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Running head: Antimicrobial susceptibility of *Brucella*
Summary

Current drug regimens for brucellosis are associated with relatively high rates of therapeutic failure or relapse. Reduced antimicrobial susceptibility of *Brucella spp.* has been proposed recently as a potential cause of therapeutic failure. The aim of this study was to evaluate the antibiotic resistance pattern of *Brucella melitensis* clinical isolates by E-test method in Hamadan, west of Iran. In a 15-month period, all patients with suspected brucellosis were enrolled. Blood Specimens were collected for diagnosis of brucellosis by BACTEC system and serological tests. Antimicrobial susceptibility of clinical isolates to seven antibiotics was assessed by the E-test method. One hundred forty-nine patients with brucellosis were diagnosed. Culture of clinical samples were positive in 38.3%, of which, 91.2% were associated with positive serological test. No significant associations were found between serology and culture method. All *Brucella* isolates were susceptible to doxycycline, streptomycin, gentamicin, ciprofloxacin and moxifloxacin. However, decreased sensitivity to rifampin and trimethoprim-sulfamethoxazole was found in 35.08% and 3.5% of isolates, respectively. Because of the high rates of intermediate sensitivity to rifampin among *Brucella* isolates, this drug should be prescribed with caution. We recommend restricting the use of rifampin for treatment of brucellosis except as an alternative drug for special situations.
Introduction

Brucellosis is a widespread zoonotic disease, with significant economic and major public health problem. Despite animal vaccination, brucellosis remains endemic in countries in the eastern Mediterranean region, including Iran (1-3). High incidence of brucellosis has been reported from Hamadan province, west of Iran (4,5).

Human brucellosis is most commonly diagnosed by serological methods such as standard tube agglutination and ELISA. However, blood culture is considered the gold standard test (6). Because of the need for biosafety level 3 precautions, antimicrobial susceptibility testing of Brucella isolates is not generally performed in clinical laboratories.

Brucella is an intracellular pathogen that involves macrophages. The treatment of brucellosis requires the use of antibiotics with ability to penetrate into the macrophages and kill bacteria. Betalactams such as penicillin and cephalosporin and also macrolide antibiotics are less effective in vivo as consequence relapses of brucellosis were seen that it is not relevant to brucella drug resistancy but it is associated with the ability of microorganisms to survive within host cells away of the antibiotics. Therefore, the selection of antibiotics with intracellular activity appears critical in the management of brucellosis (7). Several antibiotics including tetracycline, doxycycline, rifampin, trimethoprim-sulfamethoxazole, ciprofloxacin, and aminoglycosides have been used for treatment of brucellosis. Because of the high incidence of relapses after monotherapy with either antibiotic, the combination of at least two drugs is recommended.

In 1986, the World Health Organization introduced combination of two drugs, including doxycycline plus streptomycin and doxycycline plus rifampin as the standard regimen for treatment of brucellosis (8). Use of one of these drugs combined with a fluoroquinolone is not superior to the previous regimen but used as replacement therapy (9,10).
In spite of the combination therapy with a proper regimen, inappropriate response to treatment or relapse of brucellosis may occur. The relapse rate in a combination therapy with doxycycline and rifampin is nearly 10% which would be increased up to 30% in poorly compliant patients (11). Studies in endemic areas indicated that there was a possibility of increasing antibiotic resistance in brucellosis, especially resistance to rifampin. So, in a study in Egypt, high incidence of rifampin resistance has been reported (12).

With respect to the prevalence of brucellosis in Iran, especially in Hamedan province, widespread and inappropriate use of antibiotics in the community and the possibility of drug resistance infections and also the risk of recurrence and treatment failure of brucellosis, this study aimed to determine the antibiotic resistance pattern of *Brucella* isolates by E-test method in order to introduce the best treatment regimen for brucellosis.

**Materials and Methods**

One hundred forty-nine blood specimens were collected from patients with clinical diagnosis of brucellosis who referred to Infectious Diseases Section of Sina hospital in Hamedan, between May 2013 and August 2014.

