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ERGİN AYAŞLIOĞLU

SELÇUK KILIÇ

KEMALETTİN AYDIN

DİLEK KILIÇ

SEDAT KAYGUSUZ

See next page for additional authors

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Authors
ERGİN AYAŞLIOĞLU, SELÇUK KILIÇ, KEMALETTİN AYDIN, DİLEK KILIÇ, SEDAT KAYGUSUZ, and CANAN AĞALAR

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E-mail: medsci@tubitak.gov.tr

Antimicrobial Susceptibility of *Brucella melitensis* Isolates from Blood Samples

Ergin AYAŞLIOĞLU[1]
Selçuk KILIÇ[2]
Kemalettin AYDIN[3]
Dilek KILIÇ[1]
Sedat KAYGUSUZ[1]
Canan AĞALAR[1]

[1] Department of Infectious Diseases and Clinical Microbiology, Faculty of Medicine, Kırıkkale University, Kırıkkale - TURKEY

[2] Department of Communicable Diseases Research, Refik Saydam National Hygiene Center, Ankara - TURKEY

[3] Department of Infectious Diseases and Clinical Microbiology, Faculty of Medicine, Karadeniz Technical University, Trabzon - TURKEY

Aim: Brucellosis is a worldwide zoonotic disease that remains an important public health problem in rural Turkey. The aim of the present study was to identify *Brucella* species and biotypes, and to assess the antimicrobial susceptibility of isolates from blood samples.

Materials and Methods: The study included 46 *Brucella* isolates from the Kırıkkale region of central Anatolia. The identification and biotyping of the isolates were based on conventional methods. The minimal inhibitory concentration (MIC) values of tetracycline, rifampin, streptomycin, ciprofloxacin, and azithromycin were determined using the E test method.

Results: All isolates were identified as *B. melitensis* (45 isolates, biotype-3) and were sensitive to tetracycline, streptomycin, ciprofloxacin, and azithromycin. In all, 2 isolates showed intermediate sensitivity to rifampin, whereas the others were sensitive. MIC_{90} values of tetracycline, streptomycin, rifampin, ciprofloxacin, and azithromycin were 0.25 mg/l, 0.50 mg/l, 1.0 mg/l, 0.25 mg/l, and 1.0 mg/l, respectively.

Conclusions: In recent years there has been tremendous interest in the identification of *Brucella* strains and their antimicrobial susceptibility. According to antimicrobial susceptibility test results, none of the isolates in the Kırıkkale region of Turkey were resistant to the currently recommended antibiotics. The present study's findings were discussed along with a brief review of similar studies from Turkey.

Key Words: Brucellosis, tetracycline, streptomycin, rifampin, ciprofloxacin, azithromycin

Kan Örneklerinden Elde Edilen *Brucella melitensis* Suşlarının Antimikrobiyal Duyarlılık Sonuçları

Amaç: Bruselloz tüm dünyada yaygın olarak görülen ve ülkemizde kırsal alanlar başta olmak üzere önemli bir sağlık sorunu oluşturan zoonotik bir hastalıktır. Bu çalışmada, kan örneklerinden izole edilen *Brucella* cinsi bakterilerde tür/biyotip tayini yapılması ve bu suşların antimikrobiyal duyarlılıklarının belirlenmesi amaçlanmıştır.

Yöntem ve Gereç: Çalışmaya Kırıkkale ilinde kan kültürlerinden izole edilmiş 46 *Brucella* suşu alınmıştır. İzolatların identifikasyon ve tür/biyotip tayininde konvansiyonel yöntemler kullanılmıştır. Tetrasiklin, streptomisin, rifampisin, siprofloksasin ve azitromisin için minimal inhibitör konsantrasyon (MİK) değerlerinin belirlenmesinde E-test yöntemi kullanılmıştır.

Bulgular: Tüm izolatlar *B. melitensis* (45'i biyotip-3) olarak belirlenmiştir. Antibiyotik duyarlılık sonuçlarına bakıldığında, izolatların tümü tetrasiklin, streptomisin, siprofloksasin ve azitromisin'e duyarlı bulunmuştur. Kırkdört suş rifampine duyarlı, iki suş ise orta duyarlı olarak saptanmıştır. Tetrasiklin, streptomisin, rifampin, siprofloksasin ve azitromisin için $MİK_{90}$ değerleri sırasıyla 0,25 mg/l, 0,50 mg/l, 1,0 mg/l, 0,25 mg/l ve 1,0 mg/l olarak değerlendirilmiştir.

