Actual antibiotic resistance pattern of *Brucella melitensis* in central Anatolia

An update from an endemic region

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ABSTRACT

Objective: To test *in vitro* susceptibilities of *Brucella melitensis* (*B. melitensis*) blood isolates obtained from an endemic region, by broth microdilution susceptibility test.

Methods: Fifty blood isolates were tested with anti-brucella antibiotics, namely, tetracycline, gentamicin, streptomycin, ceftriaxone, ciprofloxacin, levofloxacin, ofloxacin, and rifampin. All of the clinical isolates belonged to the group of *B. melitensis* biotype-3. This study was performed at the Clinical Microbiology Laboratory of the Medical School of Ondokuz Mayis University, Samsun, Turkey, in 2005.

Results: In terms of minimum inhibitory concentration-90 (MIC_{90}) values, tetracycline (MIC_{90} 0.25 microgram/mL) and rifampin (MIC_{90} 0.5 microgram/mL) still continue to be the most effective antibiotics; however, ceftriaxone and streptomycin demonstrated higher MIC values, although they were still effective *in vitro* against *B. melitensis* strains with MIC_{90} of 8 microgram/mL.

Conclusion: All first line, and alternative antimicrobial agents could be used in various combinations in the treatment of human brucellosis. High MIC values of ceftriaxone and streptomycin are alarming, and should be closely monitored during the therapy.

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Brucellosis is an endemic zoonotic disease of the Mediterranean basin, and causes frequent, serious complications if not treated effectively. Despite extensive misuse of broad spectrum antibiotics, and the ubiquitous nature of *Brucella species* (*Brucella spp.*), the optimum therapeutic agents for brucellosis are still highly effective in many parts of the world. Our current knowledge goes back to earlier years and has not changed through the decades; that is, the most recommended antimicrobials for the treatment of brucellosis are still tetracycline, doxycycline, streptomycin, rifampin, gentamicin, trimethoprim/sulfamethoxazole, ceftriaxone, and quinolones in various combinations. According to the World Health Organization guidelines, the most preferable combination is stated to be, doxycycline plus rifampin or streptomycin.[1,2] Each of the recommended regimens has some disadvantages; the relapse rate is variable and unpredictable, and the toxicity may cause serious problems, especially in children and pregnant women.[3,4] According to the literature, little published data are available on the susceptibility patterns of *Brucella spp.*, and many of these reported data are from studies that used older strains obtained from different sources at different times, dating decades ago. Studies also suggest that the development of clinically important antibiotic resistance is less likely to occur with first line antibiotics. Screening the actual resistance pattern is also a very important issue, due to the increasing concerns regarding a potential biological warfare, and the need for defense. In various applications of alternative therapeutic regimes, doxycycline seems to be highly effective and much less expensive than most of the fluoroquinolones, and appears to have similar efficacy in most studies. Since brucellosis is not very common in the USA, where 100 to

200 cases occur each year, there has not been a standard procedure adopted for *in vitro* susceptibility testing of *Brucella spp.* by the Clinical and Laboratory Standards Institute[5] (CLSI, formerly the National Committee for Clinical Laboratory Standards), before the year 2006. Therefore, the options for treatment were not chosen to be microbiologically evidence-based. The first-line antibiotics considered to be used were of empirical nature in the management of the infection. However, some techniques including E-test on Mueller-Hinton agar and Mueller-Hinton agar, supplemented with 5% sheep blood, agar dilution method with Mueller-Hinton agar, supplemented with 1% hemoglobin and 1% PolyViteX, and broth microdilution in Mueller-Hinton broth, supplemented with 1% hemoglobin and 1% PolyViteX, broth microdilution in *Brucella* broth are used for susceptibility testing of *Brucella spp*.[6-11] On the other hand, procedures implemented for the isolation of these bacteria can be a significant hazard for the personnel working in the laboratories, where the organism is cultured.[12] Since *Brucella spp.* are facultative intracellular bacteria, an ideal combined regimen would be expected to provide high penetration rates into macrophages, plus greater stability and activity inside the acidic environment of phagolysosomes. Quinolones have shown to have good intracellular penetration and *in vitro* activity against *Brucella spp*. However, their cost and reduced activity in acidic pH could pose some problems.[4,7] Lack of bactericidal activity against *Brucella spp.*, development of resistance during therapy, and high relapse rates have also been described with quinolones. Another choice is rifampin; however, the increasing incidence of tuberculosis in some parts of the world should be taken into consideration when using rifampin on the non-tuberculosis patients in the endemic areas. It seems epidemiologically more important to prevent resistance patterns in *Mycobacterium tuberculosis*.[13,14] Ceftriaxone has a good *in vitro* activity against *Brucella spp.,* and has been suggested as an alternative parenteral therapeutic agent, but failure of ceftriaxone in the treatment of acute brucellosis has also been reported.[15] All of the facts stated above have necessitated the search for new drugs in the treatment of brucellosis, and also have shown the need of monitorized antimicrobial susceptibility tests against *Brucella spp.* to be repeated at periodic intervals, with particular care in endemic regions. This study aimed to monitor the antimicrobial susceptibilities of *B. melitensis* biotype-3, the most common *Brucella spp.* seen in our region,[16,17] to commonly used anti-*Brucella* agents.

