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Synergy Tests by E Test and Checkerboard Methods of Antimicrobial Combinations against *Brucella melitensis*

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Two different synergy testing methods, the checkerboard and the E test methods, were used to compare the in vitro efficacies of various antimicrobial combinations against 16 *Brucella melitensis* strains isolated from blood cultures. The rate of agreement of the E test and checkerboard methods was found to be 55%. The most concordant results were found for the streptomycin-doxycycline combination in 12 (75%) tests, in which four strains showed synergistic activity by E test and antagonistic activity by the checkerboard method and in which one strain showed antagonistic activity by both methods. Even though each of these methods uses different conditions and endpoints, the results of both methods frequently agreed.

Brucellosis is still an important health problem in developing countries and leads to serious economic losses (15, 19). The disease causes abortion and sterility in animals and septicemia that progresses to chronic localized infections in various organs of humans (4, 16, 19). 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 zoologic disease in Turkey (8, 11).

Because brucellae are localized intracellularly, infections with these bacteria should be treated with antibiotics that can penetrate the cell at high concentrations. The World Health Organization recommends the use of two antibiotics in combination and, in some cases, four antibiotics in combination for the treatment of brucellosis. Because of the lack of effectiveness of present therapies, relapses, and difficulties related to the antibiotics used, such as adverse effects, absorbance disturbances, and the limited ability of certain age groups to use certain antibiotics, researchers have been forced to discover new treatment regimens (2, 19).

Antimicrobial susceptibility tests are not yet standardized for brucellae, and routine susceptibility tests cannot be performed in microbiology laboratories. However, the antimicrobial susceptibilities and even the in vitro synergistic effects of antibiotics in combination against these microorganisms, which have scant growth in culture, can easily be performed by E test. The aim of this study was to evaluate the rate of agreement of two in vitro tests for synergy, E test and the checkerboard method, with various combinations of antibiotics that penetrate the cell wall against *Brucella melitensis* isolates recovered from blood.

MATERIALS AND METHODS

Sixteen strains of *B. melitensis* isolated from blood cultures at the Microbiology Laboratory of Gaziantep University from January through December 2003 were included in this study. Blood samples were collected from in- and outpatient clinics of the Medical Faculty Hospital, and they were cultured in vials of the BACTEC 9120 system (Becton Dickinson, Rutherford, N.J.) at 37°C for at least 7 days. The isolates were identified on the basis of colony morphology, staining, and slide agglutination with antiserum. Small, round, and convex colonies that grew on Mueller-Hinton agar plates (BBL, Cockeysville, Md.) under aerobic conditions within 48 to 72 h were Gram stained. Gram-negative, non-spore-forming, nonmotile coccobacilli which were oxidase and urease positive were tested for agglutination with monospecific anti-*Brucella* serum (Remel Inc., Lenexa, Kans.). Strains identified as *B. melitensis* were stored in brucella broth (Remel Inc.) at −20°C until susceptibility testing. The laboratory personnel working with these isolates wore impermeable protective clothing, gloves, and a face mask during tests with the organism.

Antimicrobial susceptibility testing of the *Brucella* isolates was performed by two different techniques: E test and the checkerboard method. No standard broth dilution test was performed with the isolates during this study. The synergy or antagonism of the drug combinations used for the treatment of brucellosis was investigated. The following antibiotic combinations were used: rifampin (RIF) and doxycycline (DOX), RIF and trimethoprim-sulfamethoxazole (SXT), SXT and DOX, streptomycin (SM) and DOX, and azithromycin (AZM) and ciprofloxacin (CIP).

E test. An inoculum equal to a 0.5 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. E test strips of RIF, DOX, SXT, SM, AZM, and CIP (AB Biodisk, Solna, Sweden) were stored at −20°C until use. The E test strips were applied to the inoculated culture plates separately by using a template, as recommended by the manufacturer, and the plates were incubated at 35°C for 48 h under aerobic conditions (Fig. 1). For testing, the combinations of E test strips were placed on the same culture medium in a cross formation, with a 90° angle at the intersection between the scales at the respective MICs for *B. melitensis*, and the plates were incubated at 35°C for 48 h. Determination of the MICs by E test were performed in duplicate, according to the recommendations of the manufacturer, and the MICs were interpreted at the point of intersection between the inhibition zone and the E test strip. The fractional inhibitory concentration (FIC) index (ΣFIC) was calculated on the basis of the resultant zone of inhibition as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC of the combination/MIC of drug A alone, and FIC B is the MIC of the combination/MIC of drug B alone.

