

***IN VITRO* SYNERGISTIC ACTIVITY OF ANTIBIOTIC COMBINATIONS AGAINST *BRUCELLA MELITENSIS* USING E-TEST METHODOLOGY**

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ABSTRACT

The treatment of brucellosis is still problematic, because of high rates of treatment failure or relapses. As the microorganism is an intracellular pathogen, treatment requires combined regimens. However, limited existing data on in vitro combinations are available for *Brucellae*. The aim of this study was to investigate the in vitro efficacy of various traditional and new antibiotic combinations against 16 *Brucella melitensis* strains. The combination effect of antimicrobial agents was evaluated by E-test synergy method to obtain a fractional inhibitory concentration (FIC) index. Co-Trimoxazole (SXT) and moxifloxacin (MXF) exhibited the lowest MIC, while Rifampin (RIF) had the highest MIC in the study. Combinations with RIF showed the best synergistic activity (100% of RIF-tetracycline (TET), and 87.5% of RIF-SXT). Synergistic activity was also detected at seven (43.7%) of ciprofloxacin (CIP)-SXT, four (25%) of TET-MXF, and two (12.5%) of TET-SXT combinations. The combinations that demonstrated additivity were TET-SXT, CIP-SXT and TET-MXF. Antagonism was observed only with the TET-Streptomycin (STR) combination in three strains (18.8%). Further work including randomized controlled clinical trials is required to fully evaluate the usefulness of these data.

Key-words: *Brucella melitensis*, antibiotics, synergism.

Brucellosis is one of the world's major zoonotic diseases and still remains a significant public health problem mainly in the Mediterranean littoral, Arabian Gulf, Indian subcontinent, in Central Asia, and in Central and South America (10,24).

Because of the intracellular survival in the phagocytic cells of *Brucella* spp., treatment of brucellosis requires not only combined regimens of antibiotics but agents that have ability to penetrate into macrophages and retain efficacy even in acidic milieu as well (24,25). The World Health Organization (WHO) Expert Committee on Brucellosis recommended treatment with a doxycycline (DOX) and Rifampin (RIF) combination for a 6-week course (10). Alternatively, rifampin could be replaced with streptomycin (STR). The high rates of therapeutic failure and relapses, toxicity and side effects of conventional antibiotic regimens have led to the investigation of new drugs and therapeutic schedules restricted largely to the addition of a

third drug (usually Co-trimoxazole -SXT) or substitution other aminoglycosides for STR (25,27). On the other hand, controlled clinical trials with other antimicrobials, including SXT, new macrolides, and β -lactams have shown inferior results or involved few patients for a proper evaluation in this setting (9). Therefore, there is no conclusive evidence on the optimum antibiotic therapy for brucellosis.

Although there are some studies on in vitro susceptibility of *Brucella* strains to newer and conventional antibiotics, the studies comparing in vitro synergistic activity of well-known traditional combination regimens are very limited (3,8,11,19,22, 26,28). The Epsilometer test (E-test), an agar diffusion method, determines not only antimicrobial susceptibility quantitatively, but assesses the synergistic activities of antimicrobial combinations against a wide variety of organisms as well (6,8, 18,22,30).

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The aim of this study was to assess the activity of conventional and newer antimicrobial combinations using E-test against *Brucella melitensis* strains.

Sixteen strains of *B. melitensis* were obtained from blood cultures between 2004 and 2005 in Gazi University Hospital in Ankara, Turkey. All of the strains were identified as *B. melitensis* biovar 3 using the following tests: the requirement of CO₂ for growth, production of urease and H₂S, sensitivity to the dyes basic fuchsin and thionin (at final concentration of 20-40 µg/ml), and lysis by the phage Tbilisi and agglutination with monospecific antisera for A and M antigens (2). The strains were stored in skim milk at -80°C and twice subcultured before starting the study.

Three *Brucella* reference strains [*Brucella abortus* 544 (ATCC 23448), *B. melitensis* 16M (ATCC 23456), and *B. suis* 1330 (ATCC 23444)] 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 control.

