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Investigation of Synergistic, Additive and Antagonist Effect of Antimicrobial Combinations Used for Brucella spp, with E-test Combination Method

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ABSTRACT

Brucella spp. is a significant cause of morbidity and mortality in human. Though combination therapy is recommended in the patients with brucellosis, there is very less data about the in vitro efficacy of the combined antimicrobials. In this study, we aimed to investigate the in vitro activities of antimicrobial combinations used against the *Brucella spp.* strains.

A total 40 human isolates of *Brucella spp.* strains were included in this study. In vitro activities of doxycycline, rifampin, cotrimoxazole, streptomycin and ciprofloxacin were studied by E-test (Bio Mérieux, France) strips. In vitro effectiveness of doxycycline-rifampin, rifampin-cotrimoxazole, doxycycline-streptomycin, ciprofloxacin-cotrimoxazole, and ciprofloxacin-streptomycin were investigated by E-test combination method, and the results were evaluated with Fractional Inhibitor Concentration Index.

Cotrimoxazole was found as the most active antimicrobial against to tested *Brucella* isolates with the lowest Minimal Inhibitory Concentration (MIC) (as, 0.016 µg/ml), while rifampin was the least active drug with highest MIC (as 1.5 µg/ml). The MIC_{50} and MIC_{90} values of the tested antibiotics were as follows: cotrimoxazole 0.032 µg/ml and 0.064 µg/ml, doxycycline 0.064 µg/ml and 0.094 µg/ml, ciprofloxacin 0.094 µg/ml and 0.75 µg/ml, streptomycin 0.25 µg/ml and 0.50 µg/ml, and rifampin 0.50 µg/ml and 0.75 µg/ml, respectively. Doxycycline-rifampin combination showed a remarkable synergistic activity to all tested strains (%100), but rifampin-cotrimoxazole combination exhibited antagonist activity to two strains (5%). The ratios of synergistic activities were as follows: ciprofloxacin-streptomycine 57.5%, rifampin-cotrimoxazole 52.5%, doxycycline-streptomycin 32.5%, and ciprofloxacin-cotrimoxazole 25%.

In this study, though cotrimoxazole was the highest in vitro active against to tested strains, its combination showed low synergistic effect with other antimicrobials. On the contrary, though rifampin showed low in vitro activity alone, it exhibited excellent synergistic effect when combined with doxycycline. Therefore, when the treatment is planned for a patient with Brucellosis, it will be benefit for testing the combination effectiveness of the drugs irrespective of their in vitro activities alone.

Key Words: Brucella, Antibiotics, Synergism, Doxycycline, Rifampicin

Introduction

Brucellosis is one of the most common zoonotic diseases in the world, and it is particularly endemic in Mediterranean countries, Arabian Peninsula, India, Africa and South America (1). It generally manifests an acute febrile disease, but in some cases, it can show a chronic progression with life-threatening complications. According to World Health Organization (WHO), *Brucella melitensis* is responsible for the majority of the human cases, and 500,000 new patients are reported every year around the world (2).

Brucella species are facultative intracellular pathogens with the ability to survive and growth in the host's phagocytic cells (3). Therefore, for the patients diagnosed with Brucellosis, WHO recommends the use of antimicrobials combination therapy regimes, having highest intracellular activity (2). Up to date, the most effective treatment protocol is reported to be the combination of doxycycline and rifampin and/or streptomycin (3,4). However, increasing resistance to these antibiotics, and the side effects of these drugs for the certain patient groups such as pregnant women and children, have limited their use, and put a requirement to explore alternative combinations (2–4). Additionally, inadequate dosing of the antimicrobials and low patient's adherence to

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treatment protocols associate with the infection relapse (5).

All these factors have impacts on the antimicrobial resistance among *Brucella* strains. Therefore, detection of antimicrobial susceptibility of *Brucella* isolates and the activity of the combination regimes will be important to successfully manage these patients. Up to date, some studies have been published, which show the in vitro efficacies of the antimicrobials against to Brucella species, however very less data is available about their combination activities.

In this study, it was aimed to investigate the in vitro synergistic, additive and antagonistic effects of traditional and new antimicrobial combination which are used in the management of brucellosis, using the E-test method.

