Identification and Determination of Antibiotic Susceptibilities of *Brucella* Strains Isolated from Patients in Van, Turkey by Conventional and Molecular Methods

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**Abstract**

**Purpose:** Brucellosis is a worldwide zoonotic disease and still constitutes a major public health problem. In this study, we aimed to identify biovars of *Brucella* strains isolated from clinical specimens taken from brucellosis patients from the Eastern Anatolia region as well determine the susceptibility of these isolates to tigecycline and azithromycin, drugs that may serve as alternatives to the conventional drugs used in the therapy.

**Materials and methods:** Seventy-five *Brucella* spp. isolates were included in the study. All strains were identified by both conventional and molecular methods. *Brucella* Multiplex PCR kit (FC-Biotech, Code: 0301, Turkey) and *B. melitensis* biovar typing PCR kit (FC-Biotech, Code: 0302, Turkey) were used for molecular typing. Antimicrobial susceptibilities of all strains were determined by E-tests.

**Results:** By conventional biotyping, 73 strains were identified as *B. melitensis* biovar 3 and two strains as *B. abortus* biovar 3. Molecular typing results were compatible with conventional methods. The MIC₉₀ and MIC₉₀ values of doxycycline were 0.047 and 0.094; tigecycline 0.094 and 0.125; trimethoprim/sulfamethoxazole 0.064 and 0.19; ciprofloxacin 0.19 for both; streptomycin 0.75 and 1; rifampin 1 and 2 and azithromycin 4 and 8. According to the MIC values, doxycycline was found to be the most effective antibiotic, followed by tigecycline, trimethoprim-sulfamethoxazole and ciprofloxacin.

**Conclusion:** Currently recommended antibiotics for the treatment of brucellosis such as doxycycline, rifampin, streptomycin, trimethoprim-sulfamethoxazole and ciprofloxacin were found to be still effective. While our results showed that tigecycline can be used as an alternative agent in the treatment of brucellosis, azithromycin has not been confirmed as an appropriate agent for the treatment.

**Key words:** *Brucella*; biotyping; antibiotic sensitivity; E-test.

**Introduction**

Gram-negative bacteria from genus *Brucella* are the causative agent of brucellosis. Brucellosis is a serious public health problem worldwide, especially in the Middle East, Mediterranean countries, South...
America and Central Asia with 500,000 new cases reported every year.\textsuperscript{2,4} Although the prevalence of brucellosis has decreased in many developed countries due to various eradication programs, it still remains an important endemic disease in Turkey, where 10,000 human brucellosis cases are reported annually.\textsuperscript{5,6} The most common symptoms of brucellosis are recurring fever, chills, shivering, fatigue, body aches, joint and back pain, anorexia and weakness. During the course of the disease, complications like spondylitis, wedge-shaped vertebral collapse, meningitis, pancarditis, bronchopneumonia, acute respiratory distress syndrome, unilateral epididymo-orchitis and uveitis can be encountered.\textsuperscript{1,7,8}

Together with the isolation and identification of \textit{Brucella} strains, conventional and molecular biotyping is very important for epidemiologic studies and eradication programs.\textsuperscript{9} According to the World Health Organization’s recommendations for the treatment of human brucellosis published in 1986, the gold standard for brucellosis therapy is doxycycline-rifampin combination for six weeks or a combination of 6 week doxycycline and 2-3 weeks streptomycin.\textsuperscript{10} The recommended drug combinations are widely used in Turkey for the treatment of brucellosis without serious complication. However, some studies have shown that the drug regimens recommendations of WHO are not implemented in clinical practice and relapses due to inadequate therapy can occur in 5-10\% of patients. Factors such as high incidence of relapse, resistance to rifampin (especially in regions with endemic tuberculosis) and the ototoxic and nephrotoxic side effects of streptomycin have led to the investigation of new therapeutic options for brucellosis.\textsuperscript{11,12} Accordingly, fluoroquinolones and macrolides are presented as new alternative antimicrobial agents for treatment.\textsuperscript{13} It has been shown that tigecycline, a novel antibiotic with activity against multidrug resistant bacteria, could be a potentially promising agent for brucellosis treatment.\textsuperscript{14,15}

In this study, we aimed to identify \textit{Brucella} strains isolated from various clinical specimens using conventional and molecular methods and to determine their antimicrobial susceptibilities to doxycycline, rifampin, streptomycin, trimethoprim-sulfamethoxazole, azithromycin, tigecycline and ciprofloxacin using E-tests.

