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Species-specific PCR for the Diagnosis and Determination of Antibiotic Susceptibilities of *Brucella* Strains Isolated from Tehran, Iran

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ABSTRACT

***Background*:** Brucellosis is an endemic zoonotic disease in the Middle East. This study intended to design a uniplex PCR assay for the detection and differentiation of *Brucella* at the species level and determining the antibiotic susceptibility pattern of *Brucella* in Iran.

***Methods*:** Sixty-eight *Brucella* specimens (38 animal and 30 human specimens) were analyzed using PCR (using one pair of primers). Antibiotic susceptibility patterns were evaluated and compared using the E-Test and disk diffusion susceptibility test. Tigecycline susceptibility pattern was compared with other antibiotics.

***Results*:** Thirty six isolates of *B. melitensis*, 2 isolates of *B. abortus* and 1 isolate of *B. suis* from the 38 animal specimens, 24 isolates of *B. melitensis* and 6 isolates of *B. abortus* from the 30 human specimens were differentiated. The MIC_{50} values of doxycycline for human and animal specimens were 125 and 10 µg/ml, respectively, Tigecycline 0.064 µg/ml for human specimens and 0.125µg/ml for animal specimens, and Trimethoprim/Sulfamethoxazole and Ciprofloxacin 0.065 and 0.125µg/ml, respectively, for both human and animal specimens. The highest MIC_{50} value of streptomycin in the human specimens was 0.5µg/ml and 1µg/ml for the animal specimens. The greatest resistance shown was to tetracycline and Gentamicin, respectively.

***Conclusion*:** Uniplex PCR for the detection and differentiation of *Brucella* at the strain level is faster and less expensive than multiplex PCR, and the antibiotics Doxycycline, Rifampin, Trimethoprim/Sulfamethoxazole, Ciprofloxacin, and Ofloxacin are the most effective antibiotics for treating brucellosis. Resistance to Tigecycline is increasing, and we recommend that it be used in a combination regimen.

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Introduction

Gram-negative bacteria of the genus *Brucella* cause brucellosis. The disease is a serious public health problem worldwide, especially in developing countries (1, 2). Brucellosis is an endemic

disease in the Middle East, the Mediterranean countries and East Asia (1). The WHO has reported every year 500,000 new cases of brucellosis diagnosed in above regions (3). This inflicts irreparable economic damages on the health systems of these countries. Due to the implementation of health programs, the prevalence of brucellosis has fallen sharply in many developed countries (4). However, brucellosis is an endemic disease in countries such as Iran, Turkey, India, etc. (5). In recent years, no accurate statistics have been published on the prevalence of this disease in Iran. Ten thousand people are annually infected with this disease in Turkey (2).

Symptoms of the disease include undulant fever, chills, fatigue, body aches, joint pain, lumbar vertebrae pain, back pain, loss of appetite and general weakness (6, 7). During the course of the disease, complications such as spondylitis, wedge-shaped vertebral collapse, meningitis, pancarditis, bronchopneumonia, acute respiratory distress syndrome, unilateral epididymo-orchitis, and uveitis may also occur (2, 8).

Following the recommendations of WHO published in 1989, the gold standard is used to treat brucellosis in Iran. In this combinatorial treatment, doxycycline- rifampin is used for six weeks, or doxycycline is taken for six weeks together with streptomycin for 2-3 weeks (9). These drugs have serious side effects for patients (10-12). Moreover, the regimen recommended by the WHO is not a practical one. There have also been reports of recurrence rates of 5-10% resulting from inappropriate treatment (13, 14). Factors such as the high rate of recurrence, resistance to rifampin (because tuberculosis is endemic in some parts of Iran) and toxic side effects (damage to the middle ear, nephrotoxicity resulting from the use of streptomycin) have led to trying new treatment options for brucellosis. That is why drugs such as fluoroquinolones and macrolides, gentamicin, ciprofloxacin, cotrimexazole, tetracycline, and erythromycin are used in Iran as new antimicrobial agents. The antibiotic tigecycline, used as a drug against multidrug-resistant bacteria, has been included in the treatment regimens in Iran (15, 16).