Determination of MIC and synergy by E-test methodology was performed in duplicate according to the manufacturer's recommendations. For the evaluation of the MICs of single antimicrobial agents, TET, RIF, SXT, MXF, CIP and STR, E-test was performed on Mueller-Hinton agar (MHA) supplemented with 5% sheep blood. MHA plates were inoculated with 0.5 McFarland turbidity standard suspensions of each isolate and E-Test strips (AB Biodisk, Solna Sweden) were placed onto each MHA plate. The results were evaluated after 48 hours of incubation in ambient air at 35°C (12).

Seven different antibiotic combinations (TET-RIF, TET-STR, TET-SXT, TET-MXF, CIP-STR, CIP-SXT, and RIF-SXT) were evaluated for 16 strains of *B. melitensis* biovar 3 by E-test prediffusion technique to obtain the fractional inhibitory concentration (FIC) index according to manufacturer's recommendations. For testing of drug combinations, after inoculation of the bacterial suspension with turbidities equivalent to 0.5 McFarland onto same culture medium, E-test strips (drug A) were applied and after 1h incubation at room temperature the first E-test strip was removed. Afterwards, an E-test strip of second antimicrobial (drug B) was applied onto the imprint of strip A, vertically transposed so MICA and MICB overlap at the same position. E-test method was performed in duplicate for all organisms and combinations. The plates were then incubated at 35°C in ambient air and MIC levels of each combination was evaluated after 48 h.

The following formulas were used to calculate the Σ FIC (the cumulative fractional inhibitory concentration 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 Σ FIC = FIC of drug A + FIC of drug B.

The results were interpreted according to FIC indexes as follows: synergistic (Σ FIC: ≤ 0.5), additive (Σ FIC: >0.5 and ≤ 1), indifferent (Σ FIC: >1 and ≤ 4), antagonistic (Σ FIC: >4).

The Minimum inhibitory concentrations (MICs) of relevant antibiotics and MIC₅₀ and MIC₉₀ levels defined as the lowest concentration of the antibiotic at which 50% and 90% of the isolates inhibited respectively, are shown in Table 1.

Table 1. MIC ranges, MIC₅₀ and MIC₉₀ values of antibiotics against *B. melitensis* biovar 3 (n=16).

Antimicrobial Agent	MIC ranges (µg/ml)	MIC ₅₀ (µg/ml)	MIC ₉₀ (µg/ml)
SXT	0.032-0.125	0.064	0.094
MXF	0.032-0.19	0.064	0.19
TET	0.047-0.25	0.094	0.25
CIP	0.064-0.25	0.125	0.25
STR	0.25-0.75	0.50	0.75
RIF	0.75-2	1.5	2

SXT: Co-Trimoxazole, MXF: Moxifloxacin, TET: Tetracycline, CIP: Ciprofloxacin, STR: Streptomycin, RIF: Rifampin.

According to the MIC₉₀ values, the most active antibiotic was SXT (MIC₉₀ 0.094 µg/ml), followed by MXF (0.19 µg/ml), CIP and TET (0.25 µg/ml), and STR (0.75 µg/ml). RIF had the highest MIC₅₀ and MIC₉₀ values.

The highest rates of synergy were observed with combinations of RIF-TET (100%) and RIF-SXT (87.5%). Synergistic activity was also detected at seven (43.7%) of CIP-SXT, four (25%) of TET-MXF, and two (12.5%) of TET-SXT combinations. Additive activity was found at nine (56.2%) of CIP-SXT, seven (43.7%) of TET-SXT and TET-MOX, one (6.2%) of CIP-STR, and two (12.5%) of TET-STR combinations.

CIP-STR and TET-STR combinations resulted in indifferent effect against 93.8% and 68.8%, respectively. Synergy was not seen with TET in combination with STR against any of the strains. The E-test synergy method for the *B. melitensis* isolates using TET plus STR revealed antagonism in three of 16 (18.8%) (Table 2).

