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Research Paper

Antimicrobial Susceptibilities of *Brucella* Isolates from Various Clinical Speciemens

Yasemin Bayram[1], Hanifi Korkoca[2], Cenk Aypak[3] ✉, Mehmet Parlak[4], Aytekin Cikman[4], Selcuk Kilic[5], Mustafa Berktas[4]

1. Van Education and Research Hospital, Department of Microbiology, 65100 Van, Turkey.
2. Muş Alparslan University, School of Health, Department of Nursing, 49100 Muş, Turkey.
3. Van Gevaş Hospital, Department of Family Medicine, 65110 Van, Turkey.
4. Van 100. Yil University, Department of Microbiology, 65100 Van, Turkey.
5. Refik Saydam National Hygiene Center, Department of Communicable Diseases Research, 06100 Ankara, Turkey.

✉ Corresponding author: Van Gevas Hospital, Department of Family Medicine, 65110, Van, Turkey. cenkaypak@yahoo.com; Tel: +90 505 6452780; Fax: +90 432 6122066

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Abstract

Purpose: Brucellosis is a worldwide zoonotic disease and still constitutes a major public health problem. In the study we claimed to identify *Brucella* species from clinical samples of patients with active brucellosis from Van region of Eastern Anatolia and to determine in vitro antimicrobial susceptibilities of these strains to commonly used anti-Brucella agents and a possible new alternative tigecycline.

Materials and Methods: A total of 56 *Brucella* isolates were enrolled the study and the identification of the isolates were based on conventional methods. In vitro activities of antimicrobials were evaluated by the E test method.

Results: All isolates were identified as *B. melitensis*. MIC_{90} values of doxycycline, streptomycin, rifampin, trimethoprim-sulfamethoxazole and tigecycline were 0.064 mg/L, 1 mg/L, 2 mg/L, 0.125 mg/L and 0.094 mg/L, respectively. Tigecycline had low MIC_{50} and MIC_{90} values against all *B. melitensis* strains; the highest MIC observed was 0.25 µg/mL.

Conclusion: Our data suggest that tigecycline can be a therapeutic alternative option for the treatment of brucellosis.

Key words: *Brucella*, antimicrobial susceptibility, E-test, tigecycline

Introduction

Human brucellosis remains the most common zoonotic disease worldwide, with more than 500,000 new cases annually [1]. It is caused by Gram-negative bacteria, *Brucella* spp. and is transmissible to humans through direct contact with infected animals, consumption of dairy products, or inhalation of aerosols [2].

Brucellosis is a multisystemic disease that shows wide clinical polymorphism. Its main clinical signs are fever, headache, anorexia, fatigue, arthritis, hepatosplenomegaly, and neurological signs [2]. The disease represents serious consequences for public health by long treatment, slow recovery and possible serious sequelae in the locomotive and nervous system [2]. Although brucellosis has been eradicated in many northern European countries, in Australia, New Zealand, and Canada due to the implementation of national surveillance program and vaccination of live-

stock, it is still hyperendemic in the Mediterranean basin, Middle East, Southwest Asia and parts of Latin America [1,3].

In Turkey, brucellosis is common, especially in East and Southeast Anatolia regions [4,5]. Among high-risk patients in the Eastern part of Turkey, seropositivity has been reported to be as high as 27.2% [6], but there have been no extensive studies done on the identification of *Brucella* species in this hyperendemic part of Anatolia.

The genus *Brucella* is an intracellular bacterial pathogen that infects 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 [2]. Therefore, a limited number of antibiotics are effective against these organisms. In 1986, the WHO has released recommendations for use of doxycycline, combined with either rifampin or streptomycin for treating human brucellosis [7]. Although this recommendation is still in function and *Brucella* isolates are generally considered susceptible to the recommended by the WHO antibiotics, sporadic cases of a kind of antibiotic resistance have been reported [8,9]. Up until 2006, in vitro antimicrobial suspectibility testing of *Brucella* spp is not standardised and not generally recommended due to risk of laboratory-acquired infection and requirement of biological safety level 3 precautions, so there are few studies on this issue in the literature [8-16]. Furthermore in vitro susceptibilities of these antibiotics may change over time and from one geographical region to another [17,18].

The side-effects of drug combination schemes, and the high incidence of relapses and therapeutic failures, have led to the investigation of new drugs to treat the disease. Fluoroquinolones, macrolides and tigecycline (TIG), a member of a new class of antimicrobials, the glycylcyclines, may serve as alternative drug choices [12-16].

This study aimed to find the most common *Brucella* species in this endemic region of Turkey since strategies for disease control and eradication derive primarily from the epidemiological characteristics of the disease and to determine the in vitro antimicrobial susceptibilities of these strains to commonly used anti-Brucella agents and a possible new alternative tigecycline.

Materials and Methods

Bacterial Strains: 56 *Brucella* isolates were collected prospectively between 2008-2009 from blood (45), synovial fluid (8), bone marrow (2), and cerebrospinal fluid (1) cultures of patients with acute brucellosis who were admitted to Van Education and Research Hospital and the hospital of the Medical Faculty of Van Yuzuncu Yil University (Van, Turkey).