This research intended to identify and differentiate *Brucella* strains in clinical human specimens and animal specimens using uniplex PCR. Sensitivity of the cultured strains to doxycycline, rifampin, streptomycin, cotrimexazole, tigecycline, ciprofloxacin, gentamicin, erythromycin, and tetracycline was then studied. Finally, antibiotic resistance in the cultured strains was compared using the E-Test with Kirby-Bauer disk diffusion susceptibility test

Materials and Methods

Specimen collection

This cross-sectional study was conducted on 30 *Brucella* isolates from human specimens and 28 *Brucella* isolates from animal specimens. The specimens were taken between April 2010 and May 2015. Twenty-eight human specimens consisting of 25 blood specimens and five CSF specimens, and 38 animal specimens including 8 liver specimens, 10 spleen specimens, and 20 blood specimens, were taken using simple random sampling.

Sampling was performed for humans by a specialist physician using syringes under sterile conditions from patients with symptoms of brucellosis such as fever, chills, fatigue, body aches, headache, joint pain, low back pain, and back pain. The animal specimens were also collected under sterile conditions by a specialist veterinarian from animals suspected of having brucellosis. Blood specimens were incubated at 37 °C for 21 d in blood culture medium (BacT/Alert, Biomerieux, France). One hundred microliters of the blood culture medium were then transferred to an agar *Brucella* culture medium (Merck-Germany)

under sterile conditions and the plates were incubated at 37 °C for 48 to 72 h.

After incubation, identification at the species level was carried out using colony morphology, Gram staining, oxidase, catalase and growth characteristics (17). Samples identified as *Brucella* species were stored in skim milk containing 10% sterilized glycerol at -80 °C until the next stages were carried out.

DNA extraction

DNA was extracted from the *Brucella* specimens using proteinase K and the phenol-chloroform method as explained in previous studies (18, 19). DNA stored at -20 °C until PCR was carried out.

Primer Design

A pair of 23 bp primers was designed able to recognize and differentiate simultaneously *B. melitensis* at 398 bp band, *B. abortus* at 520 bp band and *B. suis* at 770 bp band. The forward primer sequence was 5'-ATTGACACCTTGCCTGGACGG-3' and the reverse primer sequence 5'-GTTGAAAACCAGGGGCTGGC-3'.

Specificity, sensitivity, and reliability of the primers

To evaluate the specificity of the primers, DNA from species genetically very close to *Brucella* and non-infected blood specimens from humans, cattle, and sheep spleen DNA were used.

Serial dilution concentrations (10-1, 10-2, 10-3, and 10-4) of DNA from *B. abortus* S19 and *B. melitensis* 16M were prepared to evaluate sensitivity of the primers in PCR based on DNA concentration. Sterilized distilled water was used to dilute DNA.

PCR Amplification

Each PCR reaction mixture contained 8 µl Master mix 1X (Ampliqon Co, Denmark) that contained 1.5 mM MgCl2, 1X PCR buffer, 1 µl template DNA (0.5 µg), 0.15 mM dNTP, 1.25U Taq DNA polymerase, 10 pmol of each forward and reverse primers and sterile distilled water up to 25 µL.

PCR were performed in a GenAmp PCR system (Eppendorf, USA) according to the following program: predenaturation for 5 min at 95 0C followed by 36 cycles each containing denaturation at 95 0C for 1 min, annealing at 67 0C for 30 sec and Extension at 72 0C for 30 sec, followed by final extension at 72 0C for 5 min.

Then, The PCR products were analyzed using the electrophoresis technique on 1.5% agarose gel for 1 h at 85 volt and 25 mA, stained by SYBER green and visualized under UV transilluminator. Finally, amplification products were further evaluated by sequencing and restriction digestion procedures.

Extracted genomes of vaccine strains of *B. abortus* S19 and *B. melitensis* 16 M as positive control and suspension containing all of the reagents except template as negative control were used. All PCRs were carried out in triplicate.

