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ARCHIVES OF CLINICAL MICROBIOLOGY
ISSN 1989-8436

2015
Vol. 6 No. 1:1

Characterization of Antibiotic Susceptibility of *Brucella Spp* Isolates with E-Test Method

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Abstract

Background: Brucellosis is a worldwide zoonotic disease that remains an important public health problem especially in rural Turkey. The aim of this study is to identify Brucella species and investigate the *in-vitro* susceptibilities of clinical isolates against various antibiotics.

Methods: The study included 50 Brucella isolates obtained from clinical samples from the Cukurova University Balcali Hospital between 2010-2012. The isolates were identified by the Vitek 2 automated system. In vitro activities of doxycycline, streptomycin, rifampicin, ciprofloxacin, tigecycline, gentamycin, trimethoprim-sulfamethoxsazole, erythromycin, ampicillin, amoxicillin/clavulonic acid were evaluated against 50 Brucella isolates by the E-Test method.

Findings: All isolates were identified as Brucella melitensis. All isolates were sensitive (100%) to doxycycline, streptomycin, gentamycin, trimethoprim-sulfamethoxsazole, ciprofloxacin, ampicillin and amoxicillin/clavulonic. All 11 strains yielded intermediate sensitivity (22%) to rifampicin and one strain was resistant (%2); whereas, the others were all sensitive. Trimethoprim-sulfamethoxsazole had the lowest minimal inhibitor concentration (MIC_{50}; 0.023 ug/ml and MIC_{90}; 0.064 ug/ml) and rifampicin had the highest MIC values (MIC_{50}; 1 ug/ml and MIC_{90}; 1.5 ug/ml) against all *B. melitensis* isolates.

Conclusions: Based on these findings, the present study showed that *in-vitro* trimethoprim-sulfamethoxsazole was the most effective antibiotic against *B. melitensis*. However, enough care should be taken for the use of rifampicin which is frequently used for the treatment of brucellosis, an endemic disease in our country. Establishment of a standardized antibiotic susceptibility method for *Brucella spp* would be useful for the determination of resistance in these bacteria and an appropriate agent should be used for the treatment.

Keywords: Antibiotic susceptibility, *Brucella spp*, Brucellosis treatment, E-Test

Introduction

Human brucellosis is most frequently caused by *B. melitensis*. In addition to this, other species has also been diagnosed in human beings. Brucellosis is a widespread disease of various animal species, and causes a common zoonotic infection of humans in many countries in the world and especially in the Mediterranean areas [1-3].

The genus Brucella is divided into six classical species. Four of six Brucella species (*B. abortus*, *B. melitensis*, *B. ovis*, *B. canis*, *B. suis*, and *B. neotomae*) may cause human infection. *B. melitensis* is the most common cause of infection, followed by B. abortus and *B. suis*. *B. canis* infections are rarely described in humans [4,5].

Brucella are intracellular bacterial pathogens that infect host macrophage cells. In consequence, specialized agents that are able to penetrate the macrophages and function within their cytoplasm are required for the treatment of brucellosis [6]. According to World Health Organization (WHO) guidelines, the

recommended combination of two antibiotics can be used for the treatment of brucellosis. WHO recommended regimen is doxycycline (DOX) in combination with rifampicin (RIF) for 6 weeks. The combination of DOX and streptomycin (STR) is also effective. Although Brucella isolates are generally considered susceptible to recommended antibiotics, sporadic cases of antibiotic resistance and disease relapse have been reported [7]. Drug resistance is a particularly important issue as most people infected with brucellosis live in low socioeconomic areas of developing countries, where tuberculosis is also an endemic health problem. Thus, there are concerns over the potential increase in resistance to tuberculosis drugs due to their prolonged use in treating brucellosis [8].

Antimicrobial susceptibility tests for Brucella haven't been standardized yet, and routine susceptibility tests can't be performed in microbiology laboratories. The break point values haven't described clearly yet [9,10].

The aim of this study is to identify Brucella strains isolated from various clinical specimens and determine their *in-vitro* antimicrobial susceptibilities to DOX, STR, RIF, ciprofloxacin (CIP), tigecycline (TGC), gentamycin (GEN), trimethoprim-sulfamethoxsazole (SXT), erythromycin (EM), ampicillin (AMP) and amoxicillin/clavulonic acid (AMC) using E-test method.

