

Isolation of *Brucella abortus* Using PCR-RFLP Analysis

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Abstract

Brucella transmission and epidemiology depend on infecting species and biovar. Therefore, exact identification of the *Brucella* is important to design correct control and treatment strategies. In this study, we examined presence of other *Brucellae* in Isfahan. One hundred twenty *Brucella* isolates were collected and genomic DNA was extracted from them. *omp2a* fragment of all isolates were amplified using a pair of specific primers and the PCR products were electrophoresed and stained with EtBr. These PCR products were then restricted using *PstI* restriction endonuclease. The PCR products of all isolates had the same size of 1100bp. The banding pattern of PCR-RFLP for all of the isolates were similar to banding pattern of the *Brucella melitensis* biotype 1 except for 5 samples that demonstrated banding pattern similar to *B. abortus*. Based on our results, it is clear that biotype 1 of the *B. melitensis* is not the only *Brucella* present in Isfahan and now *B. abortus* is also present in our area. These results are very important in planning for the control of the disease as well epidemiology and even treatment of the patients.

Keywords: *Brucella abortus*, PCR-RFLP, *Omp2a*, Iran

Introduction

Brucella is small, nonmotile, nonsporulating, nontoxicogenic, nonfermenting, aerobic, Gram-negative coccobacilli that may, based on DNA homology, represents a single species (1). Conventionally, however, they are classified into seven species each of them comprising multiple biovars (2). By the discovery of new *Brucella* species, especially in wild animals, this number is increasing (3). *B. melitensis*, *B. suis* and *B. abortus*, can infect human (4), but human infection with *B. canis* has also been reported. Infection of humans with *B. ovis* and *B. neotomae* (5) and, the newly identified *B. maris*, has not been described (6). *B. melitensis* mainly infects sheep and goats (7, 8) and *B. abortus* is the major cause of abortion in cattle (9). There are some reports that even in the countries that *B. abortus* have eradicated from cattle, in some areas *B. melitensis* has emerged

as a cause of infection in this species as well as in sheep and goats (7). Still *B. melitensis* remains the principal cause of human brucellosis (10). *B. suis* is also emerging as an agent of infection in cattle, thus extending its opportunities to infect humans (11).

Brucella species and biovars have been characterized by phenotypic methods, although such methods are not always reliable (12). These species and biovars may also be characterized by their natural host and the strain's geographical origin, however, a species may infect an animal other than its natural host, and a single strain may now be found in multiple geographic locations (13). Heretofore, standard serological tests used to detect *Brucella* have required several weeks time to complete and have not been able to distinguish between species of *Brucella* (14). The methods currently available to identify spe-

cies of infecting *Brucella* require the isolation of bacteria on selective media followed by quantitative analysis of phenotypic properties of the organism. Phenotypic characterization may be based on such features as lipopolysaccharide antigens, phage typing, dye sensitivities, CO₂ requirements, H₂S production, and metabolic properties. Such methods are time-consuming (requiring 1-4 weeks) and are unreliable (15).

Recently, PCR-RFLP has provided evidence of polymorphism in a number of genes including *omp2*, *dnaK*, *htr*, and *ery* (16). The major outer membrane proteins (OMPs) of *Brucella* spp. were initially identified in the early 1980s and classified according to their apparent molecular mass. The genes encoding the group 2 porin proteins consist of two genes, *omp2a* and *omp2b*, which are closely linked in the *Brucella* genome, and share a great degree of identity (>85%). Interestingly, only *Omp2b* is expressed in laboratory grown *B. abortus* (17).

It is probable that new strains/ biovars of *Brucella* enter to a region. Information about the existing *Brucellae* in a region is essential for epidemiological, diagnosis, prevention, and treatment of the diseases. Using serological techniques, it was demonstrated that the only species of *Brucella* in Isfahan, Iran was *B. melitensis* biovar I (18). In this study, we have applied PCR-RFLP on cultured *Brucella* isolates collected from fetus and blood cultures, to study the species/ biovars present in our region.

Materials and Methods

Working with *Brucella* could be very dangerous, therefore to prevent transfer of the disease to the personnel of laboratory strong biosafety measures should be applied, at least containment level 3.

Sample collection All sheep and cattle that are suspected with *Brucella* infection had to be examined by Isfahan Veterinary Clinic Network laboratory. They use biotyping and serotyping to identify *Brucella* spp. For this study, 120 *Brucella* isolates were examined that all were

originated from Isfahan province. All of the samples were double-checked and confirmed by *Brucella* Department of Razi Institute. The samples were cultured human blood and sheep and cattle aborted embryo isolates. All *Brucella* isolates from blood and aborted fetuses have been cultured both in Razi Institute and in our laboratory, on either *Brucella* agar or tryptic soy agar at 37 °C for approximately 48 h by conventional methods (19).