Treatment failures and relapses are major problems in the management of brucellosis (13,14,20). Therapeutic failure is mostly related to pharmacokinetics and pharmacodynamics of antibiotics rather than resistance (25). Relapses usually occur in the first year after the onset of infection, but they are caused by inadequate treatment (inappropriate dosage, short-term administration, and poor patient compliance) in most of cases. In addition, pharmacokinetics and cellular immun status may play significant role in the occurrence of relapses (20,25).

Table 2. Synergy tests results of antibiotic combinations by E-test.

Strain no.	TET - RIF		TET-STR		RIF-SXT		TET-SXT		CIP-SXT		TET-MXF		CIP-STR	
	Σ FIC	Activity*	Σ FIC	Activity	Σ FIC	Activity	Σ FIC	Activity	Σ FIC	Activity	Σ FIC	Activity	Σ FIC	Activity
1	0.18	S	2.32	ID	0.51	ADD	1.50	ID	0.83	ADD	0.40	S	1.25	ID
2	0.15	S	2.08	ID	0.35	S	0.28	S	0.25	S	0.22	S	1.47	ID
3	0.30	S	2.73	ID	0.20	S	0.92	ADD	0.40	S	1.12	ID	1.71	ID
4	0.19	S	1.57	ID	0.17	S	1.48	ID	0.26	S	1.16	ID	0.77	ADD
5	0.21	S	1.70	ID	0.29	S	1.04	ID	0.58	ADD	1.03	ID	2.66	ID
6	0.25	S	2.18	ID	0.13	S	1.04	ID	0.49	S	0.82	ADD	2.05	ID
7	0.16	S	1.70	ID	0.25	S	0.82	ADD	0.53	ADD	0.79	ADD	2.01	ID
8	0.12	S	2.12	ID	0.39	S	0.54	ADD	0.59	ADD	0.56	ADD	1.53	ID
9	0.10	S	3.04	ID	0.20	S	0.30	S	0.29	S	0.24	S	1.21	ID
10	0.38	S	5.50	AG	0.29	S	0.75	ADD	0.60	ADD	1.17	ID	3.91	ID
11	0.28	S	0.53	ADD	0.23	S	1.06	ID	0.42	S	0.55	ADD	1.54	ID
12	0.18	S	0.62	ADD	0.81	ADD	1.12	ID	0.37	S	0.55	ADD	1.02	ID
13	0.31	S	6.05	AG	0.38	S	0.54	ADD	0.58	ADD	0.58	ADD	2.66	ID
14	0.12	S	2.18	ID	0.35	S	0.72	ADD	0.51	ADD	0.56	ADD	1.53	ID
15	0.27	S	4.06	AG	0.39	S	2.12	ID	0.75	ADD	1.18	ID	2.97	ID
16	0.40	S	2.75	ID	0.26	S	0.58	ADD	0.60	ADD	0.47	S	3.02	ID

TET: Tetracycline; RIF: Rifampin; STR: Streptomycin; SXT: Co-Trimoxazole; CIP: Ciprofloxacin; MXF: Moxifloxacin; S: Synergy; ADD: Additive; ID: Indifference; AG: Antagonism.

In this present study, we detected in vitro susceptibilities of antimicrobials commonly used in brucellosis and also investigated synergistic activity between some old and new antibiotic combinations by using E-test.

Up to now, the standard procedures for in vitro susceptibility testing have not been determined for *Brucella* species. Currently, methods for MIC determination are described for potential bioterrorism agents including *Brucella* species by the Clinical Laboratory Standards Institute (CLSI; formerly the NCCLS). 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 $\mu\text{g/ml}$, SXT $\leq 2/38$ $\mu\text{g/ml}$, and STR ≤ 8 $\mu\text{g/ml}$ according to the CLSI interpretive criteria (7). In our study, E-test method was used due to the fact that it was found to be reliable, reproducible, less labor-intensive, less time-consuming, and more practical than the broth micro dilution method (12,17,22,29).

