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Original Article

In-vitro antimicrobial susceptibility results of *Brucella* species; Single center study from Eastern Türkiye

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Abstract

Introduction: Brucellosis is the most common zoonotic infection worldwide. The disease imposes significant economic burdens and public health challenges. Due to documented instances of treatment failures, relapses, and resistance, regular monitoring of antimicrobial susceptibility is essential. This study aimed to evaluate the sensitivity of *Brucella* spp. against antimicrobial agents.

Methodology: The study was conducted at a tertiary state hospital from January 2023 to June 2024. Species level identification was performed using the VITEK-2 Compact system and 16S rRNA analysis. Isolated strains were cultured on Mueller-Hinton agar supplemented with 5% horse blood and β-Nicotinamide adenine dinucleotide (β-NAD), followed by testing with antibiotic discs. Antimicrobial susceptibility tests were performed according to the EUCAST 2024 version 14 recommendations. Zone diameters were categorized as susceptible, standard dosing regimen; susceptible, increased exposure and resistant.

Results: 45 *Brucella* species isolated from blood culture were included in this study. Species level determination showed *Brucella melitensis* in 36 (80%) and *Brucella abortus* in 9 (20%) patients by 16S rRNA analysis. Antimicrobial susceptibilities were as follows: gentamicin, rifampicin, ceftriaxone, tetracycline, trimethoprim-sulfamethoxazole, and streptomycin all showed 100% susceptibility. Conversely, levofloxacin had a susceptibility rate of 15.5%, and ciprofloxacin had a rate of 4.4%. No resistance was detected for levofloxacin or ciprofloxacin, and there were no significant differences in susceptibility between *Brucella melitensis* and *Brucella abortus*.

Conclusions: The study highlights increased exposure patterns for ciprofloxacin and levofloxacin, indicating potential future resistance issues. Regular monitoring of antimicrobial resistance in *Brucella* isolates is crucial for effective disease management and updating treatment guidelines.

Key words: Antimicrobial susceptibility; *Brucella*; brucellosis; zoonoses.

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Introduction

Brucellosis is caused by *Brucella* species (spp.), a genus of gram-negative coccobacilli that are facultative intracellular pathogens. This zoonotic disease can be transmitted through direct contact with infected animal tissues or secretions, ingestion of unpasteurized milk and dairy products, or inhalation of contaminated aerosols. Among the *Brucella* spp., *Brucella melitensis* is particularly notable for its aggressive nature and prevalence [1]. Additional frequently encountered *Brucella* species are *Brucella abortus*, *Brucella suis*, and *Brucella canis* [2].

Brucellosis is widely recognized as the most prevalent zoonotic disease globally, endemic in regions spanning the Middle East, Central Asia, China, India, Sub-Saharan Africa, Mexico, the Mediterranean Basin, and Central and South America. Current estimates suggest approximately 500,000 human cases occur worldwide each year [3]. This disease imposes a significant economic burden on countries in endemic regions and contributes to public health challenges [4]. Brucellosis is endemic in Türkiye, with the highest prevalence observed in the Southeastern Anatolia and Eastern Anatolia regions [5].

Individuals frequently encounter symptoms such as fever, chills, headache, muscle and joint pain, night sweats, fatigue, anorexia, weight loss, and feelings of depression. If there is an infection in a specific body area, additional symptoms related to that region may also occur [6]. The gold standard method for diagnosing brucellosis is culture to detect the causative agent [7]. Additional diagnostic methods utilized in clinical practice include serological tests such as the Rose Bengal plate test (RBPT), standard tube agglutination test (STA), Coombs gel test, and enzyme-linked immunosorbent assay (ELISA) [8]. Nevertheless, species identification can also be accomplished using polymerase chain reaction (PCR) methods [9].

