ORIGINAL PAPER

Evaluation of *in vitro* Activities of Tigecycline and Various Antibiotics against *Brucella* spp.

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Abstract

Brucellosis is a zoonosis with a worldwide distribution and remains a significant public health problem mainly in the developing world. In this study we evaluated the *in vitro* activities and synergistic effects of antibiotic combinations against blood culture isolates of *Brucella* spp. *In vitro* susceptibilities of 76 blood culture isolates of *Brucella melitensis* and one blood culture isolate of *Brucella abortus* to doxycycline, streptomycin, gentamicin, trimethoprim-sulfamethoxazole, moxifloxacin, rifampin, ciprofloxacin, and tigecycline were examined by Etest method. For 37 patients with *Brucella* spp. isolates (36 *B. melitensis*, 1 *B. abortus*), antibiotic combinations used for treatment were identified with those tested *in vitro* for synergy using Etest method. Trimethoprim-sulfamethoxazole and tigecycline were the most active of the compounds tested with MIC_{90} value of 0.094 mg/l. Among antibiotic combinations only streptomycin-rifampin combination was synergistic for one *Brucella* spp. isolate. The other antibiotic combinations revealed antagonistic or indifferent activity. Complete clinical response was achieved in all patients. Further studies are required to determine the correlation between the antimicrobial susceptibility and synergy test results with the clinical course of patients. Brucellosis can be adequately treated with existing regimens in our region.

Introduction

Human brucellosis is a multisystem disease that may involve any organ or system. Successful treatment of brucellosis requires combined antimicrobials with synergy and good intracellular penetration and should be prolonged (Young, 2005). Recently, Centers for Disease Control (CDC) listed *Brucella* as a potential biological weapon and the European Union (EU) released Bichat guidelines for clinical and bioterrorism related events of brucellosis (Bossi *et al.*, 2004; Yagupsky and Baron, 2005). Moreover, Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards [NCCLS]) released the first MIC interpretive standards for *Brucella* spp. in January 2006 (Clinical and Laboratory Standards Institute, 2006). In 1986, the World Health Organization (WHO) recommended a six-week regimen containing doxycycline plus rifampin or doxycycline plus streptomycin for the treatment of brucellosis (World Health Organization, 1986). Most cases respond to standard regimens, with fewer than 10% manifesting relapses, usually in the first year after treatment. Treatment failures are of concern especially in patients with serious or complicated brucellosis such as spondylitis, neurobrucellosis and endocarditis (Bossi *et al.*, 2004; Pappas *et al.*, 2006b).

Clinical *Brucella* isolates are generally susceptible to the antibiotic combinations recommended by WHO, however the results of *in vitro* susceptibility tests do not always predict the clinical outcome. A number of methods used for detection of *in vitro* synergy between antibiotics have been described (White *et al.*, 1996). The Etest method has been used both for *in vitro* susceptibility and synergy testing of *Brucella* spp. as it is less time consuming and less labor intensive. However

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synergy testing methods are not yet standardized (Gur *et al.*, 1999; Orhan *et al.*, 2005).

In this study, we evaluated the *in vitro* activities and synergistic effects of antibiotic combinations against *Brucella* spp. using Etest method and compared the results with clinical outcome.

Experimental

Materials and Methods

A total of 77 *Brucella* spp. strains isolated at Akdeniz University Hospital and Afyon Kocatepe University Hospital Central Laboratories between September 2001 and June 2006 were tested. The collection included 76 strains of *Brucella melitensis* and one strain of *B. abortus.* All isolates were obtained from blood using BACTEC 9240 (Becton Dickinson, U.K.) blood culture system. The isolates were collected at the individual study sites and were sent to Akdeniz University Hospital Central Laboratory for identification and susceptibility testing and were stored in stock cultures at –20°C until used. The isolates were identified to the species-level using standard classification tests, including Gram stain, growth characteristics, oxidase activity, urease activity, H_2S production, dye sensitivity such as basic fuchsin and thionin and seroagglutination. Identification was confirmed using a semi-nested real-time PCR assay targeting a 223-bp fragment of gene encoding the cell surface protein (BCSP31), specific for the Brucella genus. A class II biological safety cabinet was used to perform the tests.