Methods. Fifty strains of *Brucella spp.* were isolated from blood cultures of inpatient wards, in a tertiary training hospital in Central Anatolia, Ankara, Turkey, between September 1999 and August 2003. *Brucella* strains were isolated from the blood samples using an automated culture system (BACTEC 9050; Becton Dickinson, Sparks MD, USA). All of the isolated strains were sent to a reference center (Pendik Veterinary Research Laboratory, Istanbul, Turkey) for confirmation and biotyping. All isolates were kept in *Brucella* broth in -80°C until the work day. This study was performed at the Clinical Microbiology Laboratory of Medical School of Ondokuz Mayis University in 2005. The isolates were identified on the basis of colony morphology, CO_2 requirement, H_2S production, dye sensitivity (basic fuchsin and thionin), slide agglutination with monospecific A and M antiserum, and susceptibility to Tbilisi bacteriophage.[18] An inoculum equal to a 0.5 McFarland turbidity standard was prepared for each *Brucella* isolate from the blood agar. The following antibiotics were used; tetracycline (Sigma), gentamicin (Bilim Co, Istanbul, Turkey), streptomycin (Sigma), ceftriaxone (Eczacibasi, Istanbul, Turkey), ciprofloxacin (Bayer Turk Kimya San, Ltd, Sti, Turkey), levofloxacin (Fako Ilaclari AS, Turkey), ofloxacin (Hoechst Marion Roussel) and rifampin (Sigma). The stock solutions were prepared from standard powder forms of antibiotics. Susceptibility tests were performed using *Brucella* broth (Oxoid, UK).[19] All experiments were performed in a safety Class II cabinet, and a mask with hepafilter was used for all experiments. The well broth micro dilution method was applied by using 96 well micro titer plates. Each well was inoculated with 100 µL of *Brucella* broth, and antibiotics were diluted 2-fold. All antibiotics were tested in the concentration of 32-0.03 µg/mL. An inoculum equal to 0.5 McFarland turbidity standard (1:100 dilution) was prepared, and each well was inoculated with 100 µL of bacterial inoculums of $5x10^5$ CFU/mL. The plates were incubated for 48 hours in 5% CO_2 at 37 °C. The lowest concentration that completely inhibited visual growth was recorded and interpreted as MIC. The MICs of each antimicrobial agent were determined, and taken as basis in the calculation of MIC_{50} and MIC_{90} values.

Results. In this study, all *Brucella spp.* were identified as *B. melitensis* biotype-3. The MIC_{50} and MIC_{90} values of the antibiotics are shown in **Table 1**, and the MIC ranges were summarized in **Table 2**. While tetracycline demonstrated the lowest MIC_{90} value (0.25 µg/mL) in this study, streptomycin and ceftriaxone demonstrated the highest MIC_{90} (8 µg/mL) values. Quinolones showed lower MIC_{90} values (1-2 µg/mL) than that of streptomycin, a very frequently used antibiotic in the treatment of brucellosis and other kinds of infections observed in the area.

Discussion. Human brucellosis is an important and ongoing public health problem in Turkey, specially in Central Anatolia. Different antibiotic combinations are used for effective treatment of the disease.[6,7,20-22] However, some of the agents used in the therapy of the disease, like ceftriaxone and quinolones, are also used in the treatment of other common infections; and rifampin and streptomycin are frequently used in the treatment of tuberculosis. Susceptibility testing of *Brucella* is not routinely performed in clinical practice.