Checkerboard method. Standard powder forms of RIF (Sigma Chemical Co., St. Louis, Mo.), DOX (Sigma Chemical Co.), SXT (Hoffmann-La Roche, Inc., Nutley, N.J.), SM (Sigma Chemical Co.), AZM (Pfizer Inc., New York, N.Y.), and CIP (Miles Inc., New Haven, Conn.) were stored at 2 to 8°C until use. The stock solutions and serial twofold dilutions of each drug to at least double the MIC were prepared according to the recommendations of NCCLS immediately prior to testing (3, 10). A total of 50 μl of Mueller-Hinton broth was distributed into each well of the microdilution plates. The first antibiotic of the combination was serially diluted along the ordinate, while the second drug was diluted along the abscissa. An inoculum equal to a 0.5 McFarland turbidity standard was prepared from each *Brucella* isolate in Mueller-Hinton broth (BBL). Each microtiter well was inoculated with 100 μl of a bacterial inoculum of 5×10^5 CFU/ml, and the plates were incubated at 35°C for 48 h under aerobic conditions. The resulting checkerboard contains each combination of two antibiotics,

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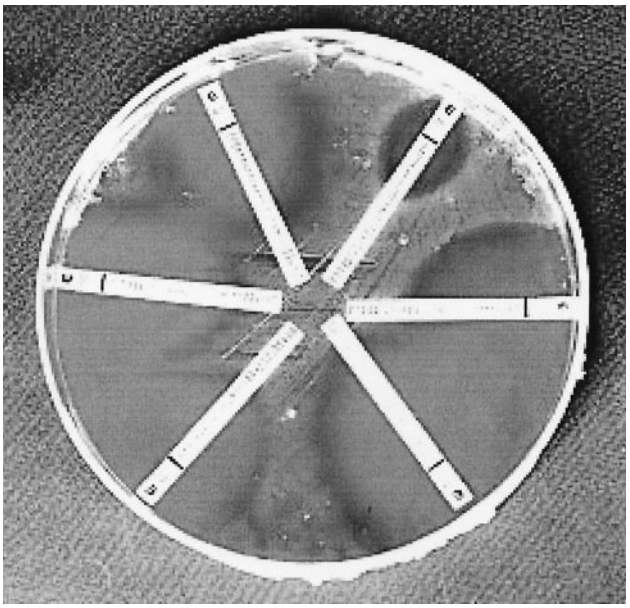

FIG. 1. Six different E test strips applied onto an agar plate.

with tubes that contain the highest concentration of each antibiotic at opposite corners (Fig. 2). According to the NCCLS guidelines for broth microdilution, the MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as detected with the naked eye. Synergy is more likely to be expressed when the ratio of the concentration of each antibiotic to the MIC of that antibiotic was same for all components of the mixture. The ΣFICs were calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the ΣFIC is ≤0.5, indifferent when the ΣFIC is >0.5 to <2, and antagonistic when the ΣFIC is ≥2.

RESULTS

During the study period, from January through December 2003, 1,287 blood samples were cultivated for bacterial growth in our laboratory. *Brucella* strains were isolated from 16 (1.24%) of the cultures, and all of them were identified as *B. melitensis.* Nine (56.25%) of the strains were obtained from male patients, and seven (43.75%) were obtained from female patients. Twelve (75%) of the strains were recovered from May through August, so there was a seasonal distribution regarding the isolation of brucellae. This is the season of the year when sheep and cows bear their young; so the production of milk, cheese, and diary products is at a maximum and migration to rural areas is increased due to the summer holidays.

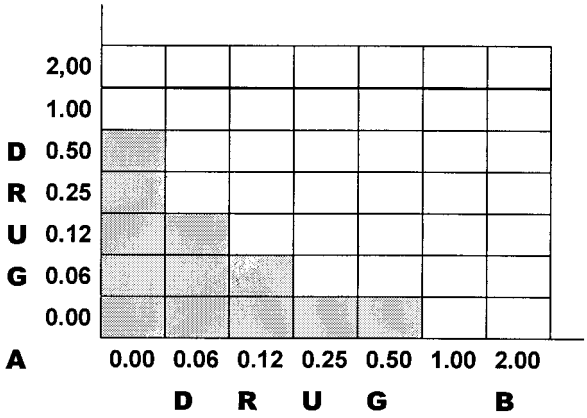

FIG. 2. The checkerboard method showing the synergy of a two-drug combination.

E test and the checkerboard method were performed in duplicate for each isolate and for all drug combinations. No discordant results were obtained between the two sets of trials. The MIC ranges, the MIC at which 50% of the isolates are inhibited (MIC_{50}s), and the MIC_{90}s of the antibiotics are shown in Table 1.