In the first part of our study, we investigated the minimum inhibitory concentration (MIC) values of antibiotics for all isolates from the two centers by Etest method. In the second part, the patient charts of 37 subjects with *Brucella* spp. isolates (36 *B. melitensis*, 1 *B. abortus*) of the 46 patients from Akdeniz University Hospital, were taken for further assessment. Demographic data, treatment protocol, as well as clinical outcome were evaluated, retrospectively. Each patient's antibiotic combinations used for treatment were identified with those tested *in vitro* for synergy using E-test method. The results were compared with clinical outcome.

The study was approved by Akdeniz University Medical Faculty Ethical Committee with B.30.2.AKD. 0.01.00.00/Etik/483 protocol number.

Antimicrobial susceptibility tests. The Etest method was performed according to manufacturer's instructions. An inoculum equal to a 1 McFarland turbidity standard was prepared from each *Brucella* isolate and 10 µl of the suspension was inoculated onto Mueller-Hinton agar plates with 5% sheep blood and Etest strips were applied to the inoculated surface.

The plates were read after 48 h incubation at 35°C under aerobic conditions according to manufacturer's recommendations. MIC values of streptomycin (STR), doxycycline (DOX), rifampin (RIF), gentamicin (GEN), trimethoprim-sulfamethoxazole (SXT), ciprofloxacin (CIP), moxifloxacin (MXF), and tigecycline (TIG) were determined.

Synergy testing. Rifampin-doxycycline, streptomycin-doxycycline, streptomycin-rifampin, trimethoprim-sulfamethoxazole-rifampin, and gentamicin-doxycycline combinations were tested against 28 (27 *B. melitensis*, 1 *B. abortus*), two, one, five and one *Brucella* spp. isolates, respectively.

Synergy tests were performed on Mueller-Hinton agar supplemented with 5% sheep blood by Etest. First, strip A was placed on the inoculated agar surface and left for one hour. The strip's position was marked on the back of the plate. Strip A was removed and strip B was placed on the imprint of A, vertically transposed so MIC_A and MIC_B overlap at the same position. Strip B was left on the agar plate and incubated for 48 h. To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC) was calculated for each antibiotic in each combination. The following formulas were used to calculate the FIC index: FIC of drug A = MIC of drug A in combination/ MIC of drug A alone. FIC of drug B = MIC of drug B in combination/MIC of drug B alone, and FIC index = FIC of drug A + FIC of drug B. Synergy was defined as an FIC index of ≤0.5. Indifference was defined as an FIC index of >0.5 but of ≤4. Antagonism was defined as an FIC index of >4.

Quality Control. Quality control isolates, *Escherichia coli* ATCC 25922 and *Streptococcus pneumoniae* ATCC 49619 were included in all runs.

Results

MIC ranges, MIC_{50} and MIC_{90} values of the antibiotics for the isolates are shown in Table I. According to their MIC_{90} values, SXT and TIG were the most active compounds against all *Brucella* spp. isolates. Among quinolones, MXF demonstrated a lower MIC (MIC_{50}: 0.125 mg/l, MIC_{90}: 0.25 mg/l) than that of CIP (MIC_{50}: 0.19 mg/l, MIC_{90}: 0.38 mg/l). MIC range of RIF was between 0.064–3 mg/l and a MIC_{90} value of 1.5 mg/l for RIF was found. DOX, STR and GEN had good activities against all isolates with a MIC_{90} value below standard CLSI breakpoints. A complete clinical response was achieved in all patients and no relapses were recorded in the patients' charts.