Sonuç: Son yıllarda, *Brucella* suşlarının identifikasyon ve antimikrobiyal duyarlılıklarının belirlenmesi üzerine büyük bir ilgi artışı vardır. Antibiyotik duyarlılık sonuçları *Brucella* suşlarında kullanılmakta olan konvansiyonel ilaçlara karşı in vitro direnç olmadığını göstermektedir. Bu çalışmadan elde edilen bulgular, Türkiye'de bu konuda yapılan benzer çalışmalar ve dünya verileri eşliğinde gözden geçirilmiştir.

Anahtar Sözcükler: Bruselloz, tetrasiklin, streptomisin, rifampin, siprofloksasin, azitromisin

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Correspondence
Ergin AYAŞLIOĞLU
Karşıyaka Sok. 32/3,
PO Box 06460,
Dikmen, Ankara - TURKEY

eayasli@yahoo.com

Introduction

Human brucellosis is a multisystemic disease characterized by a prolonged clinical course and relapses. Severe complications involving musculoskeletal, neurological, genitourinary, and cardiovascular systems may be encountered during the course of the disease (1-3). *Brucellae* are intracellular microorganisms that can survive inside macrophages by counteracting a number of host defense mechanisms. The prevention of relapses and serious sequelae may be accomplished through the combined use of antimicrobials that have the ability to penetrate into intracellular compartments and retain their efficacy, even in an acidic environment (4-6).

Tetracycline and its congeners are the most effective antibiotics for brucellosis, and constitute the basic component of any combination regimen. The recommended treatment scheme for brucellosis is the combination of doxycycline and either streptomycin or rifampin for 6 weeks. Nevertheless, some cases with severe complications may require more than 2 antimicrobial drugs and a longer treatment course (1,6,7).

The purpose of the present study was to identify *Brucella* species and biotypes in blood samples obtained from brucellosis patients from the Kırıkkale region of central Anatolia. In addition, we aimed to determine their in vitro susceptibility to 3 basic (tetracycline, rifampin, streptomycin) and 2 alternative (ciprofloxacin and azithromycin) antibiotics.

Materials and Methods

Methods

Brucellae were isolated from patients with brucellosis that were diagnosed at the Infectious Diseases and Clinical Microbiology Department of Kırıkkale University Faculty of Medicine between October 2002 and September 2004. In total, 46 isolates were included the study. *Brucella* isolates were obtained from blood using the BACTEC 9050 (Becton Dickinson, Sparks, Maryland, USA) automated blood culture system. Confirmation, identification, and antimicrobial susceptibility testing of all the strains took place at the Refik Saydam National Institute of Hygiene, Department of Communicable Diseases Research.

The identification methods, *Brucella* typing procedures, and antimicrobial susceptibility testing used in this study have been described in detail previously (8,9). Briefly, the suspected colonies on serum dextrose agar and *Brucella* agar supplemented with 5% horse serum were evaluated based on Gram stain, growth characteristics, oxidase, and catalase tests. Subsequently, nitrate reduction, motility, requirement of X and V factors, biochemical reactions, including indole, Voges-Proskauer test, citrate, and gelatin hydrolysis tests, were performed. Finally, slide agglutination was tested with anti-*Brucella* polyclonal serum. For species and biotype identification; the requirement of CO_2 for growth, production of urease and H_2S, dye sensitivity (thionine and basic fuchsin), susceptibility to Tbilisi phage, and agglutination with monospecific antisera for A and M antigens were utilized. The strains were stored in skim milk at –80 °C and subcultured twice before beginning the study.

MIC values of tetracycline, rifampin, streptomycin, ciprofloxacin, and azithromycin were determined using the E test (AB Biodisk). Antimicrobial susceptibility testing was performed by inoculating the bacterial suspension (adjusted to 0.5 McFarland) onto Mueller-Hinton agar plates supplemented with 5% sheep blood and then applying E test strips. MIC was evaluated after 48 h of incubation. *B. abortus* 19, *B. melitensis* 16M, and *B. suis* 1330 were used as reference strains. Since MIC breakpoints have not yet been established for *Brucella* spp., MIC values were interpreted according to Clinical Laboratory Standards Institute (CLSI) guidelines [formerly, the National Committee for Clinical Laboratory Standards (NCCLS)] for slow-growing bacteria (*Haemophilus* spp.) (10).