DNA extraction To extract chromosomal DNA, the cultured *Brucella* bacteria were washed off the plates in 5 ml of phenol/saline (0.1% w/v and 0.85% w/v, respectively) and the live bacteria were killed and collected by incubation at 68 °C for 2 h followed by centrifugation at 5000 rpm for 20 min. The *Brucellae* were then incubated in lysozyme solution (4 mg/ml) for 30 min at 4 °C. Then sodium dodecyl sulfate (0.5% w/v) and proteinase K (200 mg/ml) were added, and incubated at 37 °C for 1 h. After that to purify the extracted DNA from its protein contaminants, the cell lysate was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1). Finally, to increase concentration of the extracted and purified DNA, ethanol precipitation was applied. The precipitated DNA was, washed with 70%, ethanol air dried, resuspended in TE (50mM Tris- HCl, 1mM EDTA pH 8.0). The quality and quantity of the extracted DNA analyzed using UV spectrophotometry (OD_{260/280}) as well as agarose gel electrophoresis (1%) in TEA buffer (20mM Tris-acetate, 1mM EDTA pH 8.0) and ethidium bromide (0.5µg/ ml) staining. The DNA was then stored at 4 °C until use.

PCR amplification: The *Brucella omp2a* gene was used as target DNA for PCR amplification. Specific oligonucleotides were used to prime the amplification this gene locus (20). The sequences of the forward and reverse primers were: *omp2a* F 5'-CCTTCAGCCAAATCAGAATG-3' *omp2a* R 5'-GGTCAGCATAAAAAGCAAGC-3' The primers were synthesized in our laboratory using an Alf DNA synthesizer.

The reaction mixture for PCR amplification contained 50 mM KCl, 1.75 mM MgCl₂, 0.1% (w/v) Triton X-100, 0.2 mg/ml of BSA, and 10mM Tris-HCl (pH 8.5), 10mM each of the four dNTPs, 100ng of sample DNA, and 1picoM of each of the appropriate oligonucleotide primer and 1 U of *Taq* DNA polymerase (SinaGene, Iran). The PCR cycle condition was 1 cycle consisting of 45 s at 95 °C for preliminary DNA denaturation, followed by 35 cycles consisting of 30 s 95 °C for DNA denaturation, 1 min at 50 °C for DNA annealing, and 1 min at 72 °C for polymerase-mediated primer extension. This was followed with one last cycle of 72 °C for 7 min. PCR was performed in an Ependorfe Thermocycler and then 10 µl of the amplified PCR product was analyzed by agarose (1.5%) gel electrophoresis.

PCR product digestion The PCR products were then restricted using *Pst*I restriction endonuclease. Restriction enzyme was used according to the manufacturer's (Fermentas) instructions in a 20 µl reaction volume containing 8µl PCR product, 2 µl appropriate buffer, and 10 µl ddH₂O. The reaction was incubated at 37 °C for 2 h before analyzing of the digestion result on agarose (2%) gels electrophoresis and ethidium bromide staining.

Results

All 120 cultured samples that were sent to Razi Institute proved to be *Brucella*. DNA was successfully extracted from all of the sample plates. An average of about 100 µg highly pure DNA was extracted from each sample. The extracted DNA was PCR amplified as explained above. The PCR products of all 120 examined samples had the same size of 1100bp. The PCR products were then restricted using *Pst*I restriction endonuclease and the results of digestion were analyzed using gel electrophoreses. The banding pattern of PCR-RFLP for all of the isolates were similar to banding pattern of the *Brucella melitensis* biotype 1 except for 5 samples that demonstrated banding pattern similar to *B. abortus* (Fig. 1). From 120 examined samples 95 (80%)

were from aborted fetuses and 25 (20%) were from human blood cultures. These 25 positive samples were obtained from culturing more than 400 blood samples. In fact, about 25% of the blood cultures were positive. Of 95 fetus samples 92 (97%) were proved to be *B. melitensis* biotype 1 and only 3 samples (3%) were proved to be *B. abortus*. Of 25 positive blood cultures examined 24 (96%) were *B. melitensis* biotype 1 and 1 (4%) sample were *B. abortus*.