Materials and Methods

\textbf{Brucella strains} 

A total of 75 \textit{Brucella} strains, isolated in our laboratory from various clinical specimens between January 2009 and June 2011, were included in the study. \textit{Brucella} strains were isolated from blood (\(n = 71\)), CSF (\(n = 2\)), pleural (\(n = 1\)) and peritoneal fluid (\(n = 1\)). Samples were collected with syringes under sterile conditions from the patients prediagnosed with brucellosis displaying symptoms such as fever, chills, shivering, fatigue, body aches, headache, joint and back pain. Blood samples were collected into adult blood culture bottles and other sterile body fluids (CSF, pleural and peritoneal fluid) into pediatric blood culture bottles. The samples were incubated for at least 21 days in the automated blood culture system (BacT/Alert, Biomerieux, France). Positive signals were recorded and the samples were inoculated with sterile loop onto sheep blood agar, Eosin Methylene Blue (EMB) agar and \textit{Brucella} agar and incubated for 48-72 h at 37°C.

After incubation, identification at species level was performed using colony morphology, Gram staining, growth characteristics, oxidase, catalase, movement and polyvalent antisera agglutination methods. Samples with strains identified as \textit{Brucella} spp. were stored in 10\% skim milk and preserved at -80°C until conventional biotyping, molecular typing and antibiotic susceptibility tests were performed.

\textbf{Biovar Determination by Conventional Methods} 

Strains identified as \textit{Brucella} spp. were analyzed by conventional biotyping methods including the CO\textsubscript{2} requirement, H\textsubscript{2}S production, urease activity, sensitivity to thionin and basic fuchsin, phage sensitivity and agglutination with monospecific A and M antisera.\textsuperscript{16}

\textbf{Molecular Identification and Brucella melitensis Molecular Typing} 

DNA isolation: \textit{Brucella} DNA was isolated by heat-lysis method. Strains were suspended in 500 µl TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and bacterial suspensions were exposed to 100°C for 10 min to ensure cell lysis. After that, samples were centrifuged at 12,000 rpm for 10 min and an upper clear phase containing DNA was taken.

For the species identification and molecular typing of the \textit{Brucella} strains, a two-stage method was utilized. A \textit{Brucella} Multiplex PCR kit (FC Biotek Code: 0301, Ankara, Turkey) was used for differentiation of six different species in the \textit{Brucella} genus. We added 3-5 µl of the DNA sample to 45 µl of amplification mixture. For amplification, 40 PCR cycles were performed. The amplification products were separated by 1.5\% agarose gel electrophoresis and evaluated with standard molecular weight samples.

In the second stage, samples identified as \textit{B. melitensis} were analyzed using a \textit{Brucella melitensis} biovar typing PCR kit (FC-Biotek, Code: 0302, Ankara,
Turkey) for biovar differentiation of B. melitensis biovar 1, 2 and 3 in a single PCR tube.\textsuperscript{17} For this purpose, 2 μl of DNA extract was added to 45 μl of amplification mixture, and 40 PCR cycles were performed. The PCR products (10 μl) were separated by 1.3% agarose gel electrophoresis and visualized with the Alphalmager 2000 (Alpha Innotech Corporation, San Leandro, CA).

**Determination of Antimicrobial Susceptibility**

In vitro determination of antibiotic efficacy against *Brucella* strains is based on the Minimal Inhibitory Concentration (MIC) values. For this purpose, methods such as microbroth dilution, agar dilution and E-test can be used. In our study, the E-test method was utilized as it is a reliable, reproducible, less labor-intensive, less time-consuming and more practical micro-dilution method.\textsuperscript{13} Susceptibility to doxycycline, streptomycin, rifampin, trimethoprim-sulfamethoxazole, ciprofloxacin, tigecycline and azithromycin were assessed.