Antibiotic sensitivity

To determine antibiotic sensitivity of *Brucella*, the minimum inhibitory concentration (MIC) of the antibiotic is measured. For this purpose, methods such as microbroth dilution and E-Test can be used. E-Test (following the directions of the manufacturer) was used to determine the MIC values of doxycycline, rifampin, streptomycin, tigecycline, trimethoprim/cotrimexazole, tetracycline, gentamicin, and erythromycin.

Disk diffusion susceptibility tests were performed for the antibiotics ciprofloxacin, trimethoprim-sulphamethoxazole, tigecycline, tetracycline, dicloxacillin, gentamicin, streptomycin, ceftazidime, erythromycin, ofloxacin, cephradine, and rifampin.

MIC values were measured using the E-Test according to the tables of instructions in the sec-

tion on clinical directions concerning *Brucella* published by the Clinical and Laboratory Standards Institute (CLSI). These directions apply to slow-growing bacteria. Of course, the antibiotic tigecycline is not referred to in the directions but, as recommended by the US Food and Drug Administration (FDA), ≤0.25 was considered the sensitivity point and the MIC breakpoint for tigecycline (20).

All of the above steps were repeated three times to be sure of the results. In all stages of the tests, *B. melitensis* 16M, ATCC 23456, *B. abortus* S19, *E. coli* ATCC 25922 and 29213, and *S. aureus* ATCC were used for quality control testing.

Table. 1
Primer specificity

Strain	PCR identification	Strain (from human)	PCR identification
Brucella spp.	68/68*	*Pseudomonas aeruginosa*	0/1
Brucella abortus	8/68	*Campylobacter* sp.	0/1
Brucella melitensis	60/68	*Klebsiella pneumoniae*	0/1
Salmonella enterica ATCC:9270	0/2	*Listeria monocytogenes*	0/1
Agrobacterium tumefaciens PTCC 1654	0/1	*Proteus mirabilis*	0/1
Staphylococcus aureus ATCC:6538	0/1	*Salmonella enteritidis*	0/1
Shigella sonnei ATCC:9290	0/1	*Staphylococcus aureus*	0/1
Shigella flexneri ATCC:12022	0/2	*Streptococcus pneumoniae*	0/1
DNA Extraction from human Blood	0/2	*Escherichia coli* O157:H7	0/2
DNA Extraction from human Spleen	0/1	*Vibrio cholerae* PTCC 1611	0/1

Ethics approval of research

The study was approved by Research Ethics Committee of Tehran University of Medical Sciences.

Results

Distribution of specimens

Of the 68 collected specimens, 30 were human specimens (11 from females and 19 from males). The specimens were taken from patients with serum titers higher than 1:160. Five patients from Hamadan and three from Arak had been referred to medical laboratories in Tehran. Twenty-two patients were from Tehran and the rest from other cities. Thirty-eight animal specimens were also tested: 28 from sheep and 10 from cattle.

Primer specificity and sensitivity

DNA from two standard *Brucella* strains, from all specimens, from 15 pathogenic species of bacteria, from human blood, and from spleen tissue were assayed to study specificity of the primers designed for PCR. At DNA concentration of 10-20 ng/µl, the primers were only able to detect and differentiate two *Brucella* species, and nonspecific bands were not observed on gel electrophoresis. Moreover, no bands were detected after proliferation of other DNA samples (Table 1).

PCR test results

As shown in Fig. 1, this test was able to identify *Brucella* genus and species by carrying out uniplex PCR. This is a rapid test for diagnosing *Brucella* at the level of species. Based on results of uniplex PCR, of the 68 confirmed *Brucella* specimens, 8 were *B. abortus* (two in animal specimens and six in human specimens). Therefore, at the $P \leq 0.05$ level, *B. abortus* infection was more prevalent among humans than animals. Moreover, there were 60 confirmed *B. melitensis*