Three methods to detect in vitro synergy have been described: the time-kill assay (TKA), checkerboard titration technique, and E-test. However, neither is applied in routine microbiology laboratories. Whilst most synergy studies have been performed using checkerboard or TKA, these methods are too time consuming and technically challenging for routine clinical testing. Therefore, we preferred E-test method on grounds of simplicity and availability to routine clinical laboratories (18,22,23).

Since the standard protocols for antimicrobial susceptibility and the breakpoint values have not been defined up to now, the CLSI interpretive criteria for slow growing bacteria (*Haemophilus*) have been taken into consideration in order to evaluate the results of MICs determination in the literature (4,17,29). The MIC values of TET, STR, SXT, CIP, MXF interpreted according to the CLSI criteria for slow growing bacteria and potential bioterrorism agents have shown ranges below the breakpoints for sensitivity determination (7).

The MIC values of RIF range 0.75-2 $\mu\text{g/ml}$ at levels below the breakpoints for resistance determination according to the CLSI interpretive criteria, however, MIC value of 12 of our isolates of the total 16 (75%) were found to be ≥ 1 $\mu\text{g/ml}$ and these strains may be characterized as intermediately resistant by CLSI definition. MIC values of RIF in previous studies were reported to range from 0.047 to 4 $\mu\text{g/ml}$, values confirmed again by our findings (4,5,11,17,19,28,29).

SXT demonstrated the lowest MIC₅₀ and MIC₉₀ in our study and these were significantly lower than those previously observed in Turkey (4,5,17). Although higher MIC values of SXT have been determined in Turkey with MICs ranging from 0.047 to 3.0 $\mu\text{g/ml}$, in vitro SXT resistance rate was reported as low as 2% (4). However, significant rates of SXT resistance have been reported in the world (15,21).

MIC values of tetracycline were low, corresponding to in vitro susceptibility of all isolates, which is consistent with

previous reports (4,5,17,26,29,31). In vitro susceptibility to STR was also found to be in the range described previously (3,22,29). However, the MICs of STR were significantly lower than those reported by Garcia-Rodriguez *et al.* (11), Trujillano-Martin *et al.* (28), Lopez-Merino *et al.* (19) and Rubenstein *et al.* (26). These higher MIC values for STR may be related to in vitro testing method used in these studies.

CIP and MXF have shown good in vitro activity to *Brucella* strains which were comparable to that of tetracycline in our study. In previous studies from Turkey (4,5,16,17), similarly, fluoroquinolones were found very active drugs against *B. melitensis* in vitro except that a study in which higher MIC₉₀ levels of CIP (2 µg/ml) and MXF (8 µg/ml) were determined using agar dilution method (31).

There is very limited data about in vitro synergistic activity of *Brucella* strains in the literature so far though several antibiotic combinations have been used in the treatment of brucellosis such as TET-STR, DOX-STR, DOX-RIF, RIF-SXT for many years. We determined in vitro synergistic activity of seven new and old antibiotic combinations in this study. Although RIF had the highest MIC values, the best synergistic activity was observed in 100% and 87.5% of the TET-RIF and RIF-SXT combinations, respectively. In contrast, synergistic activity was not detected in the combinations with STR (TET-STR and CIP-STR). Moreover, antagonistic effect was seen only in TET-STR combination (12.5%) which it is recommended for the classical treatment of brucellosis. SXT in combination with TET and CIP led to reduced significant MICs with most strains but this was not synergistic (FICI = 0.54 - 0.92 and FICI = 0.53-0.83, respectively). In contrast to our findings, a study comparing checkerboard with E-test assays on 16 *B. melitensis* isolates did not demonstrate antagonism between STR with DOX (22). In the study by Orhan *et al.* (22) testing for synergy between RIF, DOX, STR and SXT, synergy with RIF-DOX, STR-DOX, RIF-SXT, and SXT-DOX combinations were determined by E-test method, as 93.7%, 68.7%, 37.5% and 37.5% respectively. The reasons for the discrepancies between two studies may be explained by either different E-test testing procedure used to assess the synergy or regional differences in susceptibilities of *B. melitensis* strains.

