Differentiation of Iranian Strains of *Brucella spp*. by Random Amplification of Polymorphic DNA

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Summary

101 isolates of *Brucella spp.* including the reference (n=7) and the Iranian field strains belonging to last years 1961-2003 (n=94) were biotyped and classified into 9 biotypes. Random amplified polymorphic DNA (RAPD) analysis using a ten mer-AP_4 primer was generated 72 reproduciable DNA band. The typeability was 100%. The thirteen isolates were classified into five groups, each containing 2-4 biotypes. The high DNA polymorphisms among the strains suggested that animals in Iran have been infected with genetically diverse isolates however, in the last forty years *B.abortus* biotype A3 and *B.melitensis* biotype M1 have been predominantly isolated from brucellosis cases. The results indicated that the RAPD analysis using AP_4 primer can be used to attain reproducibility amplify random fragments of DNA from Brucella genomes in order to differentiate at species and sub-species for epidemiological purpose.

Key words: *Brucella*, RAPD-PCR, polymorphism, biotyping

Introduction

Brucellosis is one of the most serious public health threatening zoonotic diseases caused by *B.abortus, B.melitensis, B.suis* and *B.canis*. Members of this genus are the main cause of contagious bacterial abortions in cattle, sheep, goats, pigs and dogs

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(Alton *et al* 1975, Alton *et al* 1988, Cloekaert *et al* 2001). Using the conventional bacteriologic methods, oxidative-metabolic tests and phage typing, the genus *Brucella* is divided into 6 species and 18 biotypes (Alton 1975, Corbel & Brinley-Morgan 1984). Recently two new biotypes among isolates of *B.maris* have been introduced (Jahans *et al* 1997). Changes in the prevalence of different biotypes could occur due to seasonal movements, contact of wild and domesticated animals, close contacts between different animals at farms, vaccination programs and culling of infected animals (WHO/FAO 1971). Attempts, based on phenotypic and molecular methods have been made to differentiate *Brucella* spp. to study the epidemiology of brucellosis. Biotyping had been used in extent for this purpose (Ekers 1978). While phenotyping method is time-consuming, labour intensive, costly and lack in discriminatintory power (Tcherneva *et al* 2000), a variety of DNA-based typing methods have been developed and applied to identity *Brucella* spp.

The phenotyping method for differentiation of *Brucella* strains has led to define 18 biotypes. Differentiation of individual strains bellows the biotype level has far been impossible. Application of nucleic acid techniques for genetic analysis of bacterial populations has improved the typeability and discriminatory power of the genotyping techniques. Pulsed filed gel electrophoresis (PFGE) has been used extensively to study the epidemiology of infections by different micro-organism. However, limited polymorphism has been found for the isolates of *Brucella* sp when they were analyzed by this technique (Allardet-Servent *et al* 1998). Random amplified polymorphic DNA (RAPD) analysis was reported as a powerful technique to differentiate the isolates of *Brucella* in a given biotype (Feket 1992, Mercier 1996).

In this study we were determined the genotypic (by RAPD-PCR) and phenotypic relationship among *Brucella* spp. isolated from different animal species in different parts of Iran.

Material and Methods

Strain. 94 isolates of *Brucella* recovered from animals and human during 1961-2003 as well as 7 reference strains were used (Table 1). All isolates were phage typed and identified to the species level using the standard biochemical and serological tests (Alton *et al* 1975, 1988, Alderik 1968).

Table 1. *Isolates of Brucella recovered from animals and human and 7 reference strains*

Place	No. of isolate	Source	Year
Ghazvin	47	Human, Cattle, Sheep, Pigs	1971-2002
Anzali	7	Cattle, Pigs	1971-1972
Shahrekurd	14	Sheep	1999-2003
Isfahan	12	Cattle, Sheep	1961-2003
Tehran	9	Cattle, Sheep	1972-2002
Other	6	Cattle, Sheep	2000-20002

DNA Eextraction. DNA was extracted using phenol-choloroform-isoamyl alchol and freeze-thawing methods with slight modification (Falamm *et al* 1984) and (Rossello-Mora & Amann 2001) respectively. In brief, the cells were grown in 2ml of *Burcella* broth for 48h then harvested by centrifugation at 10000rpm for 5min at 4°C. The pellet was washed twice with cold solution of 1M NaCl and resuspended in TE buffer (10mM Tris, 1mM EDTA pH8). The suspension was frozen and thawed three times. The crude extracts containing DNA were used as template in polymerase chain reaction (PCR) assay.