Materials and Methods

In this study; a total of 40 *Brucella spp.* strains isolated from blood cultures belonging to different patients were taken from our hospital medical microbiology laboratory.

Blood Culture and Identification: A set of 5 to 10 ml blood samples were aseptically obtained from the venipuncture, and were inoculated to blood culture bottles (Bio-Mérieux, France). The specimens were sent to microbiology laboratory of the hospital, immediately. The bottles were incubated in Bac/TAlert automatized blood culture system (Bio-Mérieux, France) until the positive signal was obtained, which was indicating a growth of microorganism occurred. Subcultures from the positive bottles were done on the tripticase soy agar with 5 to7 % blood, Eosine Methylene Blue agar, and chocolate agar mediums (Oxoid, UK), and incubated at 35^0C for 24-72 hours. The growing microorganisms were identified by conventional methods, and with Vitek2 (Bio Mériueux, France) automated bacterial identification device.

Antimicrobial Sensitivity Test and E-test Combination Method: The efficacy of doxycycline (DOX), rifampin (RIF), cotrimoxazole (SXT), streptomycin (SM) and ciprofloxacin (CIP) against these strains was studied by E-test method. The in-vitro activity of DOX-RIF, RIF-SXT, DOX-SM, CIP-SXT and CIP-SM combinations was investigated by the E-test combination method, which is the most frequently used antibiotics in clinical practice. Synergic, additive and antagonist effects were calculated by evaluating the results with the Fractional Inhibitor Concentration Index (FIC).

The MIC values of the isolates to SM, RIF, DOX, SXT, CIP were investigated by using E-test strips (BioMerieux, France). For each brucella isolate, suspensions were prepared at 0.5 McFarland distillation from breeding colonies on solid medium. 10 µl of suspension was added to the surface of Mueller Hinton Agar (Merck, Germany) containing 5% sheep blood in 120 mm plates (17). E-test strips were placed on plaques after inoculation by using an applicator. In the first stage, for each of the antibiotics to be combined, two E-test strip (antibiotic A and antibiotic B) were placed to plaques and the plaques were left at room temperature for 1 hour. Subsequently, one of the antibiotic A E-test strip was removed and an E-test strip of B antibiotics was placed instead of it which has the antimicrobial gradients overlap with the same values. Also one of the strip of B antibiotic was removed and a E-test strip of A antibiotic was placed on. Plates were incubated at 35 ° C for 24 and 48 hours in aerobic media. At the end of the first 24 hour evaluation, the MIC values of the isolate against the tested antibiotics A and B were determined. At the end of the 48-hour incubation, the MIC levels of the area where the combination was made were read. As a result, the minimum inhibitor concentration (MIC) values for the antibiotics A and B for each strain, and the MIC values obtained for the combination of AB and BA were determined. During the process, the start and end points of the first stribe were marked behind the plaque and the MIC values of the two stribes were superimposed.

The combined antimicrobials of each studied strain were determined by using the FIC index, which showed synergistic, additive or antagonistic activity. The following formula was used to determine the FIC index.

FIC (A + B) = (MIC AB / MIC A) + (MIC AB / MIC B)

The results obtained are interpreted according to the following evaluation criterion

≤0.5 synergy

> 0.5 - <1.0 additive effect

> 1.0 - <2.0 no change

≥4 antagonists (7).

MIC values of tetracycline and streptomycin were interpreted according to the breakpoint values suggested by CLSI(Clinical and Laboratory Standards Institute) for Brucella spp. Rifampin, trimethoprine-sufamethoxazole and ciprofloxacin MIC values were interpreted according to the breakpoint values of CLSI suggested by the breeders for microorganisms. In the study, B.abortus 03036, Staphylococcus aureus ATCC 25922 and Escherichia coli ATCC 25922 standard strains were used in accordance with CLSI recommendation (8).