By E test, the ΣFIC results for the combination RIF-DOX yielded synergy against 15 (93.75%) strains and indifference against only 1 (6.35%) strain. By the checkerboard method, the same drug combination showed synergy against 10 (62.5%) strains, indifference against 4 (25%) strains, and antagonism against 2 (12.5%) strains. The ΣFIC results for the other antibiotic combinations by E test and the checkerboard method and comparison of the results for synergy, indifference, and antagonism are presented in Tables 2 to 4. The results of 44 (55%) of 80 tests by both methods were in agreement. Confirmation of the results was most marked with the SM-DOX combination, for which the test results were in agreement for 12 (75%) strains. Comparisons of other antibiotic combinations and the general confirmation of the results were demonstrated (Tables 2 to 4).

DISCUSSION

Brucellosis is an important disease in developing countries, such as countries in the Middle East and the Mediterranean area. Brucellae, which are the etiologic agents of this disease, are intracellularly located pathogens. They survive in the macrophages in which they have been phagocytosed, and so they

TABLE 1. MICs of the antibiotics tested and percentage of isolates susceptible

Antibiotic	MIC range (μg/ml) by checkerboard method	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml) by E-test	% Susceptible by:	
					Checkerboard method	E test
RIF	0.5–8	1	2	0.75–3	75	93.73
SXT	0.125–8	2	4	0.064–0.125	75	100
DOX	0.06–2	0.12	0.25	0.19–0.125	68.75	75
AZM	0.5–8	1	4	0.25–8	93.75	68.75
CIP	0.125–1	0.5	0.5	0.064–1	75	93.75
SM	0.5–4	2	2	0.19–0.125	81.25	100

TABLE 2. Results obtained with antibiotic combinations by checkerboard method

Strain no.	RIF-DOX		RIF-SXT		SXT-DOX		SM-DOX		AZM-CIP	
	ΣFIC	Activity[a]	ΣFIC	Activity	ΣFIC	Activity	ΣFIC	Activity	ΣFIC	Activity
1	0.50	S	4.24	A	4	A	2.12	A	0.27	S
2	0.37	S	0.37	S	2.5	A	0.31	S	1.32	I
3	1.03	I	0.56	I	2.5	A	0.18	S	0.12	S
4	0.15	S	0.18	S	0.51	I	0.26	S	0.31	S
5	0.31	S	1.12	I	1.03	I	0.49	S	0.53	I
6	2.06	A	2.50	A	4.03	A	1.03	I	0.02	S
7	1.08	I	2.12	A	1.7	I	0.56	A	0.52	I
8	0.13	S	0.51	I	8.06	A	0.28	S	0.36	S
9	4.03	A	2.25	A	1.08	I	0.75	A	0.12	S
10	0.36	S	2.03	A	0.28	S	1.10	I	0.18	S
11	0.27	S	2.06	A	0.53	I	0.24	S	0.13	S
12	0.62	I	0.57	I	0.31	S	0.56	I	0.62	I
13	1.25	I	0.51	I	0.18	S	0.36	S	4.25	A
14	0.28	S	1.08	I	0.56	I	4.15	A	0.31	S
15	0.31	S	1.12	I	0.28	S	0.62	I	0.04	S
16	0.13	S	0.51	I	5.0	A	4.12	A	2.06	A

[a] S, synergy; A, antagonism; I, indifference.

are protected from antimicrobials, which are unable to reach them.

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 (1, 4, 19).

In order to prevent relapses and complications, patients should adhere well to the treatment protocols, otherwise the insufficient administration of the drugs will cause the phagocytosed bacteria to reinfect the host. Relapses and chronic cases will affect the quality of life of the patients and will lead to economic losses (4, 13, 14, 17).

The standard procedures for the in vitro testing of brucellae have not been determined. Proper treatment requires an in vitro method of detecting synergy between antibiotic combinations that is simple, accurate, and reproducible.

The checkerboard microtiter plate assay is used to test the activities of several drugs in combination against *B. melitensis* strains by determining the ΣFICs of all combinations tested. The use of E test in investigations of the effects of antibiotic combinations is relatively new compared to the lengths of time that the classical methods have been in use. Although synergy tests are difficult and time-consuming, E test is easily applied in the routine laboratory practice. Therefore, many studies have been conducted in recent years to test the results of this method compared with those of other in vitro susceptibility tests. The checkerboard and E test methods were reported to be in agreement in some studies (6, 7), but in some others the results of E test were found to be concordant with those of other tests for synergy (5, 12, 18).