Resistance to antimicrobial agents is rarely encountered with *Brucella* species. Therefore, routine

antimicrobial susceptibility testing for *Brucella* spp. isolated from clinical samples is not recommended [5]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST 2024 v.14) has published antimicrobial susceptibility breakpoints for *B. melitensis* for the first time, utilizing the disk diffusion method in their guidelines [10]. Until this date, the antimicrobial susceptibility testing for *Brucella* spp., as outlined in the Clinical and Laboratory Standards Institute (CLSI) M45 guideline, has primarily relied on the broth microdilution method. In this guide, *Brucella* broth agar is recommended for the antimicrobial susceptibility testing of *Brucella* strains. However, specific breakpoints for antimicrobials such as rifampicin and fluoroquinolones are not defined in this guideline [11].

Due to the intracellular nature of *Brucella* spp. and its tendency to cause chronic infections, treatment typically involves long-term administration of combination antimicrobial agents [12]. Recurrence and treatment failures can occur during the treatment of brucellosis, and the detection of resistant bacteria in endemic areas is a significant concern [13]. Therefore, it is crucial to monitor the antibiotic sensitivities of commonly used drugs in treatment [14]. In our study, we aimed to identify *Brucella* strains isolated from serum samples by molecular methods and to assess their antimicrobial susceptibility to gentamicin, rifampicin, ceftriaxone, levofloxacin, ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole, and streptomycin antibiotics using the disk diffusion method.

Methodology

This study was conducted with 45 *Brucella* strains which were isolated from blood culture samples received at the clinical microbiology laboratory, following suspicions of brucellosis, at Ağrı Training and Research Hospital between January 2023 and June 2024.

Patient selection

Individuals with a history of direct contact with tissues or secretions from *Brucella*-infected animals through conjunctiva or damaged skin, consumption of raw or unpasteurized dairy products from infected animals, or inhalation of infected aerosols, often present with symptoms such as fever, fatigue, anorexia, weight loss, headache, night sweats, and widespread muscular and joint pain. These patients were screened using the Rose Bengal test (THSK antigen, Türkiye). Patients who tested positive on the Rose Bengal test underwent standard tube agglutination (THSK antigen, Türkiye) and/or the *Brucella* Coombs gel test (BCGT, ODAK, Türkiye). Blood culture samples were obtained from patients whose titers were 1/160 or higher or who showed a 4-fold increase in titers measured at 2-week intervals. After achieving appropriate antisepsis conditions, blood cultures were obtained from the relevant anatomical sites on both upper extremities. Two sets of blood cultures, consisting of 10 cc each (totaling 40 cc), were collected in aerobic and anaerobic bottles. These samples were then transported to the clinical microbiology laboratory under appropriate conditions.

In the study, patients aged 18 years and older who exhibited symptoms, history, serological examination, and blood culture results suggestive of brucellosis were included. Only one isolate from each patient was considered for inclusion in the study. Pregnant women, children, and patients diagnosed with chronic brucellosis were excluded from participation. The demographic information of the enrolled patients, including age and gender, exposure history, symptoms, duration of symptoms, blood culture outcomes, *Brucella* spp. identified via PCR, and results from the Rose Bengal test, standard tube agglutination, and *Brucella* Coombs gel test were documented.

Sample collection and isolation of Brucella species

Aerobic and anaerobic blood culture bottles were incubated in the Render automated blood culture system (BC256 Blood Culture Systems, China). Single, tiny gram-negative coccobacilli were observed in routine Gram staining made from blood culture bottles, giving a positive signal. The samples were inoculated on the 5% sheep blood agar (Oxoid, England), eosin methylene blue agar (EMB, Oxoid, England), and chocolate agar (Merck, Germany) under aerobic conditions. They were then incubated for 24 hours at 37 °C with 5% CO_2. The blood and chocolate agar observed non-hemolytic, transparent, flat, small colonies. All isolates were detected as oxidase and catalase and urease test positive. Pure colonies obtained were identified at the species level with the VITEK-2® Compact system (Biomerieux, France). Strains identified as *Brucella* spp. were placed in a cryopreservation tube (Microbank, Canada) and stored at -20 °C for subsequent molecular typing and antimicrobial susceptibility testing.

Molecular identification

Species-level identification confirmed with polymerase chain reaction-based amplification of 16S rRNA. DNA was isolated using EurX GeneMATRIX

Table 1. Sequences of PCR primers used in this study.