Suspension of the growing bacterial colonies in Mueller-Hinton broth (Oxoid\textsuperscript{®}, Hampshire, UK) with a turbidity equivalent to that of a 0.5 McFarland standard was inoculated by rolling a sterile swab over 5% sheep blood-enriched Mueller-Hinton agar (Oxoid\textsuperscript{®}, Hampshire, UK) plates. The plates were incubated at 37°C for 48h.

Determination of the MIC was performed in accordance with the recommended reference values of the Clinical Laboratory Standards Institute’s (CLSI) guidelines for *Brucella* and slowly growing bacteria species. Tigecycline is not defined in the same guidelines and FDA recommendation of ≤0.25 as sensitive was utilized as interpretive MIC breakpoints for tigecycline.\textsuperscript{18,19}

In every stage of this study, *B. melitensis* (bv1: 16M, ATCC 23456; bv3: Ether, NCTC 10505), *B. abortus* (bv1: NCTC 10863; bv3: ATCC 23448), *B. suis* 1330 bv1 (NCTC 10316), *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 strains were used for the quality control.

This study was approved by the Non-Pharmacological Clinical Research Ethics Committee of the Yuzuncu Yil University Medical Faculty (approval No:12.01.2010/06).

**Results**

**Sample Distribution**

A total of 75 *Brucella* strains including 44 isolated from female and 31 from male patients were analyzed in this study. Samples had been sent from Infectious Disease (*n* = 38), Pediatrics (*n* = 17), Internal Medicine (*n* = 12), General Surgery (*n* = 2), Thoracic Surgery (*n* = 2), Orthopedics (*n* = 2), Gynecology (*n* = 1) and Brain Surgery (*n* = 1) departments of Yuzuncu Yil University, Faculty of Medicine, Training and Research Hospital.

**Conventional and Molecular Typing Results**

Seventy-three (97.3%) of *Brucella* strains were identified as *B. melitensis* biovar 3 and the rest of them (2.7%) were identified as *B. abortus* biovar 3 by conventional biotyping. The results of agarose gel electrophoresis and multiplex PCR were in accordance with conventional biotyping. Agarose gel electrophoresis image of *B. melitensis* strains is shown in Figure 1.

**Results of Antibiotic Susceptibility Testing**

According to antibiotic susceptibility testing by E-test, all strains were found to be susceptible to doxycycline, tigecycline, trimethoprim-sulfamethoxazole, ciprofloxacin, streptomycin. Forty of the 73 *B. melitensis* strains were susceptible to rifampin, 33 strains were intermediate-resistant to rifampin and 34 strains were resistant to azithromycin. The two strains identified as *B. abortus* were resistant to azithromycin; one was sensitive, and another intermediate-resistant to rifampin. The antibiotic susceptibilities of *Brucella* strains are shown in Table 1.

According to MIC\textsubscript{50} and MIC\textsubscript{90} values, doxycycline was the most effective antibiotic against *B. melitensis* strains. After doxycycline, the most effective antibiotics were tigecycline, trimethoprim-sulfamethoxazole and ciprofloxacin, respectively. The highest MIC\textsubscript{50} and MIC\textsubscript{90} values had azithromycin, rifampin and streptomycin, respectively. The MIC ranges and MIC\textsubscript{50} and MIC\textsubscript{90} values of the antibiotics used in this study are given in Table 2.
Table 1. The distribution of antibiotic susceptibility of Brucella strains.

| Antibiotics | B. melitensis | B. abortus | The limit of sensitivity |
|-------------|---------------|------------|-------------------------|
|             | (S) | (I) | (R) | (S) | (I) | (R) |                     |
| Doxycycline | 73  | -   | -   | 2   | -   | -   | ≤1                     |
| Tigecycline | 73  | -   | -   | 2   | -   | -   | ≤0.25                  |
| TMP-SXT*   | 73  | -   | -   | 2   | -   | -   | ≤2/38                  |
| Ciprofloxacin | 73 | -    | -   | 2   | -   | -   | ≤1                     |
| Streptomycin | 73 | -   | -   | 2   | -   | -   | ≤8                     |
| Rifampin    | 40  | 33  | -   | 1   | 1   | -   | ≤1*                    |
| Azithromycin | 39 | -    | 34  | -   | -   | 2   | ≤4                     |

*TMP-SXT: Trimethoprim/sulfamethoxazole, S: Sensitive, I: Intermediate sensitive, R: Resistant, * Rifampin I: 2, R: ≥4.