Synergy test results of antibiotic combinations against *Brucella* spp. isolates (36 *B. melitensis*, 1 *B. abortus*) from 37 patients are shown in Table II. In our study synergy tests were performed using Etest

Table I
MIC ranges, MIC_{50} and MIC_{90} values of antibiotics against *Brucella* spp. isolates (n = 77)

Antibiotic	MIC_{50}*	MIC_{90}*	Range	CLSI breakpoint*
Doxycycline	0.047	0.125	0.016–0.19	≤1
Streptomycin	0.5	1	0.064–1.5	≤ 8
Gentamicin	0.25	0.5	0.064–0.75	≤4
Trimethoprim-sulfamethoxazole	0.047	0.094	0.016–0.125	≤2/38
Moxifloxacin	0.125	0.25	0.032–0.25	–
Rifampin	0.75	1.5	0.064–3	–
Ciprofloxacin	0.19	0.38	0.064–0.5	–
Tigecycline	0.064	0.094	0.023–0.5	–

* MIC values and CLSI breakpoints are expressed in mg/l.

Table II
Synergy test results of antibiotic combinations against *Brucella* spp. isolates

Antibiotic combinations	Synergy test results n (%)			
	Antagonism	Indifference	Synergy	No of strains
RIF-DOX	25 (89.3)*	3 (10.7)	–	28
STR-DOX	2 (100)	–	–	2
STR-RIF	–	–	1 (100)	1
SXT-RIF	3 (60)	2 (40)	–	5
GEN-DOX	–	1 (100)	–	1
Total n (%)	30 (81.1)	6 (16.2)	1(2.7)	37(100)

* Twenty-four isolates of *B. melitensis* and one isolate of *B. abortus* RIF, rifampin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; STR, streptomycin; DOX, doxycycline.

method. The combination of RIF-DOX yielded antagonism against 89.3% and indifference against 10.7% of the isolates. The STR-DOX combination exhibited antagonism against all of the isolates tested. The STR-RIF combination was tested in one isolate and was found synergistic. For the combination of SXT-RIF we found antagonism against 60%, and indifference against 40% of the isolates. The GEN-DOX combination exhibited indifference against one tested isolate (Table II). Demographic data, clinical characteristics of patients and synergy test results of individual patient's antibiotic combinations are presented in Table III.

Discussion

As shown in several earlier studies, *Brucella* spp. are generally susceptible to traditional drugs used for the treatment of brucellosis (Bosch *et al.*, 1986; Qadri and Ueno, 1993; Baykam *et al.*, 2004). However strains with decreased susceptibility to RIF and STR have been reported (Baykam *et al.*, 2004; Lopez-Merino *et al.*, 2004). In our study all strains were susceptible to STR (MIC_{90}: 1 mg/l) and DOX (MIC_{90}: 0.125 mg/l) according to CLSI interpretive breakpoints of ≤8 mg/l and ≤1 mg/l, respectively. Furthermore, MIC_{90} value of STR in our study is lower than the

MIC_{90} values of previous studies ranging from 2 to 4 mg/l (Lopez-Merino *et al.*, 2004; Turkmani *et al.*, 2006; Khan *et al.*, 1989; Rubinstein *et al.*, 1991; Akova *et al.*, 1999). However, DOX was found to have a higher MIC_{90} value when compared to those previously reported (Baykam *et al.*, 2004; Akova *et al.*, 1999; Bodur *et al.*, 2003). Several other studies found even higher MIC_{90} values for DOX for *Brucella* isolates (Trujillano-Martin *et al.*, 1999; Yamazhan *et al.*, 2005). GEN had a good activity against all isolates with a MIC_{90} value below standard CLSI breakpoint. This finding agrees with data from previous studies (Qadri and Ueno, 1993; Turkmani *et al.*, 2006). RIF breakpoints have not been established for *Brucella* spp. by CLSI. MIC of RIF for our isolates ranges between 0.064–3 mg/l. MIC values of RIF ranging from 0.02 to 16 mg/l have already been reported (Bosch *et al.*, 1986; Qadri and Ueno, 1993; Lopez-Merino *et al.*, 2004; Turkmani *et al.*, 2006; Khan *et al.*, 1986; Bodur *et al.*, 2003; Al-Orainey *et al.*, 1991). MIC values of SXT ranging from 0.032 to >32 mg/l have been reported previously (Bosch *et al.*, 1986; Qadri and Ueno, 1993; Baykam *et al.*, 2004; Lopez-Merino *et al.*, 2004; Turkmani *et al.*, 2006; Khan *et al.*, 1986; Bodur *et al.*, 2003; Al-Orainey *et al.*, 1991). CLSI determined interpretive breakpoints for SXT in 2006 (Clinical and Laboratory Standards Institute, 2006).