Results

The most effective antimicrobial to the tested strains was found as SXT with the lowest MICs as 0.016 μg/ml, and RIF was found as the least active agent with highest MICS, as 1.5 μg/ml. MIC_{50} and MIC_{90} values of antibiotics were found as; SXT 0.032 μg/ml and 0.064 μg/ml, DOX 0.064 μg/ml and 0.094 μg/ml, CIP 0.094 μg/ml and 0.75 μg/ml, SM 0.25 μg/ml and 0.50 μg/ml, RIF 0.50 μg/ml and 0.75 μg/ml, respectively.

The combination of RIF-SXT showed antagonists (5%) effect in two strains, while the DOX-RIF combination showed synergistic effect for all tested strains (100%). The synergistic effect rates of the combination of CIP-SM and RIF-SXT against strains were 57.5% and 52.5%, respectively, and the synergistic effect rates of DOX-SM and CIP-SXT combinations were 32.5% and 25%, respectively.

In our study, the highest in vitro activity was detected to SXT, and the highest MIC level was found for RIF. However, the synergistic effect of SXT in combination with other antibiotics was low. The RIF-DOX combination showed a synergistic effect against all strains. The MIC_{50} and MIC_{90} levels obtained from the antibiotics studied by the e-test method are shown in Table 1, and the in-vitro activities of the combinations used are shown in Table 2.

Discussion

The genus *Brucella*, which causes chronic infections in the reticuloendothelial system, is a significant cause of morbidity and mortality in humans (2,3). In our country, 50-60% of Brucellosis cases are between the ages of 20-50, 10-15% of in children and 10% of over 65 years (9). According to Turkish Health Ministry, brucellosis is seen as endemic in our country. In 2002, the number of reported cases was 17744 while in 2009; 9324 cases were reported. From 2002 to 2009, there was a reduction of about 50% in the cases of brucellosis (10,11)

WHO recommended following combinations for the treatment of brucellosis, such as DOX-RIF and DOX-SM/gentamicin, or alternatively RIF-quinolone, DOX-SXT combination, or DOX or minocycline monotherapy (3). Combination of SXT-gentamycin or RIF-gentamycin is recommended for children under 8 years diagnosed with acute brucellosis. Furthermore, RIF monotherapy or RIF-SXT combination therapy is recommended for pregnant women with brucellosis. In case of any focal infection (i.e., endocarditis, spondylitis, meningitis, paraspinal abscess), additional CIP or ofloxacin to SM or gentamicin, or SXT may be used in addition to DOX-RIF (3, 4).

In vitro resistance for the many drugs used in the treatment of brucellosis has not been reported. Even after the recurrence, no resistance was also reported in the isolated strains. Thefore, recurrence or treatment failures are believed to be due to the intracellular localization of the bacteria. In most cases, inadequate treatment (low dose administration) or frequent interruption in the medication due to patient's low adherence are reported to be exact causes of the treatment failures (2, 5, 25).

The most sensitive antimicrobial agent in Turkey is reported as SXT. Kilic et al. (12) conducted a study of SXT MIC_{50} value as 0.064 μg/ml, and MIC_{90} value was found to be 0.094 μg/ml. Baysan et al. (13) reported SXT MIC_{50} and MIC_{90} as 0.047 μg/ml and 0.094 μg/ml, respectively; whereas Ascen et al (14) reported as 0.012 μg/ml and 0.023 μg/ml orderly. Our finding about SXT MIC_{50} value was 0.032 μg/ml, and MIC_{90} value was determined as 0.064 μg / ml. These finding were very similar to the data of our country.

In some studies, RIF MIC_{50} and MIC_{90} values are reported as ≤1. Şengöz et al. (15) a value of MIC_{50} 0.75 μg/ml; MIC_{90} value 1 μg/ml, Eşel et al (16) 0,50 μg/ml and 1 μg/ml, Altay (17) 0,75 μg/ml and 1 μg/ml, and Baykam et al (18) 0.75 μg/ml and 1 μg/ml, were reported the RIF MICs, respectively. These data are also in accordance with our findings. In our study, we found the highest MIC_{50} and MIC_{90} values (as 0.5 μg/ml and 0.75 μg/ml) for RIF. In our study, we found the highest MIC_{50} and MIC_{90} values (as 0.5 μg/ml and 0.75 μg/ml) for RIF