In this study, tetracycline had the lowest MIC_{50} and MIC_{90} values (0.125-0.25 µg/mL). Similar results had been reported by Bodur et al[21] from the same area. Rubinstein et al[11] found minocycline to be the most active antibiotic against *B. melitensis* among conventional anti-brucella agents (rifampin and streptomycin). The findings of this study also imply that combination therapies with doxycycline should still depend on the first line regimens in the treatment of human brucellosis. On the other hand, streptomycin demonstrated the highest MIC values, but when used in combinations with other anti-brucella agents, it has high effectivity and low relapse rates. This may be related to its *in vivo* effectivity as reported before.[23] Especially in osteoarticular brucellosis with sacroileitis, a significantly high cure rate can be achieved through combination therapies using streptomycin.[24] It should be considered that streptomycin has less intracellular activity with high MICs that might cause problems in the treatment of brucellosis, and might lead to therapeutic failures. All 3 quinolones tested in this study have low MICs with nearly similar values. According to these results, quinolones can be used in the treatment of brucellosis as part of a combination. Although quinolones are capable of intracellular penetration and concentrate within phagocytes, such agents might be suitable to be used against infections caused by *Brucella spp*., however, they appear to lack effectiveness at low pH values found in phagolysosomes. Previous clinical reports also indicated that acidity impairs the activities of quinolones.[8,25] So far, Arda et al[26] indicated that levofloxacin is ineffective in the treatment of experimental murine brucellosis as monotherapy, or in combination with rifampicin. Nevertheless, quinolones might play a role in combination therapy in cases where intolerance or resistance precludes the use of one of the commonly used antibiotics.[27] Ceftriaxone may be considered a second-line therapy for brucellosis.[28] In our study, MICs of ceftiaxone demonstrated variable values of 0.25-8 µg/mL. For this reason, antibiotic susceptibility of *Brucella* isolates to ceftriaxone should be carefully determined in clinical isolates. Several studies showed that rifampin had excellent anti-brucella activity. This fact, together with its good intracellular penetration and clear synergism in combination with doxycyclin, made it a good first line drug for the treatment of brucellosis.[23] High activity of rifampin in the phagolysosomes of macrophages has also been documented.[8] Rifampin is frequently used in tuberculosis patients, however,

Table 1 - In vitro activities of antimicrobial agents against *Brucella melitensis* biotype-3.

Antibiotics	MIC range (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
Tetracyclin	<0.03-0.25	0.125	0.25
Gentamycin	0.25-2	1	2
Streptomycin	2-8	4	8
Ceftriaxone	0.25-8	4	8
Ciprofloxacin	0.25-1	1	1
Levofloxacin	0.25-1	1	1
Ofloxacin	0.25-2	1	2
Rifampin	0.25-2	0.5	0.5

MIC - minimum inhibitory concentration.

Table 2 - Minimum inhibitory concentration (MIC) ranges of *Brucella melitensis.*

Antibiotics	Number of occurrences at indicated MIC (µg/mL)											
	32	16	8	4	2	1	0.5	0.25	0.125	0.06	0.03	<0.03
Tetracycline	0	0	0	0	0	0	0	16	28	5	0	1
Gentamycin	0	0	0	0	22	20	7	1	0	0	0	0
Streptomycin	0	0	7	37	6	0	0	0	0	0	0	0
Ceftriaxone	0	0	8	25	12	1	3	1	0	0	0	0
Ciprofloxacin	0	0	0	0	0	27	20	3	0	0	0	0
Levofloxacin	0	0	0	0	0	35	14	1	0	0	0	0
Ofloxacin	0	0	0	0	25	17	7	1	0	0	0	0
Rifampin	0	0	0	0	1	4	25	20	0	0	0	0

there is great risk of a potential selective pressure on *Mycobacterium tuberculosis* if solely used on other infections.[1]

In conclusion, *in vitro* activities of the conventional anti-brucella antibiotics were tested by the broth microdilution method, and tetracycline, rifampin, and quinolones demonstrated the lowest MICs. Therefore, these agents are still the first line regimens to treat brucellosis, when other clinical features (age, affected systems, hepatic and renal functions, combination therapies) of the patients were considered. All antibiotics demonstrated low MICs except ceftriaxone, and streptomycin, hence susceptibility of strains to these agents should be tested, especially in cases of treatment failure. However, susceptibilities to the biotypes of the said agents need to be studied further.

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