Table III
Characteristics of brucellosis patients and synergy test results of antibiotic combinations

Patient No	Sex[a]	Age (years)	Complications	Administered drugs	Synergy test result[b]	Clinical outcome
1	M	35	spondylitis	RIF+DOX	A	Cure
2	F	59	spondylitis	RIF+DOX	A	Cure
3	F	49	spondylitis	RIF+DOX	A	Cure
4	F	39	arthritis	RIF+DOX	A	Cure
5	F	20	arthritis	RIF+DOX	A	Cure
6	M	65	arthritis	RIF+DOX	A	Cure
7	F	44	arthritis	RIF+DOX	A	Cure
8	F	13	arthritis	RIF+DOX	A	Cure
9	F	13	none	RIF+DOX	A	Cure
10	M	40	none	RIF+DOX	A	Cure
11	M	46	none	RIF+DOX	A	Cure
12	F	39	none	RIF+DOX	A	Cure
13	M	70	none	RIF+DOX	A	Cure
14	M	53	none	RIF+DOX	A	Cure
15	F	33	none	RIF+DOX	A	Cure
16	M	39	none	RIF+DOX	A	Cure
17	M	42	none	RIF+DOX	A	Cure
18	M	51	hepatitis	RIF+DOX	A	Cure
19	F	26	abortus	RIF+DOX	A	Cure
20	M	53	epididymo-orchitis	RIF+DOX	A	Cure
21	M	24	none	RIF+DOX	A	Cure
22	F	28	none	RIF+DOX	A	Cure
23	F	67	none	RIF+DOX	A	Cure
24	F	27	neurobrucellosis	RIF+DOX	A	Cure
25	F	37	neurobrucellosis	RIF+DOX	A	Cure
26	F	67	neurobrucellosis	RIF+DOX	I	Cure
27	M	33	none	RIF+DOX	I	Cure
28	F	27	arthritis	RIF+DOX	I	Cure
29	M	5	none	RIF+SXT	A	Cure
30	M	2	arthritis	RIF+SXT	A	Cure
31	M	2	arthritis	RIF+SXT	A	Cure
32	M	53	none	RIF+SXT	I	Cure
33	M	5	none	RIF+SXT	I	Cure
34	M	71	none	DOX+STR	A	Cure
35	M	66	spondylitis	DOX+STR	A	Cure
36	F	26	none	RIF+STR	S	Cure
37	F	66	none	DOX+GEN	I	Cure

[a] F: female, M: male; [b] A: antagonism, I: indifference, S: synergism.
RIF: rifampin, GEN: gentamicin, SXT: trimethoprim-sulfamethoxazole, STR: streptomycin, DOX: doxycycline.

All isolates in our study were susceptible to SXT with MIC values ranging from 0.016 to 0.125 mg/l.

An alternative approach may be the use of quinolones for the treatment of brucellosis. Ciprofloxacin and ofloxacin have been used clinically for the treatment of human brucellosis in various combinations. *In vitro* susceptibility testing results exist for several other quinolones including gatifloxacin, levofloxacin, norfloxacin and pefloxacin (Falagas and Bliziotis, 2006; Pappas *et al.*, 2006a). In our study, we found a MIC_{90}

value of 0.38 mg/l for CIP which is consistent with data from previous reports (Qadri and Ueno, 1993; Lopez-Merino *et al.*, 2004). Our MIC_{90} value for MXF (0.25 mg/l) was lower than those previously reported (0.5–8 mg/l) (Lopez-Merino *et al.*, 2004; Trujillano-Martin *et al.*, 1999; Yamazhan *et al.*, 2005).