Table 1. MIC values of antibiotics, levels of MIC_{50} and MIC_{90}

Antibiotic	MIC50 (μg/ml)	MIC90 (μg/ml)	Value Range (μg/ml)	CLSI Breakpoint
DOX	0,064	0,094	0,023-0,19	≤1
RIF	0,50	0,75	0,25-1,5	ND
SXT	0,032	0,064	0,016-0,094	≤2
SM	0,25	0,5	0,064-0,75	≤8
CIP	0,094	0,125	0,047-0,25	ND

Table 2. In vitro activity results of the combinations

Antibiotic combinations	Synergy Test Results (n%)				
	Antagonism	Indifferent	Additive Effect	Synergy	Total Strain
DOX-RIF	-		-	40 (100%)	40
RIF-SXT	2 (5%)	5 (12,5%)	12 (30%)	21 (52,5%)	40
DOX-SM	-	11 (27,5%)	16 (40%)	13 (32,5%)	40
CIP-SXT	1 (2,5%)	9 (22,5%)	20 (50%)	10 (25%)	40
CIP- SM	-	3 (7,5%)	14 (35%)	23 (57,5%)	40

The MIC_{50} and MIC_{90} values of DOX were found low in many studies conducted in Turkey and abroad. In Altay's (17) study, the MIC_{50} and MIC_{90} values were found 0.023 and 0,064 µg/ml. Şengöz et al. (14) reported MIC_{50} and MIC_{90} values as 0.096 µg/ml and 0.032 µg/ml; and Kılıç et al (12), as 0.094 µg/ml and 0,25 µg/ml, respectively. Furthermore, Baysan et al (13) reported 0,047 µg/ml and 0,125 µg/ml, orderly; and Baykam et al. found as 0.03 µg/ml and 0.06 µg/ml, respectively. In a study by Rolain et al (19), DOX MIC_{50} and MIC_{90} values were found as 0,06 µg/ml and 0,25 µg/ml; Rubinstain et al (20) determined as 0,25 µg/ml and 0,4 µg / ml, and Hoe (21) found as 0,125 µg/ml and 0.125 µg/ml, respectively. In our study, DOX MIC_{50} and MIC_{90} values were found as 0,064µg/ml and 0,094 µg/ml, orderly.

Lubani et al. (22) studied clinically with oxytetracycline and DOX; recurrence rate was inversely proportional to treatment duration. Montejo et al. (23) reported a recurrence rate of 14% after six weeks of DOX therapy. The use of DOX alone in the treatment of brucellosis should be considered according to the latest information and new studies should be included in this treatment (5).

Considering the MIC values of CIP in our study, the efficacy against Brucella isolates was determined to be as low as SXT and DOX. The MIC_{50} and MIC_{90} values of CIP in studies carried out in Turkey and abroad were found as fellows: Alışkan et al. (14) found as 0.094 µg/ml 0.125 µg/ml, Turan et al. (24) found as 0.094 µg/ml and 0.19 µg/ml, 0.094 µg/ml and 0,19 µg/ml in Baykam's study (18), and Rubinstain et al (20) reported 0.4 µg/ml and 0.8 µg/ml, respectively.

New generation fluoroquinolones are antimicrobials of interest in the treatment of brucellosis due to their good oral bioavailability, high tissue concentrations, intracellular penetrations and in vitro activities on Brucella species. In the works done; the high recurrence of fluoroquinolone use alone is found, the use of brucellosis alone is not appropriate, but due to the fact that the in vitro efficacy against Brucella isolates is assumed to be very good, it is interpreted as it can be used in combination treatment regimens(4-6,25).

In our study, MIC_{50} for SM was 0.25 µg/ml, and MIC_{90} was 0,5µg/ml. In Altay's study (17), it was reported the value of MIC_{50} at 0,50 µg/ml, and MIC_{90} value was reported as 0,75µg/ml. Furthermore, Rolen et al. (19) reported these values as 1 µg/ml and 2 µg/ml, and Şengöz et al (15) found them as 0,5µg/ml and 0,75 µg/ml, respectively.