The demographic data of all patients including age, sex, occupation, belonging to rural area, exposure to animals, consumption of unpasteurized dairy products, history of brucellosis, and history of antibiotics therapy documented.

Blood specimens were cultured in BACTEC automated blood culture system (9050 BD Company, USA). For nine patients with arthritis, synovial fluid and a bone marrow aspiration culture were also performed in addition to blood cultures, that the joint fluid cultures were positive in only two cases.
The specimens incubated at 37°C for 7 to 30 days. Each positive specimen was subcultured in Mueller Hinton plus 5% blood and *brucella* agar mediums. Differential tests including catalase, oxidase, urease and reactions with monospecific sera were performed to identify the organisms.

All of the 149 clotted blood specimens were centrifuged at 3000×g for 10 minutes due to obtained the sera and the Wright, Coombs Wright and 2ME (2-Mercaptoethanol) agglutination tests were performed to detect *brucella* antibodies.

Antibody titers equal to or greater than 1:160 were considered as positive for Wright and Coombs Wright tests, and titers equal to or greater than 1:40 were considered as positive for 2ME test.

Specimens with positive cultures were examined for drug susceptibility testing by E-test (Liofilchem, Italy). All of the plates incubated at 37 °C with 10% CO2 for 3 days and the minimum inhibitory concentration (MIC50 and MIC90) of the antibiotics including doxycycline, streptomycin, gentamicin, rifampin, ciprofloxacin, moxifloxacin, and trimethoprim-sulfamethoxazole to *Brucella* species were determined by E-test according to the CLSI guidelines. MIC50 and MIC90 levels were defined as the lowest concentration of the antibiotic at which 50% and 90% of the strains were inhibited, respectively. When MIC breakpoint for *Brucella* were not established, guidelines for fastidious bacteria (*Hemophilus influenza*) were used (13). The reference strain *Escherichia.coli* ATCC 25922 was used as quality control for susceptibility testing.

Data were analyzed using SPSS version 17 software. Statistical significance was determined by χ2 and Fisher’s Exact tests. The *P*-value less than 0.05 were considered significant. The study conformed to the provisions of the declaration of Helsinki and was approved by the Ethical Committee of Hamedan University of Medical Sciences (approved number: P-16359-202).

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Results

One-hundred forty-nine brucellosis patients were enrolled in this study. The age range of patients was 11 to 82 years (mean 41.1±17.2 years), 105 were males (70.5%) and 44 were females (29.5%). With regard to job status of the 149 patients studied, 68 (45.6%) were farmers, 39 (26.2%) were housewives, 28 (18.8%) were employed, 8 (5.4%) have administrative jobs, 4 (2.7%) were butcher, one patient was veterinarian and one was veterinary staff. According to residence area, 109 (73.2%) and 40 (26.8%) were living in rural and urban areas, respectively.

Seventeen of patients (11.4%) had history of recent brucellosis with a mean interval of 8.32 month (SD: 42.23) and 132 patients (88.6%) were new cases of brucellosis. And also, 128 patients (85.9%) had consumed unpasteurized dairy products.

The mean interval between onset of disease and diagnosis was 43.3 days (SD: 56.9). The frequency of symptoms and signs in 149 brucellosis patients are shown in Table 1. Based on clinical findings, fatigue, myalgia and fever were the most common complaints of patients.

The most common osteoarticular involvement was sacroiliitis (45%), of which, 27% were bilateral. Oachitis was found in 20 (13.4%) patients, of which, 30% were bilateral, and only one patient had concurrent endocarditis and orchitis.

Laboratory findings included anemia (36.9%), normal leucocyte count (82.6%), positive C-reactive protein (87.9%), increased erythrocyte sedimentation rate (53.7%), and elevated alanine transpherase (37.5%). Serological tests including Wright and 2ME were positive in 132 (88.6%) and 131 (88.5%) patients, respectively.