White et al. (18) compared the checkerboard method, the time-kill method, and E test to investigate the effects of four different antibiotic combinations against *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* strains. There was 44 to 88% agreement between the time-kill and checkerboard methods and 63 to 75% agreement between the time-kill and E test methods. The rate of agreement between the checkerboard and E test methods was found to be 75%.

TABLE 3. Results obtained with antibiotic combinations by E test

Strain no.	RIF-DOX		RIF-SXT		SXT-DOX		SM-DOX		AZM-CIP	
	ΣFIC	Activity[a]	ΣFIC	Activity	ΣFIC	Activity	ΣFIC	Activity	ΣFIC	Activity
1	0.08	S	0.6	I	2.45	A	0.36	S	0.25	S
2	0.06	S	1.02	I	0.31	S	0.12	S	0.73	I
3	0.09	S	0.03	S	0.30	S	0.10	S	1.47	I
4	0.3	S	0.47	S	3.30	A	0.12	S	0.12	S
5	0.12	S	1.04	I	1.50	I	0.24	S	0.55	I
6	1.37	I	1.18	I	0.30	S	0.62	I	0.44	S
7	0.2	S	0.81	I	2.17	A	0.4	S	0.13	S
8	0.28	S	2.94	A	0.56	I	0.4	S	0.37	S
9	0.06	S	0.03	S	0.26	S	8.1	A	0.06	S
10	0.21	S	0.52	I	2.20	A	1.7	I	0.54	I
11	0.04	S	0.01	S	1.25	I	0.05	S	0.13	S
12	0.28	S	3.96	A	3.20	A	1	I	0.08	S
13	0.05	S	0.01	S	0.24	S	0.3	S	0.08	S
14	0.29	S	2.68	A	1.50	I	0.4	S	0.13	S
15	0.14	S	1.52	I	0.30	S	1.12	I	0.13	S
16	0.04	S	0.45	S	2.40	A	0.3	S	0.13	S

[a] S, synergy; A, antagonism; I, indifference.

TABLE 4. Comparison of results by E test and checkerboard method[a]

Activity	No. (%) of strains									
	RIF-DOX		RIF-SXT		SXT-DOX		SM-DOX		AZM-CIP	
	CB	ET	CB	ET	CB	ET	CB	ET	CB	ET
Synergy	10 (62.5)	15 (93.7)	2 (12.5)	6 (37.5)	4 (25)	6 (37.5)	7 (43.7)	11 (68.7)	10 (62.5)	12 (75)
Indifference	4 (25)	1 (6.25)	8 (50)	7 (43.7)	6 (37.5)	4 (25)	4 (25)	4 (25)	4 (25)	4 (25)
Antagonism	2 (12.5)		6 (37.5)	3 (18.7)	6 (37.5)	6 (37.5)	5 (31.2)	1 (6.25)	2 (12.5)	
Total	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)

[a] CB, checkerboard method; ET, E test.

Bonapace et al. (5) evaluated the effects of the combinations trovafloxacin-tobramycin and cefepime-piperacillin against 10 *Acinetobacter baumannii* strains by the checkerboard, time-kill, and E test methods. They found that the rate of agreement between the results of the checkerboard and E test methods was 63%.

Göksel (9) tested the combinations meropenem-amikacin and meropenem-ciprofloxacin for synergy against multiple-drug-resistant *Pseudomonas aeruginosa* strains by the checkerboard and E test methods. He found that in 26.7% of the tests there was only minor disagreement between the two methods and observed no major disagreements.

In our study the rate of agreement between the checkerboard and E test methods was 55%. The rates of agreement obtained with the combinations RIF-DOX, RIF-SXT, SXT-DOX, SM-DOX, and AZM-CIP were 63.75, 31.25, 13.75, 62.5, and 56.25%, respectively.

Even though each of these methods uses different conditions and endpoints, there was frequent agreement between the results of the two methods. Further comparisons of the E test technique with the checkerboard method for the determination of synergy are warranted. The checkerboard method is difficult and time-consuming for routine antimicrobial synergy testing, but we suggest that E test can easily be applied to antibiotic susceptibility testing of *B. melitensis* strains, as it is less labor-intensive and less time-consuming. The standardization of these techniques for routine laboratory testing is needed because of the common use of combination therapies against the growing numbers of multiple-drug-resistant strains.

The antibiotic synergy test results observed in our study clearly corresponded to the therapy for brucellosis given at our hospital: DOX at 100 mg orally twice a day combined either with RIF at 600 mg/day orally or with SM at 1 g/day intramuscularly for 6 to 8 weeks for adults. The majority of our patients were cured by this protocol, they tolerated the treatment well, and no relapses occurred.

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