Table 2. The MIC values (µg/ml) for Brucella melitensis strains (n:73).

| 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 | 2 | 3 | 4 | 6 | 8 | 12 | 16 |
|-------------|---------------------|-------|-------|-------|-------|-------|-------|-------|------|------|------|----|-----|---|---|---|---|---|---|---|
| Doxycycline |                     | 7     | 12    | 27*   | 18    | 8*   | 1     |       |      |      |      |    |     | 1 |   |   |   |   |   |   |
| Tigecycline |                     | 5     | 23    | 27    | 14    | 4    |       |       |      |      |      |    |     |   |   |   |   |   |   |   |
| TMP-SXT     |                     | 2     | 4     | 11    | 10    | 12   | 13    | 1     | 3    | 1    | 3   |    |     |   |   |   |   |   |   |   |
| Ciprofloxacin |                 | 22    | 46    | 4     |       |   |       |       |      |      |      |    |     |   |   |   |   |   |   |   |
| Streptomycin |                     |       |       |   |       | 27   | 24    | 19    | 3    |      |      |    |     |   |   |   |   |   |   |   |
| Rifampin    |                     | 1     | 3     | 14    | 22    | 21   | 11    | 1     |      |      |      |    |     |   |   |   |   |   |   |   |
| Azithromycin |                     | 1     | 1     | 3     | 5     | 11   | 18    | 18    | 9    | 6    | 1   |   |     |   |   |   |   |   |   |   |

*MIC: Minimum inhibitory concentration, TMP-SXT: Trimethoprim/sulfamethoxazole, *: MIC<sub>50</sub>, #: MIC<sub>50</sub>.

Discussion

*Brucella melitensis* is the most frequently reported cause of human brucellosis worldwide and it is commonly isolated from patients. In several countries, it is reported as endemic.1 In Turkey, concordantly, *B. melitensis* is the main cause of human brucellosis.2 In most of the studies conducted both abroad and in Turkey, *B. melitensis* has been reported as the only isolated species.20-22 However, the results of some studies have confirmed the presence of *B. abortus* in Turkey.23 In previous biotyping studies, it has been shown that the most common variation is *B. melitensis* biovar 3.9 From the 75 strains isolated in our study, 73 were identified as *B. melitensis* biovar 3 and two strains as *B. abortus* biovar 3.

As *Brucella* spp. are intracellular microorganisms, at least one antibiotic from the combination used for the treatment should be an antibiotic with a good intracellular penetration.24 Doxycycline is ranked as a gold standard drug by the World Health Organization (WHO) and it became the most commonly prescribed tetracycline derivative in the treatment of *Brucella* infections because of its superior pharmacokinetics profile.8 In many studies, doxycycline was found to be the most effective antibiotic against *Brucella* strains.13,21,25 In our study as well, doxycycline was compared to other antibiotics used in therapy and was found to be the most effective antibiotic with the lowest MIC<sub>50</sub> and MIC<sub>50</sub> values.