Fifty-seven (38.3%) culture of blood specimens and two specimens of joint fluid cultures were positive, of which, 52 specimens (91.2%) were associated with positive serological test. All of Brucella isolates were B.melitensis on biological testing. There was not any significant correlation between culture positivity and complication of brucellosis including sacroiliitis,
spondylitis, and epididymoorchitis. Also, no significant association was found between serological testing and culture.

The MIC50 and MIC90 values of Brucella isolates are shown in Table 2. Doxycycline and gentamicin had the lowest and rifampin had the highest MIC50 and MIC90 values. According to the MIC values, 35.8% of isolates were intermediate sensitive to rifampin. A small percentage of isolates (3.5%) had intermediate susceptibility to trimethoprim-sulfamethoxazole (Table 3).

Discussion

The diagnosis of brucellosis on nonspecific symptoms of the disease is a continuing problem for doctors especially in endemic areas. Fever, chills, sweating, headache, fatigue, loss of appetite and muscle and joint pain are nonspecific symptoms of brucellosis and also arthralgia, myalgia, back pain, anorexia are the most common complaints of patients. In this study, the most common symptom was fever (81.9%) and the most common osteoarticular complication was sacroiliitis (45%). According to various studies, the frequency of osteoarticular complications has been reported in 10 to 80% of patients (14,15). In a previous study in our region, the frequency of osteoarticular complications was 28/5%, of which, 75/7% had sacroiliitis (16).

Laboratory diagnosis is based on culture, serology and PCR. Serological test is global standard of diagnostic methods and usually used for diagnosis of brucellosis. In the absence of serological response, blood culture or PCR are useful to confirm the diagnosis. In the present study, 57 (38.3%) of blood specimens were culture positive, of which, 52 specimens (91.2%) were associated with positive serological test, and five specimens (8.8%) were negative serologically. Considering the lack of association between positive cultures and antibody titers, the blood cultures have same credit at low or high serological titers. Also,
contrary to early predictions, in this study, there was not any significant relationship between the presence of fever and positive blood culture. Accordingly, it can be recommended to perform blood culture in all patients with clinical suspicion of brucellosis and negative or low serological antibody titers, even in the absence of fever.

In this study, Brucella isolates were susceptible to all antibiotics except rifampin and trimethoprim-sulfamethoxazole, in which, decreased susceptibilities were found in 35% and 3.5% of isolates, respectively.

In contrast to the present study, other studies carried out in Peru (17) and China (18) reported no resistance to rifampin. However, intermediate sensitivity to rifampin was reported in some of the areas of Turkey (19,20) and also in Malaysia (21). Resistance to rifampin was higher in Egypt (12) and Kurdistan, west of Iran (22).

In a study of 355 isolates of B. melitensis in Egypt, 64% of isolates showed reduced susceptibility to rifampin and 2% to trimethoprim- sulfamethoxazole (12). Moreover, in another study of 231 isolates of B. melitensis in Qatar, increased MIC of rifampin was reported in 48% of isolates. All isolates were sensitive to doxycycline, streptomycin, gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole (23).

Increased MIC of rifampin was reported in 70% of 41 brucella isolates in Malaysia. 21

Another study of 27 isolates of Brucella species including B. abortus and B. Canis in Brazil, showed decreased susceptibility to rifampin and trimethoprim-sulfamethoxazole in 10 and 5 isolates respectively (24). In addition, a study of 147 B. abortus strains from cattle in Brazil reported reduced susceptibility to rifampin in 36% and resistant to rifampin in 2% of isolates (25). Reports of such a high prevalence of reduced sensitivity among Brucella isolates from livestock could be a warning alarm for increasing incidence of rifampin-resistant brucellosis in community.
According to our results and the aforementioned reports of the high frequency of intermediate susceptibility to rifampin among *brucella* isolates, this drug should be prescribed with caution in patients if necessary. Moreover, because both brucellosis and tuberculosis are endemic in our region, and rifampin is a first-line anti-tuberculosis agent, widespread use of rifampin may induce rifampin-resistant tuberculosis (26). Accordingly, we suggest that rifampin could be reserved as an alternative drug for treatment of brucellosis.