Identification methods: Identification of species was made on the basis of the requirement of CO_2 for growth, production of urease and H_2S, sensitivity to the dyes basic fuchsine and thionine (at final concentrations of 20-40 μg/ml), and agglutination with monospecific antisera for A and M antigens [19]. The strains were stored in skim milk at –40°C and subcultured twice before the susceptibility tests.

Antimicrobial susceptibility testing: Minimum inhibitory concentration (MIC) of doxycycline (DOX), rifampin (RIF), streptomycin (STR), tigecycline (TIG) and trimethoprim-sulfamethoxazole (TMP-SMZ) were determined by E-test (Biomerieux, Sweden) method on Mueller-Hinton agar (Oxoid, Basingstoke, UK) supplemented with 5% sheep blood and interpreted after 48 hours of incubation at ambient air. Mueller-Hinton agar supplemented with 5% sheep's blood was inoculated with suspensions of the test organism equivalent 0.5 McFarland turbidity, and E-test strips were applied onto culture plates. The plates were incubated in ambient air at 35°C and read after 48 hours. The MIC was interpreted as the value at which the inhibition zone intercepted the scale on the E-test strip. MIC_{50} and MIC_{90} levels defined as the lowest concentration of the antibiotic at which 50% and 90% of the isolates inhibited, respectively. The Clinical Laboratory Standarts Institute (CLSI; formerly the NCCLS) breakpoints for TMP-SMZ, STR, DOX were employed for the results. Three *Brucella* reference strains (*B. abortus* 544, *B. melitensis* 16M, and *B. suis* 1330) were used as controls for identification, biotyping and antimicrobial susceptibility testing. In addition to these *Brucella* reference strains, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 were also used as the quality control strain for susceptibility testing.

Results

All isolates were identified as *B. melitensis*. In vitro activities of DOX, STR, RIF, TMP-SMZ, and TIG against these isolates were evaluated.

The MIC values of DOX, STR and TMP-SMZ interpreted according to the CLSI criteria for potential bioterrorism agents and interpretive criteria for slow growing bacteria (*Haemophilus*) has been used to evaluate the results of MICs of TIG. The MIC_{50} and MIC_{90} values of relevant antibiotics are shown in Table 1.

Table 1. MIC range, MIC_{50} and MIC_{90} values of antimicrobial agents.

Antimicrobial	E-test MIC (µg/ml)			CLSI breakpoints (µg/ml)		
	MIC ranges	MIC_{50}	MIC_{90}	S	I	R
DOX[a]	0.023-0.125	0.047	0.064	≤ 1	-	-
TIG[b]	0.019-0.25	0.064	0.094	ND[f]		
TMP/SMZ[c]	0.064-0.25	0.064	0.125	≤ 2	-	-
STR[d]	0.064-1.5	1	1	≤ 8	-	-
RIF[e]	0.5-2.0	1.5	2	ND[f]		

a:Doxycycline; b: Tigecycline; c: Trimethoprim/ sulfamethoxazole (only the trimethoprim portion of the 1/19 drug ratio is displayed); d:Streptomycin; e: Rifampin;

f: not displayed in CLSI table for *Brucella* spp.

According to MIC_{90}, DOX (0.064 µg/ml) was found to be the most active agent, followed by TIG (0.094 µg/ml), TMP-SMZ (0.125 µg/ml), STR (1 µg/ml) and RIF (2 µg/ml) respectively. All isolates were found to be sensitive to DOX, STR and TMP-SMZ. The MIC values of TIG interpreted according to the CLSI criteria for slow growing bacteria, has shown ranges below the breakpoints for sensitivity determination. The highest MIC of TIG against *Brucella* isolates was 0.25µg/ml.

Discussion

Brucellosis is endemic in Turkey and approximately 10,000 cases of human brucellosis are reported annually [5]. Brucellosis and its complications are still serious public health concern in Eastern Anatolia. Although the diagnosis of brucellosis can be made only by the isolation of causative agent; *Brucella* spp. are difficult to isolate and the procedures are time consuming and expensive [8,20]. Moreover, *Brucella* spp. are so highly infectious that the attempts at isolation and identification of *Brucella* from clinical specimens are not routinely performed [8,20-22]. Therefore, the epidemiology of brucellosis has not been extensively studied, and limited data are available about the prevalence and species most commonly encountered in Eastern Anatolia. This is the first study which identifies *Brucella* species and their susceptibility pattern in this region. Our findings are in accordance with the previous reports from different regions of Turkey, Mediterranean and South America basin which have revealed that human brucellosis is almost exclusively caused by *B.melitensis*, accounting for 99% of total cases [8-16,22-25].