RAPD-PCR. Primer AP4 (5′-TCACGCTGCA-3′) previously used for typing of vancomycin resistant *Enterococci* in RAPD analysis (Barbier 1996), was used. RAPD-PCR was performed in a final volume of 25 µl of reaction mixture including 2.5µl PCR buffer contained 2.5mM $MgCl_2$ (pH8.6, Roche Diagnostic, Germany), 0.5 µl of 10mM dNTPs (Fermentas, UAB, Lithuania), 1µl of 10pmol of primer (TIB, MOL BIOL Germany), 1.25unit of *Taq* polymerase (Fermentas, UAB, Lithuania), 2µl of crude genomic DNA and 18.75µl of distilled water. PCR was

conducted using a thermal profile consisting of an initial 10min denaturation at 95°C, 45 cycleS of 10s at 94°C, 10s at 35°C, 2min at 72°C and a 10min final extension at 72°C in a mastercycler (Eppendorf, Germany). The PCR products were analyzed by electrophoresis on 1.5% agarose gels. The 100bp ladder (Roche Diagnostics, Germany) was used as molecular weight marker and tubes containing distilled water free of template were used as negative control of the experiments. Gels were stained in a 0.5μg/ml ethidium bromide solution for 15min. and photographed by Bio-Doc (UVP, UK).

Analysis of RAPD data. Each isolate was scored for the presence or absence (1 or 0) of each band. The raw data matrix of 1's and 0's was then used to generate a dissimilarity matrix. The denderogram was generated using unweighted pair-group method using arithmetic averages (UPGMA) and phentree programs (Burr 1970).

Results

Of 101 isolates of *Brucella*, 44 were identified as *B.abortus*, 47 as *B.melitensis* and 10 as *B.suis*. These isolates were differentiated into biovars A1, A2, A3, A5, M1, M2, M3, S1 and S2. The biotype characteristics and number genotypes for the isolates are shown in table 2. Amplification of template DNA with AP_4 primers produced 72 different genotypes. All isolates shared a 700bp DNA fragments in RAPD-PCR. Depending on the strains used, the RAPD analysis produced 1-11 DNA bands ranging from 50bp to 2642bp in size (Figures 1 and 2). Eight isolates including 4 reference strains and 4 field isolates belonged to biotype A1. The reference strain 544 and S19 strains produced a specific DNA band with 980bp and 350bp that was absent in other strains of biotype A1. Five patterns were obtained by RAPD-PCR with different biotypes including: biotypes M1, S1, S2 in genotypes 1; A2, A3 in genotype 2; A2, A3, M1, Rev.1 in genotype 3; A3 and A5 in genotype 4, and M1, M2, S2 in genotype 5. A dendrogram demonstrates the low genetic distances among them (Figure 3).

Table 2. *Identification, biovar distribution, and number of RAPD types among Brucella isolates*

***Brucella* spp.**	**Biovar**	**No. of isolate**	**No. of profile**	**Animals (n)**
B.abortus	A1	8	6	Pig (3), Cattle (1), Reference(4)
	A2	2	2	Pig (1), Cattle (1)
	A3	29	21	Pig(21), Cattle(7), Sheep(1)
	A5	5	5	Pig (3), Cattle (2)
B.melitensis	M1	35	28	Sheep (23), Pig (6), Cattle (3), Reference (2), Human (1)
	M2	2	2	Sheep, Cattle
	M3	1	1	Pig
	M1 (Rev.1)	9	8	Sheep (9), Reference (1)
B.suis	S1	4	3	Pig (4)
	S2	6	5	Pig (6)
Total		101	72	

Discussion

Several methods had been used for typing of *Brucella* strains to understand the epidemiology of brucellosis. Such methods have been used to biologically characterize the native strains of *Brucella* spp. to investigate the seasonal changes in the population of the organism in certain regions and also to find the introduction of new strains through the importation of livestock (Vizcaino *et al* 2000). The application of molecular techniques has had a revolutionary impact on the diagnosis of infectious diseases. They are highly sensitive and specific methods for identifying pathogens. RAPD technique has provided a powerful tool for the direct analysis of the genomes and for epidemiological studies of many pathogens.