Due to the sensitivity of isolates to doxycycline, aminoglycosides, and quinolones, it seems that the use of a regimen containing doxycycline with an aminoglycoside or a quinolone is more preferable than trimethoprim-sulfamethoxazole and significantly rifampin-based treatment.

In conclusion, culture of blood and the clinical specimens are useful for definitive diagnosis even in patients with low titers of antibodies. Because of the high frequency of intermediate sensitivity to rifampin among *brucella* isolates, this drug should be prescribed with caution. We recommend restricting the use of rifampin for treatment of brucellosis and reserve it as an alternative drug for special situations.

**Conflicts of interest**

All contributing authors declare no conflicts of interest.

**Acknowledgments**

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supported by the Vice-chancellor of Research and Technology, Hamedan University of Medical Sciences.

References


Table 1: The frequency of symptoms and signs in brucellosis patients (N: 149).

<table>
<thead>
<tr>
<th>Symptom</th>
<th>N (%)</th>
<th>Signs</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fatigue</td>
<td>141 (94.6%)</td>
<td>Sacroiliitis</td>
<td>67 (45%)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>132 (88.6%)</td>
<td>Spondylitis</td>
<td>45 (30.2%)</td>
</tr>
<tr>
<td>Fever</td>
<td>122 (81.9%)</td>
<td>Orchitis*</td>
<td>20 (19.0%)</td>
</tr>
<tr>
<td>Sweating</td>
<td>112 (75.2%)</td>
<td>Pulmonary findings</td>
<td>22 (14.8%)</td>
</tr>
<tr>
<td>Weight loss</td>
<td>112 (75.2%)</td>
<td>Peripheral Arthritis</td>
<td>17 (11.4%)</td>
</tr>
<tr>
<td>Anorexia</td>
<td>110 (73.8%)</td>
<td>Hepatomegaly</td>
<td>7 (4.7%)</td>
</tr>
<tr>
<td>Backache</td>
<td>102 (68.5%)</td>
<td>Lymphadenopathy</td>
<td>4 (2.7%)</td>
</tr>
<tr>
<td>Headache</td>
<td>94 (61.3%)</td>
<td>Splenomegaly</td>
<td>2 (1.3%)</td>
</tr>
<tr>
<td>Althralgia</td>
<td>69 (46.3%)</td>
<td>Meningitis</td>
<td>1 (7%)</td>
</tr>
</tbody>
</table>

*Reported in 105 male patients
Table 2: The MIC values of antibiotics against 57 Brucella isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>MICRange (μg/ml)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (μg/ml)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (μg/ml)</th>
<th>CLSI Breakpoints for Brucella (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>0.012-0.750</td>
<td>0.250</td>
<td>0.500</td>
<td>≤0.5</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.016-0.640</td>
<td>0.470</td>
<td>0.470</td>
<td>≤4</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.094- 0.750</td>
<td>0.250</td>
<td>0.380</td>
<td>≤1*</td>
</tr>
<tr>
<td>Moxifloxocin</td>
<td>0.032-0.750</td>
<td>0.190</td>
<td>0.500</td>
<td>≤1*</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.125-2/000</td>
<td>1/000</td>
<td>1.500</td>
<td>≤8</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.094-0.750</td>
<td>0.380</td>
<td>0.750</td>
<td>≤4</td>
</tr>
<tr>
<td>Rifampin</td>
<td>0.500-3/000</td>
<td>1.000</td>
<td>2.000</td>
<td>≤1*</td>
</tr>
</tbody>
</table>

*CLSI breakpoints for slow-growing bacteria (Haemophilus spp.)
Table 3: Antimicrobial susceptibility of 57 *Brucella* isolates.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Susceptible</th>
<th>Intermediate</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>55</td>
<td>96.49</td>
<td>2</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>57</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>57</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>57</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>57</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>57</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Rifampin</td>
<td>37</td>
<td>64.91</td>
<td>20</td>
</tr>
</tbody>
</table>