In this present study, we also performed in vitro susceptibilities of *B.melitensis* against commonly used antimicrobials and a novel compound tigecycline. Antimicrobial susceptibility testing for *Brucella* spp is not generally recommended for routine microbiology laboratories except in life-threatening organ involvement, and in case of treatment failure and relapse [21]. Another problem with such testing is the lack of standardization. Methods for MIC determination are described for potential bioterrorism agents including *Brucella* species by the CLSI. The CLSI proposes the microbroth dilution method using *Brucella* broth for *Brucella* spp. The breakpoints used for interpretation as susceptible were as follows: TET/DOX ≤1 µg/ml, TMP-SMZ ≤2 µg/ml, and STR ≤8 µg/ml according to the the CLSI interpretive criteria [26]. 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 [20]. E-test method was found to be reliable, reproducible, less labor-intensive, less time-consuming, and more practical than the broth micro dilution method [11,24,27]. Therefore E-test method was used in this study. E-test could be performed on two different culture media: the Mueller-Hinton agar plates widely used for antibiotic susceptibility testing and the Brucella agar plates commonly used in the laboratory as Brucella growth medium. Although no significant differences were observed between two culture media, we preferred the Mueller-Hinton agar plate in this study because clearer inhibition zones are visible and the calibrated carrier strip indicating the MIC can be more easily read [25].

TET and its derivatives are among the most effective drugs against brucellosis [2]. DOX has become the most commonly prescribed tetracycline derivative in the treatment of brucella infections because of its superior pharmacokinetic features [28]. In the present study, among the tested antibacterial agents, DOX was found to have the lowest MIC_{50} and MIC_{90} values which is consistent with previous reports [8,10,11,22-24,27,29]. Conversely in a Mexican study, Lopez-Merino et al. found the MIC values for TET were higher than in *Brucella* strains isolated in Turkey [9] which demonstrates the antibiotic susceptibility

patterns of *Brucella* strains appear to vary geographically.

Another drug of choice in the treatment regimen of brucellosis is RIF and it was found to be the only antibiotic with increased activity in acidic environmental conditions [27]. In our study, the highest MIC values were determined for RIF among the studied antimicrobials. As MIC values of RIF in previous studies were reported to range from 0.047 to 4 µg/ml, its values confirmed again by our findings [8,10-12,22-25]. Memish et al. reported an in vitro resistance rate of 3.5% for RIF [31]. These findings should be taken into consideration for the potential emergence of RIF resistance of *Brucella* spp. in the region. Another concern for RIF using widespread in the long treatment regimens like brucellosis may cause an increase in RIF resistance in *M. tuberculosis* because both brucellosis and tuberculosis can simultaneously exist in the same countries in many parts of the world [32]. Furthermore experimental studies suggested that the development of mycobacterial resistance to RIF may lead to development of resistance to other antimicrobials as well [32]. The resistance rate of RIF against *M. tuberculosis* was reported as 15–58% in Turkey [33]. The burden of such resistance for public health must be considered.

TMP-SMZ containing regimens is considered to be suitable oral regimens that may be of significantly lower cost than traditional combinations in certain developing countries and mostly prescribed in brucellosis for children and pregnant women [2]. In our study MIC_{50} and MIC_{90} values for TMP-SMZ were lower than those previously observed in Turkey [8,10,11] and conforming the results of Kilic et al. [16]. In vitro TMP-SMZ resistance rate was reported 2% in Turkey [8]. However, significant rates of TMP-SMZ resistance have been reported in the world [31,34].

Although streptomycin is known to be one of the most active agent against brucellosis, its adverse effects, such as ototoxicity, nephrotoxicity, and parenteral administration, preclude its wider use [24,29]. In our study susceptibility to STR was found to be in the range described previously [8,10,12,24,29].

This is one of the few studies which, determines the in vitro activity of TIG, a new glycylcycline compound, against *Brucella* strains. We found that TIG was more effective than RIF, TMP-SMZ and STR but was not as effective as DOX. Dizbay *et al*. reported TIG was more effective than RIF, SXT, STR, and DOX [8]. Also Kilic *et al*. found TIG had the least MIC_{50} and MIC_{90} values compared to TET, and fluoroquinolones against *Brucella* strains isolated in Central Anatolia [13]. These are in contrast with our findings and might be due to the strain specific susceptibility. As MIC_{50} and MIC_{90} values of TIG in these two previous studies were reported to be 0.064 and 0.125 µg/ml respectively, values of them confirmed again by our findings.

Although TIG has similar properties to TET, it has been reported that it is more potent than TET [35,36]. TET is the mainstay of anti-brucellosis regimen. Therefore, Pappas et al. suggested replacing DOX with more potent TIG might increase efficacy and reduce treatment duration [37]. On the other hand, parenteral administration of TIG, the conservation of TIG because of promising results of its use in the treatment of multiresistant bacterial infections, and overall cost were considered as limitations of such a therapy [12].

In conclusion, there is no significantly important resistance problem for classically recommended antibiotics targeted to *Brucella* species in Turkey, but antibiotic susceptibility patterns of Brucella spp. appear to vary geographically. Therefore, we suggest, regional periodic assessment of susceptibility of strains to antimicrobials. The results of this in vitro study suggest TIG as a therapeutic option in the treatment of brucellosis. Clinical trials are warranted to assess the real therapeutic potential of TIG in human brucellosis, particularly in countries with higher prevalence of antibiotic resistance.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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