Despite the existence of high prevalence of brucellosis in Iran, no data was available on the genetic background of the strains involved. The present study is the first one that uses RAPD-PCR to analyze the population of *Brucella* strains in our country. This technique was used to differentiate the isolates at sub-species level. The typeability of the RAPD was 100% and its discriminatory power is higher than phenotyping technique. Similar results reported when PCR-based typing techniques were used in parallel with biotyping (Mercier *et al* 1996). Previously repetitive

element sequence based PCR (REP-PCR) had been used to analyze different biotypes of *Brucella* spp. Although this technique is useful for identification of species, some limited success in differentiation of isolates at sub-species level have been remained (Tcherneva 1996).

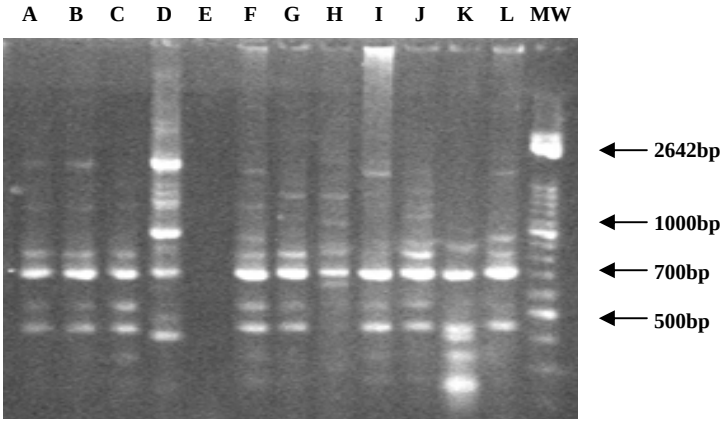

Figure 1. *DNA polymorphism among different biotypes of Brucella produced by RAPD-PCR. Lanes A, B and J: the field strains of B.melitensis biotype M1; C: B.abortus strain S.99; D: Campylobacter fetus; E: negative control; F: B.melitensis H.38; G: B.melitensis Rev.1; H: B.melitensis16M; I: B.abortus strain RB51; K: B.abortus strain S19; L: B.abortus strain 544; MW: molecular weight marker*

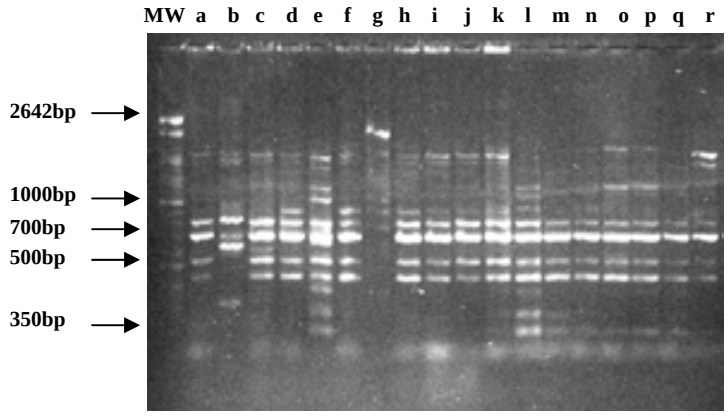

Figure 2. *PCR fingerprinting of Brucella strains produced by RAPD-analysis using AP_4 primer. Lanes a-d and g: field strains of B.melitensis; e-f and m-r: filed strains of B.melitensis biotype M1; h-i: mixed phenotypes recovered from aborted fetuses; j, k: B.melitensis Rev.1; l: B.abortus S19; MW: molecular weight marker*