Materials and Methods

A total of 50 Brucella strains isolated from various clinical specimens at the Central Laboratory of Cukurova University Balcali Hospital between January 2010 and October 2012 were included in this study. Brucella strains were isolated from blood (n=45), CSF (n=2), nephrostomy (n=1), abscess (n=1) and synovial fluid (n=1). Blood cultures were incubated in vials of the BACTEC 9240 system (Becton Dickinson, Rutherford, NJ) at 37°C for 7 days. Positive signals were recorded and the samples were inoculated into 5% sheep blood agar (COS; bioMerieux) twice, and incubated with and without 5% CO2 for 48-72 h at 37°C. After incubation, Gram-negative coccobacilli which were oxidase and catalase positive were identified by the Vitek 2 automated system. The strains identified as *B. melitensis* were stored in microbank tubes at -20°C until susceptibility testing. On the other hand, the isolates were tested for agglutination with monospecific anti-Brucella serum (Remel Inc., Lenexa, Kans.). All Brucella isolates were identified as *B. melitensis*.

Testing antimicrobial susceptibility

Antimicrobial susceptibility testing of the Brucella isolates to ten antibiotics- DOX, STR, RIF, CIP, TGC, GEN, SXT, EM, AMP and AMC- was performed by E-test method. E-test strips were stored at -20°C until use. An inoculum equal to a 0.5 McFarland turbidity standard was prepared from each Brucella isolate, and bacterial suspension was inoculated onto Mueller-Hinton agar plates supplemented with 5% sheep blood. The E-test strips were applied to the inoculated culture plates separately as recommended by the manufacturer, and the plates were incubated at 37°C for 48 h under aerobic conditions. Determination of the MIC was performed in accordance with the recommended reference values of the Clinical Laboratory Standards Institute's (CLSI) guidelines to DOX, STR, GEN, SXT for *Brucella spp* and RIF, CIP, AMP, AMC for slow-growing bacteria (*Haemophilus spp*.). The MIC_{50} and MIC_{90} values, which indicate that the relevant concentration inhibits the growth of 50% or 90% of the bacteria, respectively, of the tested population were determined. All tests were performed by biosafety level 3 cabinets. Such testing carries the risk of contagious among laboratory personnel.

Reference strains

The reference strains Escherichia coli ATCC 25922 and Staphylococcus aures ATCC 29213 were used as quality controls.

Results

Thirty-three (66%) of the 50 strains were obtained from male patients, and seventeen (34%) were obtained from female patients. Samples had been sent from infectious disease (n=21), pediatrics (n=12), gastroenterology (n=2), brain surgery (n=2), orthopedics (n=2), hematology (n=2), internal medicine (n=1), general surgery (n=1), cardiology (n=1), rheumatology (n=1), otorhinolaryngology (n=1), urology (n=1), physical therapy and rehabilitation (n=1), burn unit (n=1) and cardiovascular surgery (n=1) departments of Cukurova University Balcali Hospital.

Using the BACTEC 9240 automated blood culture system, we detected all cultures positive for *B. melitensis* within six days of incubation. Moreover, 39 of 45 (87%) blood cultures were detected positive within first three days of incubation (**Figure 1**).

According to antibiotic susceptibility testing, 38 of the 50 B. melitensis strains were susceptible to RIF, 11 strains were intermediate-resistant and one strain was resistant to RIF. All strains were found to be suspectible to other antibiotics (**Table 1**). EM and TGC were included in the present study for research purposes only. Those agents aren't defined by CLSI standards.

According to MIC_{50} and MIC_{90} values, SXT (MIC_{50}; 0.023 ug/ml and MIC_{90}; 0.064 ug/ml) was the most effective antibiotic against *B. melitensis* strains. After SXT, the most effective antibiotics were GEN (MIC_{50}; 0.047 ug/ml, MIC_{90}; 0.094 ug/ml) and DOX (MIC_{50}; 0.064 ug/ml, MIC_{90}; 0.094 ug/ml), respectively. The highest MIC_{50}

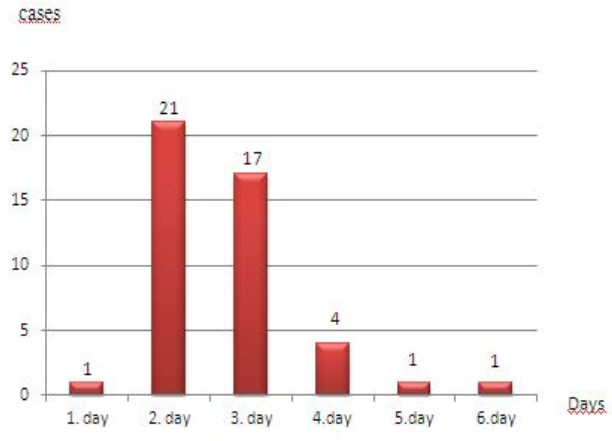

Figure 1 Blood cultures day of generation, BACTEC 9240 (Becton-Dickinson)

and MIC_{90} values had EM and RIF respectively. EM is ineffective *in-vivo* for brucellosis treatment (**Table 2**).

