 pattern 2]. *B. melitensis* (biovars 1 and 3) is the predominant species, associated with sporadic cases and outbreak in humans and sheep. In cattle the biovar of the *Brucella* strains indicated the presence of 20% isolates of *B. abortus* biovar 1, 2, 4 and *B. melitensis* biovar 1,2,3 [Figure and Table 1 pattern 2], 70% *B. abortus* biovar 3,5,6

B. melitensis biovar 1, 2 [Figure and Table 1 pattern 1], 10% *B. melitensis* biovar 1 [Figure 1 pattern 3]. In particular, *B. abortus* biovar 3,5,6 was significantly associated with cattle (70% versus 5% in sheep and goat), while *B. melitensis* biovar 1 was significantly associated with human and sheep and goat (62% versus 10% in cattle).

Table 1: RFLP patterns detected when PCR amplified *omp2a* gene of the human and livestock *Brucella* strains digested by Pst1 enzyme

	P_1	P_2	P_3
Species	B. abortus Biovar 3,5 B. melitensis Biovar 1, 2	B. abortus Biovar 1, 2, 4 B. melitensis Biovar 1, 2, 3	B. melitensis Biovar 1
Human (%)	35	5	62
Sheep and Goat (%)	5	22	62
Cattle (%)	70	20	10
Average (%)	36.67	15.64	45

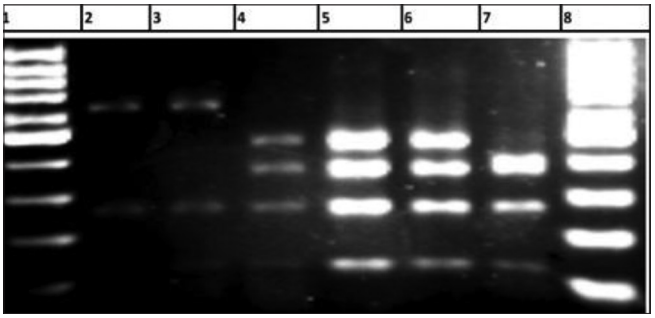

Figure 1: Restriction patterns of PCR amplified *omp2a* genes of the human and livestock *Brucella* strains digested by Pst1 enzyme

DISCUSSION

Human brucellosis is caused by infection with certain species of the genus *Brucella* and is characterized by bacterial persistence and inflammation of many host tissues.[3] Neutrophils are one of the predominant cell types present in the infiltrate of these inflamed tissues, and due to their potential effect on the inflammatory response and tissue damage, direct activation of neutrophils by *Brucella abortus* might contribute to the pathology associated with human brucellosis.[4] *B. abortus* expresses outer membrane lipoproteins *(Omp)* with inflammatory properties on a variety of cell types.[4]

B. melitensis is the most important zoonotic agent, followed by *B. abortus* and *B. suis*. The occurrence of brucellosis in human is directly related to the status of animal brucellosis in a particular geographical region.[15,16] Hence, defining a case as brucellosis with the causative species will give more clues about prevalence, treatment, and control measures rather than merely diagnosing as brucellosis.[16] *Brucella* species vary from country to country, however the most common isolated species are *B. melitensis and B. abortus.*[16,19] Although *B. abortus* is not usually associated with human brucellosis, it is the most common cause of the infection in general. Its clinical picture is less severe than

the one seen in infections caused by *B. melitensis*.[14] *B. melitensis* is the most frequently reported cause of human brucellosis and it is commonly isolated from the patients.[17] As the most virulent type, *B. melitensis* is related with severe, acute diseases. In some regions it is reported as endemic. In Iran, brucellosis was first recognized in 1949[19] and is now endemic throughout the country. The annual reported incidence of human diseases is 26551 cases in 2005. The prevalence of brucellosis among sheep/goats was 10.2% and among cows was 17.5% in the 1990s. The long-term serological studies have indicated that 5% of sheep and 0.8% of cattle was infected with brucellosis.[20] In our country, concordantly, *B. melitensis* is known to be the main cause for human brucellosis.[6,20] Due to differences in the pathogenicity of *Brucella* species and biovars, with a view to epidemiology of brucellosis, recognition of *Brucella* biovars is important and hence typing of various strains is the main task of control centers of brucellosis and must be performed continuously.[9,13,15] In this study, the genetic profiles of *B. melitensis* and *B. abortus* isolates from humans and animals were analyzed by DNA polymorphisms at the *omp2* locus. The *omp2* locus contains two gene copies (named *omp2a* and *omp2b*) coding for porin proteins and has been found particularly useful for molecular typing and identification of *Brucella* at the species, biovar, or strain level.[22] On the basis of restriction patterns (from 13 restriction enzymes) of the PCR-amplified omp2a genes *Brucella* strains could be classified into different groups [Figure 1].[24] How ever PCR-RFLP of *omp2a* and *omp2b* was not able to accurately identify our isolates, which were identified as *B. melitensis* biovar 1 or 3 and *B. abortus* biovars [Figure 1 Patterns 1, 2].[17,21] In the province of Isfahan, brucellosis is an endemic disease and the main sources of brucellosis in animals and humans are infected sheep and cattle. *B. melitensis* biovars 1 is the predominant species, associated with sporadic cases and outbreak in humans. Isolates of *B. abortus*, primarily biovars 1, 2, 3 are also associated with sporadic human brucellosis.[23,24]

CONCLUSION

Our findings confirm abundance of *B. melitensis*, particular biovar 1 in human and sheep are identical but *B. abortus* biovar 3 as the etiological agent of cattle brucellosis most frequently isolated in Isfahan area and suggest, therefore, that sheep and goat populations are the principal cause of human brucellosis.

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