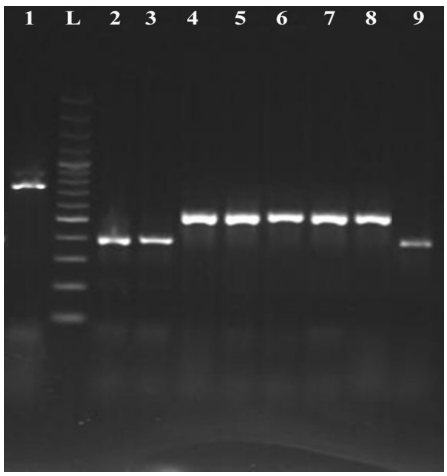

Fig. 1
Agarose gel electrophoresis of *B. melitensis, B. abortus and B.suis* strains. (M: DNA molecular marker, bp: base pair). Lane 1: animal *B. suis*, 770 bp, Lane 2: *B. melitasis* 16M 398 bp, Lane 3: animal *B. melitensis* and Lane 9: Human *B. melitasis.* Lane 4: *B. abortus* S19, 520 bp. Lane 5: animal *B. abortus.* Lane 6-8: human *B. abortus*, negative control not shown in Fig. 1

specimens (36 in animal specimens and 24 in human specimens): there were no significant differences between the patients and the animals with respect to the prevalence of the disease. Moree over, one *B. suis* isolated from animal samples.

Results of antibiotic sensitivity determined by the disk diffusion susceptibility test

Based on the directions of the company manufacturing the disks and CLSI guidelines, inhibition zone diameters were measured. All human specimens were sensitive to ciprofloxacin, trimethoprim-sulphamethoxazole, tigecycline, tetracycline, dicloxacillin, gentamicin, streptomycin, ceftazidime, ofloxacin, cephradine, and rifampin. Only three specimens exhibited intermediate susceptibility to rifampin. All 30 human specimens and 35 animal specimens were resistant to erythromycin. Moreover, among the 38 animal

Table. 2
Antibiotic sensitivity of human and animal specimens obtained by employing the E-Test

Antibiotics	*Brucella* from human isolates			*Brucella* from animal isolates			Breakpointc for susceptibility *(µg/ml)
	(S)	(I)	(R)	(S)	(I)	(R)	
Doxycycline	30	-	-	38	-	-	1≥
Tigecycline	30	-	-	34	-	4	***ND
TMP-SXT[a]	28	1	1	38	-	1	0.5≥
Ciprofloxacin	30	-	-	38	-	-	1≥
Streptomycin	30	-	-	38	-	-	8≥
Rifampin	30	-	-	38	-	-	**1≥
Tetracycline	21	5	4	38	-	-	1≥
Gentamicin	25	2	3	36	1	1	***ND

TMP-SXT: Trimethoprim/sulfamethoxazole, S: Sensitive, I: Intermediate sensitive, R: Resistant, *: Standard breakpoints are from CLSI guidelines for slowly growing bacteria (Haemophilus spp.), **: Rifampin I: 2, R: ≥4. ***ND, not defined by CLSI standards

Table. 3
The MIC values (µg/ml) for *Brucella* strains isolated from humans (n: 30)

Antibiotics	MIC results (µg/ml)																		
	0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8
Doxycycline	-	-	-	-	5	5	15a	5b	-	-	-	-	-	-	-	-	-	-	-
Tigecycline	-	-	-	-	11a	-	11b	8	-	-	-	-	-	-	-	-	-	-	-
TMP-SXT	-	-	-	-	7	-	19a	2b	-	-	1	-	1	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	4	-	19a	7b	-	-	-	-	-	-	-	-	-	-	-
Streptomycin	-	-	-	-	-	-	-	-	-	4	5a	5	4b	-	8	4	-	-	-
Rifampin	-	-	-	-	-	-	4	1	-	-	4	12a	5	1	3	-	-	-	-
Tetracycline	-	-	-	-	6	-	-	10a	-	-	7	6	1	-	-	-	-	-	-
Gentamicin	-	-	-	-	5	-	20a	-	-	-	2	-	1	2	-	-	-	-	-

MIC: Minimum inhibitory concentration, TMP-SXT: Trimethoprim/sulfamethoxazole, a : MIC_{50}, b : MIC_{90}.