Tigecycline is a broad-spectrum glycylcycline antimicrobial agent and seems to have excellent *in vitro* activity against many Gram-positive and Gram-negative microorganisms (Nathwani, 2005). Pappas *et al.*

suggested a shorter duration of treatment with the use of TIG as a single agent (Pappas *et al.*, 2005). MIC_{90} value of TIG was 0.094 mg/l for our isolates. There are conflicting data about the MIC of TIG against *Brucella* in Turkey. In a study of Turan *et al.* the MIC of TIG was found to be lower than those of RIF and CIP but greater than that of DOX (Turan *et al.*, 2007). TIG was found to be superior to DOX and its MIC_{90} values were the lowest in the study of Dizbay *et al.* (2007). This new compound may have a use in the treatment of brucellosis in the future, especially in complicated or serious forms of disease. However it is costly, intravenously administered and its wide use may lead to development of resistance for other serious nosocomial pathogens in countries where brucellosis is endemic (Pappas *et al.*, 2006b).

Time-kill, checkerboard and Etest are most extensively used *in vitro* methods for detecting synergy (White *et al.*, 1996). There are few reports about *in vitro* combination studies for Brucellae (Orhan *et al.*, 2005; Rubinstein *et al.*, 1991; Akova *et al.*, 1999). Akova *et al.* (1999) tested activities of RIF-DOX and STR-DOX by checkerboard method against 20 *B. melitensis* isolates. They found that RIF-DOX combination was synergistic for 17 isolates, additive for two isolates and indifferent for one isolate. The combination STR-DOX has showed synergistic effect for 18 isolates and was indifferent for two isolates. Dizbay *et al.* in their study reported TIG-RIF and DOX-RIF combinations demonstrated synergistic activity in all strains. They didn't detect synergy for DOX-STR combination in any strain, but found that 18.75% of their isolates exhibited antagonism for that combination (Dizbay *et al.*, 2007). On the other hand, Orhan *et al.* found a high rate of synergy for DOX-STR combination. They also reported that the results of Etest and checkerboard methods agreed with a rate of 55% in 16 *B. melitensis* isolates (Orhan *et al.*, 2005). Rubinstein *et al.* determined *in vitro* susceptibilities of 86 clinical isolates of *B. melitensis* to minocycline, streptomycin, co-trimoxazole, rifampin and six fluoroquinolones and also studied the possible synergistic effects of several combinations of these antibiotics. They found that no combination exhibited synergy against any of the tested strains by checkerboard test (Rubinstein *et al.*, 1991).

Differences in agreement among synergy testing methods have been reported previously most of which have not been clinically validated. Rubinstein *et al.* demonstrated the discrepancy between the results of checkerboard and time-kill methods in evaluating the antibiotic susceptibility of *B. melitensis* and concluded that time-kill studies have a better correlation with clinical results and therefore may be more appropriate to predict therapy results (Rubinstein *et al.*, 1991). However, Ariza *et al.* (1986) reported that synergism

for the combinations used could not be evaluated appropriately because of very low MICs of tetracycline and DOX for all the isolates. Similarly, all our isolates chosen for synergy testing had very low MICs of DOX, STR, SXT, and GEN according to their CLSI breakpoints. In our study, performance of time-kill studies on the isolate for which synergy was observed as well as some of the antagonistic organism/drug combinations might strengthen our findings with Etest. Further studies are needed to clarify this issue.

In endemic regions, conventional antibrucellar antibiotics are cheap, easy to use and mostly well tolerated. We believe that brucellosis can be adequately treated with existing regimens, with protracted administration of appropriate drug combinations to minimize the percentage of relapses even for the most complicated brucellosis patients.

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