Results

All 46 isolates sent to the reference laboratory were identified as *B. melitensis.* All but one (biotype-1) of the isolates (biotype-3) agglutinated with both anti-A and anti-M monospecific sera.

MIC_{50} and MIC_{90} values of the relevant antibiotics are shown in Table 1. All isolates were sensitive to tetracycline, streptomycin, ciprofloxacin, and azithromycin. In all, 44 isolates were sensitive and 2 isolates showed intermediate sensitivity to rifampin based on the criteria for slow-growing bacteria.

Table 1. The antimicrobial susceptibility of 46 *B. melitensis* isolates from blood samples.

Antibiotics	MIC Range (mg/l)	MIC_{50} (mg/l)	MIC_{90} (mg/l)
Tetracycline	0.023-0.38	0.125	0.25
Streptomycin	0.125-0.50	0.25	0.50
Rifampin	0.125-1.5	0.50	1.0
Ciprofloxacin	0.047-0.50	0.125	0.25
Azithromycin	0.064-1.5	0.75	1.0

Conclusions

In recent years a vast quantity of data regarding the identification and in vitro antimicrobial susceptibility of *Brucella* strains has been accumulated; publications from Turkey (6, 9, 11-14), as well as the present study, are summarized in Table 2. In Turkey, *B. melitensis* is the most common species and the majority of isolates are biotype-3. *B. abortus* was identified as a causative agent in only 1 study (5 isolates) (12). Future molecular biological studies, rather than the conventional methods previously utilized, will lead to the exact genetic classification of *Brucella* spp.

Routine in vitro antimicrobial susceptibility testing of *Brucella* spp. is not generally recommended (2,12). Such testing carries the risk of contagiousness among laboratory personnel and requires biological safety level 3 precautions (2,15). Moreover, in vitro antimicrobial susceptibility does not always predict clinical efficacy. Treatment failure in brucellosis is related to such factors as inappropriate dose, short-term administration, insufficient intracellular penetration of the drug, and poor patient compliance, rather than drug resistance (7,15,16); however, antimicrobial susceptibility testing may be recommended in cases of life-threatening organ

Table 2. Summary of Brucella antimicrobial susceptibility test results of Turkish studies.

Researcher		Akova et al.	Bodur et al.	Baykam et al.	Kose et al.	Sengoz et al.	Yamazhan et al.	Ayaslioglu et al.
Region		Ankara	Ankara	Ankara	İzmir	İstanbul	İzmir	Kırıkkale
Isolate #		43	41	42	11	43	44	46
B. melitensis		43	41	37	11	43	44	46
(biotype 3)		(ND)	(39)	(29)	(10)	(ND)	(ND)	(45)
B. abortus		0	0	5	0	0	0	0
Method		Microdilution	E test	E test	E test	E test	Agar dilution	E test
MIC_{90} (mg/l)	DOX	< 0.125	0.064	0.064	0.047	0.090	0.50	0.25*
	STR	2.0				0.75		0.50
	SXT		0.38	1.5	1.0			
	CRO		0.38	0.50	0.50			
	RIF	2.0	0.75	1.0	0.75	1.0		1.0
	CIP	2.0	0.25	0.19	0.25	0.38	2.0	0.25
	AZM	1.0					32.0	1.0

DOX: doxycycline; STR: streptomycin; SXT: trimethoprim/sulfamethoxazole; CRO: ceftriaxone; RIF: rifampin; CIP: ciprofloxacin; AZM: azithromycin.
ND: not determined.
*E strip for tetracycline was used.

involvement (i.e. brucella endocarditis and meningitis) and in the event of treatment failure and relapse (16).