Primers	Sequence (5'-3')	Size of Product (bp)	Reference
27F	5'AGAGTTTGATCMTGGCTCAG 3'	1470	15
1492R	5' TACGGYTACCTTGTTACGACTT	1470	15

PCR: Polymerase chain reaction.

Bacterial & Yeast DNA isolation kit (Poland) following the manufacturer's recommendations. The amplification of the 1470-bp location of the 16S rRNA gene was performed using 27F 5' AGAGTTTGATCMTGGCTCAG 3' and 1492R 5' TACGGYTACCTTGTTACGACTT universal primers (Table 1) [15]. The cycles are initial denaturation at 95 °C for 5 minutes, 30 cycles of 95 °C for 45 seconds, annealing at 57 °C for 45 seconds, extension at 72 °C for 1 minute, and final extension at 72 °C for 5 minutes. The amplified products were run and viewed in a 1.5% agarose gel (Sigma, St. Louis, MO, USA). Spectrophotometric measurement was performed in the Thermo Scientific Nanodrop 2000 (USA) device to check the amount and purity after DNA isolation. In the purification phase of the PCR product, MAGBIO "HighPrepTM PCR Clean-up System" (AC-60005) purification kit was used for the single band samples obtained and purified in accordance with the kit procedures. ABI 3730XL Sanger sequencing device (Applied Biosystems, Foster City, CA) and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, MA, USA) were used. The isolates were identified by comparing the DNA reference isolates with data stored in the GenBank using the Basic Local Alignment Search Tool (BLAST + 2.15.0, http://www.ncbi.nlm.nih.gov/BLAST) program.

Antimicrobial susceptibility of Brucella species

Before analysis, strains stored at -20 °C in cryopreservation tubes (Microbank, Canada) underwent two subcultures. Ultimately, the strain was suspended in isotonic fluid to achieve a 0.5 McFarland turbidity standard and then inoculated onto Mueller-Hinton agar supplemented with 5% defibrinated horse blood and β-Nicotinamide adenine dinucleotide (β-NAD) (20 mg/L) (RTA, Türkiye). Additionally, discs containing gentamicin, rifampicin, ceftriaxone, levofloxacin, ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole, and streptomycin (Bioanalyse, Türkiye) were placed on the agar. The plates were incubated for 48 hours at 35 ± 1 °C with 5% CO_2. These eight antibiotics were assessed for susceptible standard dosing regimen, susceptible increased exposure, or resistance according to the EUCAST 2024 v.14 clinical breakpoints. Quality control strain *Streptococcus pneumoniae* ATCC 49619 was used for standardization.

Statistical analysis

Frequency (n) and percentage (%) were reported for categorical (qualitative) variables, while mean (x), median, standard deviation, minimum, and maximum values were provided for numeric (quantitative) measurements. The normality of numeric variables was assessed using the Shapiro-Wilk test. For normally distributed variables, independent samples t-test was employed to compare between groups. For variables that did not follow a normal distribution, the Mann-Whitney U test was used. Data analysis was conducted using SPSS 26 (IBM SPSS Statistics for Windows, Ver 26.0. Armonk, NY: IBM Corp, USA), and results were deemed statistically significant at $p < 0.05$.

Ethical approval

The research was allowed by the Scientific Research Ethics Committee of Ağrı İbrahim Çeçen University with the decision dated 28.03.2024 and numbered 117.

Results

Blood samples were collected from a total of 45 eligible participants for the study. Among them, 21 (46.7%) were women, and 24 (53.3%) were men. The average age was calculated to be 41.3 (± 15.6) years. 38 (84.4%) participants reported a history of animal husbandry, while 39 (86.7%) participants reported exposure to raw milk and dairy products. Only one participant's disease exposure history could not be ascertained. None of the participants had previously been diagnosed with brucellosis. 42 of 45 patients were treated as outpatients, and 3 of 45 patients were treated as hospitalized.

Upon admission, the most prevalent symptom among participants was muscle and joint pain, reported by 39 individuals (86.7%), followed by fatigue in 34 participants (75.6%) and fever in 31 participants (68.9%). Among male participants, 4 individuals (16.6%) experienced testicular pain. Table 2 outlines the presenting symptoms. The average duration from hospital admission to the onset of symptoms was 3.69 ± 2 weeks (min: 1 week, max: 8 weeks).