While isolates against antibiotics used in the treatment of brucellosis are generally clinically sensitive, in-vitro synergy testing may not always be clinically compatible. Time-kill, checkerboard and E-test are very common in-vitro synergistic methods (7, 13). There are only a few studies that have identified the in-vitro antimicrobial combination activity against Brucella isolates. Although the E-test method used to determine the synergistic effects of antimicrobials and in-vitro sensitivities is an appropriate method, a standard synergy test method has not been developed (7,12,20,26).

In our study, the highest in-vitro synergistic efficacy was determined against DOX-RIF (100%) combination as Kılıç et al. (12) did with E-test combination method. The DOX-RIF synergic efficacy was found to be 94% by the E-test method and 63% by the checkerboard method by Orhan et al. Akova et al (27) used 20 *Brucella spp.* had a synergistic effect in 17 of the isolates, an additive effect in two isolates, and an indifferent effect in an isolate.

In our study, the synergistic effect of the combination of RIF-SXT was 52.5%, additive effect 30%, indifferent effect 12.5%, antagonistic effect was about 5%. Kılıç et al. (12) reported a synergistic efficacy of 88%, additive efficacy of 13%. Turkmani et al. (28) reported a synergistic

efficacy of 38%, additive efficacy of 19% and indifferent efficacy of 44%.

The synergistic efficacy of our combination of DOX-SM was 32.5%, additive efficacy 40%, and undifferentiated efficacy 27.5%. While Kılıç et al. (12) did not show synergic activity, additive activity was 12.5%, undifferent activity was 68.75% and antagonistic activity was 18.75%. Al-Dahouk D et al. (29) did not observe a synergistic effect in the combination of DOX-RIF and DOX-SM with the time-kill method in combination with DOX and SM. Dizbay et al (30) found no synergistic effect between DOX-SM and 19% reported antagonism. However, Orhan et al. (26) reported 44% with the Checkerboard method, 69% with the E-test method, 31% with the Checkerboard method and 6% with the E-test method. Akova et al (27) found a 90% synergistic effect with the Checkerboard method.

The synergistic activity of our CIP-SXT combination was found as 25%, additive efficacy 50%, indifferent efficacy 22.5%, and antagonistic efficacy 2.5%. Kılıç et al. (12) found 44% of synergic activity and 56% of additive activity and did not report undifferentiation and antagonistic activity. Although CIP-SXT is not used as a first line in the treatment of brucellosis, it can be combined with alternative treatment or first-line treatment when recurrence and side effects occur in classical treatment (12,25).

In some studies, DOX-RIF has been reported to be the most commonly used treatment combination in patients (5,6). Treatment of the combination of SXT and RIF gave good results in children, but not enough results were obtained in adults due to the risk of this treatment of developing resistance to RIF (6, 12, 22).

Despite the high MIC_{50} and MIC_{90} values of RIF in Brucella isolates, the second most effective synergistic and additive effect was determined in combination with RIF-SXT. In clinical trials, the recurrence rate of RIF-SXT combinations given to children under eight years of age was determined to be 4-8%, but no recurrence was observed when the treatment duration was extended to 8 weeks (22).

There are very limited in vitro studies about Brucella susceptibility to combination therapies. Instead, resistance to antibiotic combinations, and resistance to recurrent cases have been mostly reported in clinical studies (5,6). In our study, the antagonistic effects of CIP-SXT and RIF-SXT were 2.5% and 5%, respectively. The antagonistic effect of DOX-SM combination was reported as 19% in the study performed by Kilic et al. (12). Orhan et al. (26) found that antagonistic efficacy of the DOX-SM combination with the E-test method was 6%, and 13% in the checkerboard method, and antagonistic effect of the RIF-SXT combination was 7%.

Although our study found that SXT was the most effective and RIF was the least effective agent, the combination of DOX-RIF showed the best synergistic effect in the in vitro environment.

If clinical treatment is planned and the agent is isolated, testing the effectiveness of the antimicrobial combination that is intended to use will be benefit for the successful treatment. In this context, we believe that in-vitro detection of combinatorial activities as well as single antibiotic susceptibility in antimicrobial susceptibility testing against Brucella species would be beneficial.

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