Table. 4
The MIC values (µg/ml) for *Brucella* strains isolated from animals (n: 38)

Antibiotics	(MIC results (µg/ml																		
	0.016	0.023	0.032	0.047	0.064	0.094	0.125	0.19	0.25	0.38	0.5	0.75	1	1.5	2	3	4	6	8
Doxycycline	-	-	-	8	8	-	18a	-	-	-	2	2	-	-	-	-	-	-	-
Tigecycline	-	5	6	8	5	-	10a	-	-	-	2	1	1	-	-	-	-	-	-
TMP-SXT	-	-	5	-	12a	-	20b	-	-	-	-	-	1	-	-	-	-	-	-
Ciprofloxacin	-	-	1	10	5	2	20a	-	-	-	-	-	1	-	-	-	-	-	-
Streptomycin	-	-	-	-	2	-	-	-	-	-	-	5	14a	4	7	1	5	-	-
Rifampin	-	-	5	-	16a	2	12b	-	-	-	2	-	1b	-	-	-	-	-	-
Tetracycline	-	-	8	-	2	2	22a	3	-	-	-	1	-	-	-	-	-	-	-
Gentamicin	-	-	-	7	3	2	23a	1	-	-	1	-	1	-	-	-	-	-	-

MIC: Minimum inhibitory concentration, TMP-SXT: Trimethoprim/sulfamethoxazole, a: MIC_{50}, b : MIC_{90}.

specimens, four were also of intermediate sensitivity to tigecycline and three were resistant to rifampin.

The only significant difference in sensitivity to antibiotics at $P \leq 0.05$ was to erythromycin, and the animal and human specimens in this study exhibited no significant differences in sensitivity to the antibiotics.

Results of determining MIC values and antibiotic sensitivity using the E-Test

Table 2 shows results concerning antibiotic sensitivity of human and animal specimens obtained by employing the E-Test.

Doxycycline was the most effective antibiotic against *Brucella*, followed by tigecycline, while streptomycin and rifampin were the least effective (Table 3 & 4).

Discussion

Brucellosis is an endemic disease in developing countries (21). High prevalence of the disease accounts for a large part of annual treatment costs in health systems, and inflicts considerable economic losses on the livestock industry (22). Rapid and definitive diagnosis of this disease saves time and treatment costs and allows physicians to select appropriate treatment strategies. PCR is one of the reliable techniques for this purpose and its use is increasing compared to serological diagnostic tests because it yields fewer false-positive and false-negative test results (23). This study introduced a test for diagnosing brucellosis, and for differentiating *Brucella* at the strain level that is more efficient than other methods because it performs PCR only once and uses two primers, while in previous researches one pair of primers was used for identifying *Brucella* species and another pair for differentiating the strains (18, 24).

The introduced test uses a pair of primers that allow researchers to identify simultaneously *Brucella* genus and species thus eliminating the need to use the multiplex PCR technique, which is a common molecular method for diagnosing *Brucella*. The probability of infection is greater in multiplex PCR because several pairs of primers are used, and the costs of manufacturing and storing the primers are higher compared to the method employed in this study. Moreover, this test is able to differentiate *B. melitensis* and *B. abortus* from *B. suis*, while in similar studies sometimes up to six pairs of primers were used simultaneously to identify these three species.

Results of differentiating *Brucella* species in this study were similar previous studies, where *B. melitensis* (an endemic species of *Brucella* in

Central Asia) continued to be the main cause of brucellosis in humans and animals. The results also showed there was a significant difference in the extent of *B. abortus* infection in humans and animals: although the samples in this study were selected by the simple random selection method, all animal *B. abortus* isolated found in sheep.

This study investigated and determined sensitivity of *Brucella*, as an intracellular microorganism, to antibiotics in addition to introducing a rapid diagnostic strategy. According to the WHO guidelines for the treatment of the disease caused by these bacteria, at least one antibiotic of high cellular penetration must be used (9). Doxycycline, classified as a golden treatment medicine, is recommended by this organization (9). This drug, which is a derivative of tetracycline but with improved pharmacokinetics and better performance, is the most commonly prescribed medicine for the treatment of brucellosis. Doxycycline was the most effective medicine for the treatment of brucellosis (11, 12). We showed that it was the most efficient among the studied antibiotics because it had the lowest MIC50 and MIC90.