Discussion

Brucellosis is still an important health problem in developing countries and leads to serious economic losses. The disease causes abortion and sterility in animals and septicemia those progresses to chronic localized infections in various organs of humans. Although brucellosis has been eradicated from animals in some developed countries, 500,000 new cases are reported yearly throughout the world, and it is still a widespread zoonotic disease in Turkey [9,11].

Brucella spp are highly infectious pathogens. Routine *in-vitro* antimicrobial susceptibility testing of Brucella spp. is not generally recommended [12-14].

Such testing carries the risk of contagiousness among laboratory personnel and requires level 3 biosafety precautions [6,12,14]. Additionally, there is no standardized method for susceptibility testing recommended by CLSI for these microorganisms [6].

In-vitro efficacy of antibiotics against *Brucella spp.* has usually been based on the determination of MIC values by micro broth dilution, agar dilution, and E-test methods. The disc diffusion method has not been recommended [14]. Most studies from Turkey utilized the E-test method and usually the results are similar [6,15]. E-test is a reliable, reproducible, and practical as well as less labor-intensive and time-consuming than other methods for antimicrobial susceptibility testing of Brucella strains [10,16]. Brucella agar, Muller-Hinton agar, and Muller-Hinton broth supplemented with 1% Polyvitex, or a combination of 1% Polyvitex and 1% haemoglobin, and Muller-Hinton agar supplemented with 5% sheep blood agar are the media used for antibiotic susceptibility testing of Brucella [10,14].

To achieve effective treatment, antimicrobials that can penetrate the cell at high concentrations should be chosen, and the duration of the therapy should be set properly [9]. DOX; has become the most commonly prescribed tetracycline derivative in the treatment of Brucella infections because of its superior pharmacokinetic features [17]. We found that DOX was not as effective as SXT. DOX had the highier MIC values than SXT.

SXT is an agent recommended for the treatment of brucellosis. It is used in combination with RIF in pregnant women and children under 8 years old, who cannot use tetracycline. A combination of SXT, DOX and RIF is successfully used in the treatment of Brucella endocarditis, which is the brucellosis complication with the highest mortality rate [18]. A study from Egypt by Maksoud et al., reported that SXT is an effective antibiotic with low MIC levels (MIC_{50}; 0.047 µg/ml and MIC_{90}; 0.19 µg/ml) [7]. Our study showed that SXT had the lowest MIC_{50} and MIC_{90} values. SXT was found to be the most effective antibiotic [19]. As well as our study, Aliskan et al. reported SXT as the most effective antimicrobial agent with the lowest MIC_{50} and MIC_{90} values [20].

Table 1 Antibiotic susceptibilities of *B. melitensis* isolates.

Antimicrobial agents	Susceptible		Intermediate susceptible		Resistant	
	n	%	n	%	n	%
DOX	50	100	-	-	-	-
STR	50	100	-	-	-	-
RIF	38	76	11	22	1	2
CIP	50	100	-	-	-	-
GEN	50	100	-	-	-	-
SXT	50	100	-	-	-	-
AMP	50	100	-	-	-	-
AMC	50	100	-	-	-	-

Table 2 MIC ranges, MIC_{50} and MIC_{90} values of ten antibiotics against *B. melitensis* isolates.

Antimicrobial agents	MIC ranges (ug/ml)	MIC_{50} (ug/ml)	MIC_{90} (ug/ml)	CLSI Breakpoints for Brucella (µg/ml)		
				S	I	R
DOX	0.047-0.19	0.064	0.094	≤1	-	-
STR	0.25-0.5	0.25	0.38	≤8	-	-
RIF	0.38-4	1	1.5	≤ 1*	2*	≥ 4*
CIP	0.094-0.19	0.125	0.19	≤ 1*	-	-
GEN	0.032-0.125	0.047	0.094	≤4	-	-
SXT	0.008-0.38	0.023	0.064	≤ 2/38	-	-
EM**	0.25-2	1.5	2			
AMP	0.064-0.5	0.125	0.38	≤1*	2*	≥ 4*
AMC	0.032-0.094	0.064	0.094	≤ 4/2*	-	≥8/4*
TGC**	0.019-0.125	0.094	0.125			

*CLSI breakpoints for slow-growing bacteria (*Haemophilus spp.*).
**Not defined by CLSI standards

RIF is a potent antibiotic in the treatment of Brucella infections, and it is widely accepted in the best first-line therapy [21]. Depending on its concentration, this antibiotic can have bacteriostatic or bactericidal effects. RIF can have bactericidal activity against slow and irregularly growing Mycobacterium tuberculosis organisms and it also plays a significant role in the treatment of Brucella species [22]. Several studies showed that RIF had excellent anti-Brucella activity, which accounts for its good intracellular penetration and clear synergism in combination with therapies which are recommended by the WHO antibiotics for the treatment of brucellosis [23].