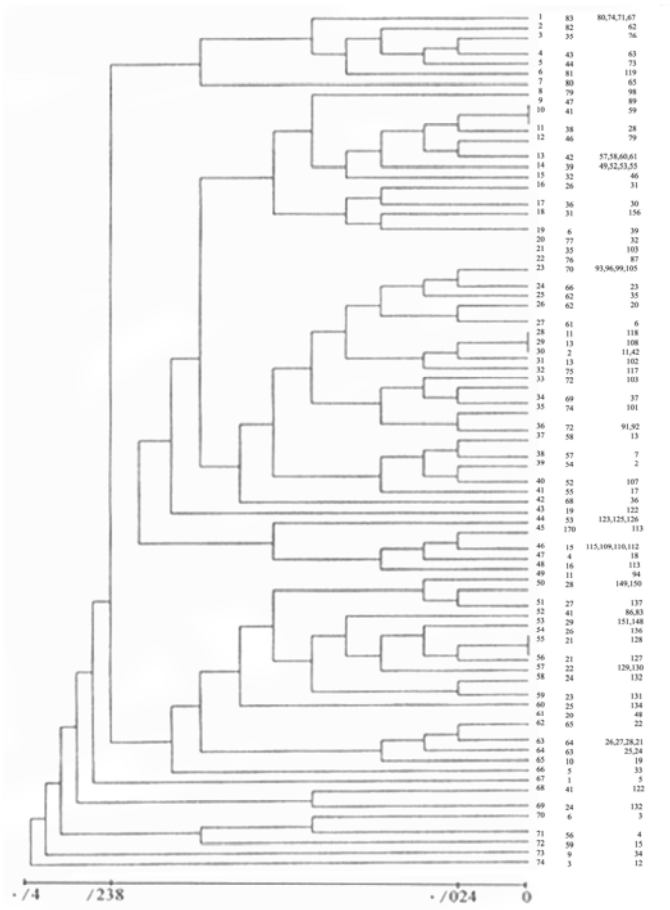

Figure 3. *Dendrogram showing the number of genotypes among the strains of Brucella spp and the genetic distances at which they are separated. Genotypes 2, 10, 18, 21, 24-26, 40, 41, 47, 50-70 and 72 belong to filed isolates B.melitensis cultured from cases with brucellosis in Tehran, Isfahan, Ghazvin, Gilan and Shahrkurd during 1961-2003; genotypes 51-54, 57-60 and 68 have been isolated from aborted sheep following vaccination with Rev I during 2001-2003; genotypes 3-9, 12, 14-17, 19, 22, 23, 28-29, 33-36, 43, 48, 49 are different biotypes of B.abortus isolated from cattle and pig during 1971-2003 period at different areas of Tehran, Ghazvin, Gilan provinces; 11, 20, 42, 45, 46 are B. suis biotypes 1 and 2; 27. The reference strains Rev.I, S. 99, H38, S19, 544, 16M and RB51 are distributed in genotypes 27, 37, 38, 39, 44 and 71 respectively. Isolates that clustered in types 3 and 4 or 20 and 21 are separated from each other at a genetic distance of 0.095. The maximum genetic distance between the isolates of Brucella spp. was found 0.238 by RAPD*

In this study the 101 isolates were classified into five RAPD groups according to their distinctive DNA fingerprint patterns. Strains of *B.melitensis* have been isolated from cases with brucellosis in both human and animals (Zowghi *et al* 1988). Despite the heterogenecity among isolates within biotype M1, certain isolates (n=5) with identical DNA profiles were found at different areas such as Shahr-Kurd, Karaj and Tehran. All biotypes of *B.abortus* have been isolated from animals. The biotype A3 was the most frequent phenotypes of *B.abortus* and it is endemic biotype (Zowghi 1982). The results of RAPD analysis on 29 field isolates of biotype A3 showed that isolates with identical RAPD patterns could infect sheep, pig and cattle in different areas. It suggests that clonal spread of certain genotypes with biotype A3 had occurred at different provinces.

RAPD analysis of 5 isolates of biotype A5 showed that they belong genetically to two clones, each containing 2 and 3 isolates. The minor genetic changes among the isolates within each clone may be due to frequent transmission and passages between different animals. The RAPD-PCR using AP_4 primer was sensitive enough to differentiate the reference strains of biotype A from the field strains. This is particularly important as it could distinguish the vaccinated cattle from the infected ones. It was useful to distinguish two reference strains Rev.1 from each other as well as from field strains of *B.melitensis* and 16M strain. The isolation of strains of *B.suis* of type S1 and S2 was limited to the 1970s. The piggeries shut down after the revolution in 1978 and after that isolation of *B.suis* was not reported from Iran (Ardalan & Ebadi 1977). DNA polymorphism data showed that the RAPD pattern of *B.suis* isolates is not similar, indicating those pigs had been infected with different genotypes at the time. DNA based typing used in this study showed considerable diversity in the population of *Brucella* spp. in Iran. It may suggest that the microorganism has been widespread in point of time and location in the country. Transmission to different animals and passages of strains probably has created such heterogeneity in a single species with clonal population (Bricker 2003). Further

genetic studies with other oligonucleotide primers are necessary for the differentiation of *Brucella* spp. isolated from Iran.

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