An additional problem with such testing is the lack of standardization. In vitro efficacy of antibiotics against *Brucella* spp. has usually been based on the determination of MIC values by micro broth dilution, agar dilution, and E test methods. The disc diffusion method has not been recommended (4). Most studies from Turkey utilized the E test method and usually declared concordant results (9,11-13). The E test is said to be more reliable, reproducible, and practical, as well as less labor-intensive and time-consuming than other methods (9,17). A previous study that compared the E test to the microdilution test reported no significant difference between MIC end-points (17); however, Akova et al. (6) detected somewhat higher MIC values with the microdilution method than with the E test method and Yamazhan et al. (14), using the agar dilution method, noted even higher MIC values for all the tested antimicrobials. These discordant findings may be attributed to either regional differences in the susceptibility of *Brucella* strains or to the different methodologies used to assess the MIC values.

Tetracycline and its derivatives are among the most effective drugs against brucellosis (1,7). Despite their widespread use in eradicating *Brucella* infections, there is no problem associated with tetracycline resistance (11-16,18). Doxycycline has become the tetracycline analogue of choice for treating brucellosis because of its superior pharmacokinetics, lipophilic ability, and bioavailability (16). Among the antibacterial agents used to treat brucellosis, doxycyline has shown the lowest MIC_{90} values (9,11-14). In the present study, tetracycline and ciprofloxacin shared the lowest MIC values among the antibiotics tested. Although streptomycin is known to be active against brucellosis (6,13,15), its disadvantages, such as ototoxicity, nephrotoxicity, and parenteral administration, preclude its wider use. Even though rifampin is somewhat toxic, it has the advantage of oral administration (19,20). In the present study none of the isolates were resistant to rifampin, although 2 showed intermediate sensitivity. In a previous study that assessed the antimicrobial susceptibility of *Brucellae* at different pH levels, rifampin was the only antibiotic with increased activity in acidic environmental conditions (6). In clinical trials, the doxycycline-rifampin combination was as effective as the doxycycline-streptomycin combination in the majority of *Brucella* infections, excluding Brucella spondylitis, for which streptomycin is recommended (20). Fluoroquinolones represent candidate therapeutic alternatives for brucellosis because of their excellent bioavailability, intracellular penetration, and ability to achieve optimum tissue concentrations (6,21). In vitro studies of quinolones have demonstrated favorable antimicrobial activity against *Brucella* spp. (11-13). Our study revealed compatible results, suggesting that in vitro ciprofloxacin was as effective as tetracycline against *B. melitensis* strains. Nonetheless, fluoroquinolones have the disadvantage of reduced activity in acidic environments (6,16). It was also demonstrated that ciprofloxacin monotherapy increases the probability of relapse (22,23). There are also some studies that assessed quinolones as components of combination regimens, suggesting efficacy equal to classically recommended combination schemes (24-26). Currently, published randomized clinical trials do not support the use of quinolone-based combinations as a first-line therapy (27).

Azithromycin is a new macrolide with good intracellular penetration and in vitro activity against *Brucellae* (28). Acidic environmental conditions also impair the activity of macrolides (6). The present study found favorable in vitro activity of azithromycin against *Brucella* strains. Yamazhan et al. reported high MIC values for both azithromycin and fluoroquinolones with the agar dilution method, and proposed increased resistance of *Brucella* strains to both antimicrobial agents (14). Their finding may be explained by regional and methodological differences. Large-scale clinical trials involving azithromycin in human brucellosis are lacking. A small clinical trial (29) refuted the efficacy of an azithromycin-gentamycin combination in human brucellosis; however, in vitro results are encouraging and warrant further clinical study of azithromycin for *Brucella* infections.

Reports from Turkey revealed that, in vitro, several antibiotics are effective against *Brucella* isolates and that the problem of resistance seems minor. Our results are consistent with a recent study from Greece that evaluated the antimicrobial susceptibility of 74 *B. melitensis* isolates using the E test (15). Lopez-Merino et al. from Mexico reported that in vitro antibacterial activity of quinolones approximated that of tetracyclines, and exceeded that of streptomycin and rifampin (30). The authors encouraged the evaluation of these drugs in clinical trials.

In conclusion, classically recommended therapeutics for brucellosis show favorable in vitro antibacterial activity profiles. Ciprofloxacin also seems to be promising, as it had anti-*Brucella* activity equal to that of tetracyclines in our study. Azithromycin is effective in vitro, although its in vivo efficacy remains to be confirmed. Future large-scale, randomized, double-blind in vivo comparison studies that assess these antibacterial agents in various combination protocols are warranted.

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