As synergy testing methods are not yet standardized for reproducibility and interpretation, it is extremely difficult to compare these methods' results from different studies. Three Etest methods have been recently described: (i) a prediffusion technique used in this study; (ii) which Etest strips were placed side by side; and (iii) a second Etest strip was placed on the agar in a cross formation, with a 90° angle at the intersection between the scales at the respective MICs for the organism. Only the prediffusion method was reproducible, and has shown higher agreement rates with TKA than those of other E-test synergy methods (30). The third E-test synergy testing method was applied by Orhan *et al.* and antagonism was difficult to detect with their method as previously reported (6,30). In

addition, controversial results for classical combinations used in the therapy were also obtained from killing studies. In the study by Al-Dahouk *et al.* (1) testing for killing activity between DOX-RIF and DOX-STR, it was shown that the combination of DOX with STR did not exhibit killing activity. This finding is in agreement with our result in which no synergistic effect of TET plus STR was observed.

Despite the fact that quinolones are not recommended in first-line therapy for brucellosis, we detected 43.7% and 25% synergistic activity and 56.2% and 43.7% additive activity in CIP-SXT and TET-MXF combinations, respectively. These results may lead one to consider to use quinolones as an alternative choice if toxicity occurs in the classical combinations or as part of a second-line regimen in patients who fail to respond or develop disease relapse after therapy.

In conclusion, though Etest synergy method is relatively new and the use of the Etest strips for synergy has yet to be standardized, prediffusion technique has the potential to be a useful screening test for the determination of synergy due to easier to perform and time-efficient. The combinations with RIF-DOX and RIF-SXT appeared to be most effective (synergy 100% and 87.5%, respectively) by E-test prediffusion technique. Antagonistic activity was only seen in TET-STR combination, which it is commonly used in treatment of brucellosis. Although clinical association with these results are yet to be determined, it can be postulated that, other combinations especially with RIF or quinolones could be replaced in treatment failure or late response to the treatment with TET-STR. Considering the fact that in vitro antimicrobial susceptibility does not always predict clinical efficacy, in vivo studies and controlled clinical trials are warranted to confirm these considerations.

RESUMO

Atividade sinérgica *in vitro* de combinações de antibióticos contra *Brucella melitensis* através da metodologia E-test

O tratamento da brucelose é problemático devido à alta frequência de tratamentos mal sucedidos e recidivas. Por tratar-se de um patógeno intracelular, o tratamento requer procedimentos combinados. Entretanto, existem poucos dados sobre combinações *in vitro* para *Brucellae*. O objetivo deste trabalho foi investigar a eficiência de vários tratamentos tradicionais e novas combinações de antibióticos contra 16 isolados de *Brucella melitensis*. O efeito combinado foi avaliado através do método do E-test para obtenção do FIC (índice de concentração inibitória fracional). Co-trimoxazol (SXT) e moxifloxacina (MXF) apresentaram o MIC mais baixo, enquanto rifampicina (RIF) apresentou o MIC mais alto. Combinações com RIF mostraram a melhor atividade sinérgica (100% para RIF-tetraciclina e 87,5% para RIF-SXT). Atividade sinérgica

foi também detectada para sete (43,7%) combinações de ciprofloxacina (CIP-STX), quatro (25%) de TET-MXF e duas (12,5%) de TET-SXT. As combinações que apresentaram efeito aditivo foram TET-SXT, CIP-SXT e TET-MXF. Antagonismo foi observado somente para a combinação TET-estreptomicina (STR) em três isolados (18,8%). Mais pesquisas utilizando ensaios clínicos randomizados controlados são necessárias para avaliar a utilidade desses dados.

Palavras-chave: *Brucella melitensis*, antibióticos, sinergismo

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