The use of this antibiotic has led to four of the 38 animal specimens having MIC values close to 1 μg/ml, which shows increased resistance to doxycycline. Our study showed that the use of this antibiotic to treat animals should be reviewed because resistant bacteria are easily transmitted from animals to humans, and it is predicted that in future resistant strains to doxycycline will be detected in humans.

Use of antibiotics of the Aminoglycoside family such as gentamicin together with tetracycline is also used to treat brucellosis in Iran (6). Cephalosporins such as streptomycin are used in brucellosis treatment. Streptomycin was an effective medicine for treating brucellosis (25, 26). Our study indicated that the use of streptomycin was still effective, but this antibiotic has very toxic effects on the nervous system and causes hearing disorders during treatment and, hence, is not an appropriate drug for the treatment of brucellosis. These results agree with those reported by previous researchers (27, 28).

Gentamicin resistance pattern was also analyzed in our study. Nowadays, use of this drug is restricted due to its high toxic side effects. In this study, four specimens showed complete resistance, and one specimen intermediate resistance, to gentamicin. It seems that the use of the streptomycin and gentamicin regimen should be reviewed because it has many side effects and resistance to the regimen is increasing.

At present, a combination of rifampin and doxycycline is considered the best oral treatment strategy for brucellosis (29). Our results support previous studies (2, 30). Rifampin had higher MIC values in the research carried out by these researchers in 1999 and 2013 compared to averages of previous years. Moreover, the MIC value of this antibiotic in our study was higher compared to those reported by them. Therefore, we conclude that resistance to rifampin is increasing, and we predict that in future research isolates resistant to rifampin will be detected. In our research, we identified three isolates with intermediate resistance to rifampin.

The sulfamethoxazole-trimethoprim regimen is used to treat brucellosis in animals. Moreover, the cotrimexazole-rifampin regimen is administered for treatment of children with brucellosis (31). Our study showed that two *Brucella* specimens were completely resistant to this regimen and one specimen exhibited intermediate resistance to it. Considering the complete and intermediate resistance of these two specimens to this regimen, we suggest its use be reviewed.

Other progressive drug regimens on the treatment of brucellosis include the use of ciprofloxacin together with a member of the Quinolone family such as ofloxacin. Fluoroquinolones are antibiotics with high cellular penetration, and

they were efficient against *Brucella* (32). In the disk-diffusion susceptibility test in our research, all human and animal specimens were sensitive to these antibiotics. Studies conducted by other researchers also confirmed this and, therefore, we suggest they be used for treating brucellosis.

The synthetic member of the new generation of tetracyclines called tigecycline is among the drugs that are used in single-drug regimens at present to treat brucellosis and, since it has few side effects (occasional vomiting and nausea have been reported) is use is increasing by the day (33). The mechanism of action of tigecycline against *Brucella* is similar to that of tetracycline, and our research showed that resistance to it is increasing and is getting close to resistance to tetracycline (34). In this study, four animal specimens were completely resistant to tigecycline, four human specimens were also completely resistant, and five exhibited intermediate resistance to, tetracycline. Single-drug therapy for brucellosis using tigecycline has increased resistance to it, and it is better to use tigecycline in combination treatment. Injection of this drug requires hospitalization, which increases treatment costs. Results concerning the sensitivity pattern to this antibiotic showed that the causal agent of brucellosis is more sensitive to doxycycline compared to tigecycline.

Conclusion

This study introduced a rapid method of identifying and differentiating *Brucella* at the level of species with the sensitivity of 99% and specificity of 100%. It is faster, costs less, and is more accurate than serological studies and the multiplex PCR.

The gold standard treatment of brucellosis recommended by the WHO is still efficient in Iran. This study also showed resistance to tigecycline is increasing, and it predicts strains resistant to rifampin will soon be reported. The ciprofloxacin and ofloxacin regimen is also suggested as an effective regimen for treating brucellosis because all the studied specimens were sensitive to it.

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Conflict of interest

The authors declare that there is no conflict of interests.

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