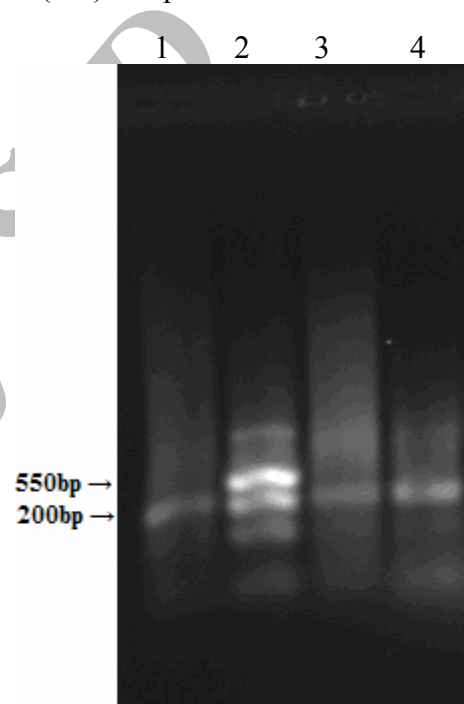


Fig. 1: *Pst*I restriction digestion of PCR products from a 1100bp region of *omp2a* gene. Lane 1) *B. melitensis* biotype 1, standard strain provided by Razi Institute of Iran. Lane 2) *B. abortus*. Lanes 3 and 4) *B. melitensis* biotype 1. Lanes 2, 3 and 4 were from our clinical samples

Discussion

Brucella infects hundreds of thousands of humans every year and therefore is an important health problem in developing countries. It also is the main cause of abortion in livestock and results in a great economy loss in these countries. Therefore, to control the disease in the endemic area, epidemiological information is of great importance (21). It should be noted that *Brucellae* have definite host preferences, for

example, *B. melitensis* mainly infects sheep but *B. abortus* main host is cattle and therefore epidemiological information can help to control infection in sheep and cattle as well as human (22). *Brucella* always has been present in Iran and the main plans to control this disease were ewes and cattle vaccination and slathering of infected cattle (23, 18). This programme was not completely successful and we have faced an outbreak of the disease since 2002.

To solve this disease problem first it should be detected in livestock and secondly proper vaccination with a safe vaccine should be applied (24). Because of ecological differences between species of *Brucella* characterization of the *Brucella* spp. in an area can help to choose the correct and safe vaccine as well time of vaccination. It also should be noted that there are some reports that vaccine strains, both Rev1 and Rb51, can infect human and can induce abortion in ewes and cattle. Therefore, determination of origin of infection in human and ewes/ cattle is also of great importance (24).

The PCR technique has increasingly been used as a supplementary method in *Brucella* diagnosis (10, 12, 14, 16, 25, 26). Recently, a molecular biotyping approach has been proposed based on restriction endonuclease polymorphism in the genes encoding the major 25- and 36-kDa outer membrane proteins of *Brucella* (17, 20, 27). In *Brucellae* that are pathogen for human, there are 2 genes *omp1* and 2 in this locus. The *omp2* gene (28) exists as a locus of two nearly homologous repeated copies that differ slightly among *Brucella* spp. and biotypes, called *omp2a* and *omp2b* (29, 20). In this study, we have applied PCR-RFLP to identify *Brucella* spp. present in our region using *omp2a* and *b* gene polymorphism. As mentioned above, this locus was also used by others to distinguish between species or strains of *Brucella* and it is demonstrated that using polymorphism in this locus is a powerful tool for *Brucella* genotyping. A 1100bp piece of the *omp2a* was amplified and the PCR products were restricted using *Pst*I restriction endonuclease (20, 28). We were able to distinguish be-

tween *Brucella* species or biotypes by the application of these enzymes. Our study detected two *Brucella*, *B. melitensis* type 1 and *B. abortus*. Detection of *B. abortus* might be because of the method we have applied. Routine laboratory method for *Brucella* diagnosis is serological tests. These tests are not sensitive enough to distinguish between *B. melitensis* and *abortus*.

In areas with no proper control on sheep and cattle traffic between cities or even countries, entering a new species/ biovar of *Brucella* is always possible and therefore to be aware of the *Brucellae* that are present in the area, a permanent monitoring system is essential. Based on banding pattern of PCR-RFLP of the *omp2a* fragment, it can be resulted that unlike what the previous studies had demonstrated that only biotype 1 of the *B. melitensis* exist in Isfahan now we report that *B. abortus* is also present in our area. These results are very important in planning for the control of the disease as well as epidemiology of the disease and even treatment of the patients.

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