RIF demonstrated the highest MIC values (0.38-4 µg/ml), with 22% of the isolates showing reduced susceptibility and 2% probable resistance, according to CLSI criteria for slow-growing bacteria. To our knowledge, this is the first report of resistance to RIF among *B. melitensis* isolates from Adana. The emergence of strains of intermediate sensitivity and resistance to RIF is likely due to the frequent usage of RIF as an antitubercular agent in long-term, multi-drug tuberculosis therapy in Turkey, which is accepted as an endemic region for tuberculosis. Some previous studies show that RIF has been intermediate-sensitive. In Adana, Aliskan et al. found that, from 65 isolates containing *B. melitensis* strains isolated from bone marrow and blood, 8 showed intermediate sensitivity to RIF. In Van Parlak et al., found that, from a total of 75 strains, 34 were found to have intermediate sensitivity to RIF [19]. Reduced susceptibility in 158 isolates (45%) was demonstrated by Maksoud et al in Egypt [24]. In another study conducted in Peru, only one Brucella isolate demonstrated reduced susceptibility to RIF [18].

Since decreasing suspectibility to RIF has been reported in many parts of the world, we suggest periodic assessment of susceptibility of strains to those antibiotics used most frequently in treatment, for an early detection of any drug resistance, especially in areas of endemicity [1].

Aminoglycosides penetrate human cells rather poorly, but have shown some intracellular activity after prolonged incubation [24]. In the present study, all Brucella isolates were susceptible to STR and GEN in agreement with previous studies from various countries [6,7,24,25]. In our study, MIC50 ve MIC90 (0.25 µg/ml and 0.38 µg/ml) values of STR is relatively higher than GEN (0.047 µg/ml and 0.094 µg/ml). STR and GEN have been used clinically for the treatment of human brucellosis in tetracycline combinations.

Several studies focused on quinolones activity against *Brucella*, because these agents appeared as an attractive alternative drug choice for human brucellosis treatment [6]. Although fluoroquinolones had shown a high bactericidal activity against *Brucella in-vitro*, the *in-vivo* effectiveness of these antibiotics remains controversial [4,6]. MIC_{50} and MIC_{90} values were evaluated together and CIP was found to be one of the active agents by Köse et al. [3]. Our study revealed compatible results, suggesting that *in-vitro* CIP was as effective against *B. melitensis* strains.

TGC is a broad-spectrum glycylcycline antimicrobial agent and has been shown to be effective *in-vitro* against aerobic and anaerobic Gram-positive and Gram-negative microorganisms. Its activity against Brucella spp. has been investigated in several studies. As resistance breakpoints are not available for this agent in Brucella spp., *in-vitro* efficacies can be compared using MIC_{50} and MIC_{90} values. Baysan et al. reported 0.064 mg/l and 0.094 mg/l and Altun et al. reported 0.047 µg/ml and 0.094 µg/ml respectively MIC_{50} and MIC_{90} values for TGC [6,17]. Dizbay et al. reported TGC was more effective than RIF, SXT, STR, and DOX [13,17].

MIC50 and MIC90 values of TGC were 0.094 ug/ml and 0.125 ug/ml respectively for our isolates. We found that TGC was more effective than STR, CIP and RIF but was not as effective as SXT, GEN and DOX. There are conflicting data about the MIC of TGC against *Brucella* in Turkey. Some *in-vitro* studies are needed to determine the efficacy of TGC in the treatment of brucellosis.

The role of macrolides in brucellosis treatment also remains controversial [6]. MIC values of EM ranged from 0.25-2 ug/ml, indicating reduced activity. EM, AMP, and AMC acid were included in the study for research purposes only, as those agents are ineffective *in-vivo* for brucellosis treatment. Subsequently, the low MIC values of AMP (MIC_{50}; 0.125 ug/ml, MIC_{90}; 0.38 ug/ml) and AMC (MIC_{50}; 0.064 ug/ml, MIC_{90}; 0.094 ug/ml) found in our isolates do not correspond to any therapeutic purpose.

Conclusion

Brucellosis remains a major public health problem in countries with low socioeconomical status. The necessity to keep RIF for tuberculosis treatment and the requirement of alternative drug therapy for specialized cases entails the research for other antibiotic usage. Our findings should alert us to the potential emergence of RIF's resistance of Brucella in the region. Antibiotic susceptibility patterns of *Brucella spp.* can differ from one geography to another. The establishment of a simple and reliable method for Brucella susceptibility testing would be useful for an early detection of any drug resistance that may be developed. Therefore, we suggest, regional periodic assessment of susceptibility of strains to antimicrobials.

Competing Interests

There are no competing interests

Funding

This work was supported by a grant from Scientific Research Projects Coordination Unit, Cukurova University (TF2010D9).

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