Aminoglycosides combined with tetracyclines are still commonly used for brucellosis treatment. The most preferred aminoglycoside for brucellosis is streptomycin. Other studies from different parts of the world have also determined streptomycin as one of the most effective antibiotics in treatment of brucellosis. However, as streptomycin exerts toxic effects upon the eighth nerve and carries the risk of the side effect of ototoxicity during therapy, an alternative treatment regimen is suggested.8,26 MIC values for streptomycin obtained in the present study were higher when compared with other tested antibiotics tested and in accordance with results from previous studies from Turkey.13,22,27

A combination of rifampin and doxycycline is the best oral therapy for brucellosis nowadays.28 In previous studies, rifampin has been presented together with less active antibiotics and existence of intermediate-sensitive strains has been reported. Aliskan et al.29 found that, in an endemic region, from 65 isolates containing *B. melitensis* strains isolated from bone marrow and blood, 8 showed intermediate sensitivity to rifampin. In our study, the MIC<sub>50</sub> value of the rifampin was higher compared to the other tested antibiotics, and from a total of 75 strains, 34 were found to have intermediate sensitivity to rifamp-
pin. The emergence of strains of intermediate sensitivity is likely due to the frequent usage of rifampin as an antitubercular agent in long-term, multi-drug tuberculosis therapy in Turkey, which is accepted as an endemic region for tuberculosis.7

Trimethoprim-sulfamethoxazole is another agent recommended for the treatment of brucellosis. It is used in combination with rifampin in pregnant women and children under 8 years old, who cannot use tetracycline. A combination of trimethoprim-sulfamethoxazole, doxycycline and rifampin is successfully used in the treatment of Brucella endocarditis, which is the brucellosis complication with the highest mortality rate.30 Previous studies have identified trimethoprim-sulfamethoxazole as an effective antibiotic with low MIC levels.20 Aliskan et al.29 reported trimethoprim-sulfamethoxazole as the most effective antimicrobial agent with the lowest MIC50 and MIC90 values. In our study, trimethoprim-sulfamethoxazole was found to be the most effective antibiotic after doxycycline according to the MIC50 and it was the third most effective antibiotic, after doxycycline and tigecycline according to the MIC90.

Azithromycin is another antibiotic with good tissue penetration features that can be used in combination with rifampin, especially during pregnancy.24 In many studies, azithromycin was found to be an effective antibiotic against Brucella strains.27,31 In the present study, however, azithromycin’s MIC values were high. Additionally, when compared to other tested antibiotics, azithromycin’s activity was the lowest and resistance was reported in 36 strains. This might be due to the regional differences in antimicrobial susceptibilities between countries.

Ciprofloxacin and ofloxacin are the leading quinolones effective in the treatment of brucellosis. Fluoroquinolones easily penetrate into the cells and they are efficient against Brucella spp.1,8,12 Previous studies confirmed fluoroquinolones as effective against Brucella.5,20,21,28 The MIC values of ciprofloxacin obtained in our study were found to be similar to the results of previous studies. Due to high relapse rate associated with ciprofloxacin monotherapy, it should be used only in combined regimens.28

A semi-synthetic member of a new generation tetracycline group, tigecycline, may be of importance for the treatment of brucellosis in the future.14 Tigecycline’s activity against Brucella strains is similar to that of tetracycline; however, the development of resistance to this antimicrobial agent is more difficult. From a pharmacological point of view, monotherapy for brucellosis with tigecycline appears possible.12

With the exception of nausea and vomiting, tigecycline’s side effects are mild and the only restrictive factor for its usage is that administration can be provided only by intravenous infusion which requires the hospitalization of patients.15 Additionally, the high cost of tigecycline therapy is yet another factor that limits its wider usage. The reported results suggest tigecycline as a promising therapeutic option although it is not as effective as doxycycline.5,13,32 In the present study, tigecycline was found to be the most effective antibiotic after doxycycline. According to this result, tigecycline is an appropriate agent for the treatment of brucellosis. However, its administration by intravenous infusion and the consequent need for hospitalization of patients limits its usage to cases with no response to treatment or to complicated cases.

In conclusion, our results have revealed that B. abortus antigens in addition to B. melitensis should be used in conventional and molecular biotyping of brucellosis in eastern Turkey. The World Health Organization’s recommendations for brucellosis treatment published in 1986 still appear to be effective in Turkey. Although azithromycin does not seem to be an appropriate agent for the treatment of brucellosis in our region due to its high MIC values and presence of resistant strains, tigecycline could be considered in cases with no response to treatment or in complicated cases.

Competing Interests

The authors have declared that no competing interest exists.

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