All samples tested positive in the Rose-Bengal plate test. Among the participants, 28 serum samples were assessed using the standard tube agglutination method, and 17 serum samples were evaluated using the *Brucella* Coombs gel test. The standard tube agglutination test revealed titers of 1/160 in 5 samples (17.9%), 1/320 in 3 samples (10.7%), 1/640 in 4 samples (14.3%), and 1/1280 in 16 samples (57.1%). In the *Brucella* Coombs gel test, 2 samples (11.8%) exhibited a titer of 1/320, 2 samples (11.8%) had a titer of 1/640, and 13 samples (76.5%) showed a titer of 1/1280. The VITEK-2® Compact system identified *B. melitensis* in 30 blood samples (66.6%) and *Brucella* spp. in 15 blood samples (33.4%). The molecular 16S rRNA sequence analysis showed a similarity of > 99% compared to the reference sequence. Based on the 16S rRNA analysis conducted to ascertain the species of *Brucella* agents, *B. melitensis* was identified in 36 patients (80.0%), while *B. abortus* was detected in 9 patients (20.0%) (Table 2).

Zone diameters were assessed for gentamicin, rifampicin, ceftriaxone, levofloxacin, ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole, and streptomycin using the disk diffusion method. The antimicrobial susceptibility results were as follows: all 45 isolates showed 100% sensitivity to gentamicin, rifampicin, ceftriaxone, tetracycline, trimethoprim-sulfamethoxazole, and streptomycin. Levofloxacin exhibited sensitivity in 15.5% (7 out of 45) of the isolates, while ciprofloxacin was sensitivity in 4.4% (2 out of 45) of the isolates. No resistance was observed in the levofloxacin and ciprofloxacin discs. Table 3 presents the zone diameters and antimicrobial susceptibilities for the antimicrobial agents. There were no differences observed in antimicrobial susceptibility between *B. melitensis* and *B. abortus*.

Table 2. Demographic and clinical results of brucellosis infected patient.

	N (%)
Symptoms	
Muscle and joint pain	39 (86.7%)
Fatigue	34 (75.6%)
Fever	31 (68.9%)
Night sweats	31 (68.9%)
Anorexia	30 (66.7%)
Back pain	25 (55.6%)
Weight loss	20 (44.4%)
Abdominal pain	15 (33.3%)
Headache	14 (31.1%)
Gender	
Women	21 (46.7%)
Men	24 (53.3%)
The standard tube agglutination test titers	
1/160	5 (17.9%)
1/320	3 (10.7%)
1/640	4 (14.3%)
1/1280	16 (57.1%)
***Brucella* Coombs gel test titers**	
1/320	2 (11.8%)
1/640	2 (11.8%)
1/1280	13 (76.5%)
Identification of VITEK-2® Compact system	
B. melitensis	30 (66.6%)
Brucella spp.	15 (33.4%)
Identification of 16S rRNA analysis	
B. melitensis	36 (80.0%)
B. abortus	9 (20.0%)

Discussion

Brucellosis, a zoonotic disease prevalent worldwide and in Türkiye, can impact various organ systems and present with various clinical manifestations. Due to its ability to mimic several other diseases, it often features in the differential diagnosis of various medical conditions [16]. The principal objective of treatment is to alleviate symptoms, prevent complications, and

Table 3. Antimicrobial susceptibility results.

	Antibiotics	Concentration µg/disk	Range Min-Max (mm)	Range Mean (mm)	Sensitive (%)	Intermediate (%)	Resistant (%)	Zone diameter breakpoints (mm)	
								S ≥	R <
***Brucella melitensis* (n:36)**	Ceftriaxone	CRO-30	38-60	49,78	100	0	0	**30**	**30**
	Rifampicin	RA-5	24-36	27,64	100	0	0	**20**	**20**
	Streptomycin	S-10	36-60	44,5	100	0	0	**15**	**15**
	Trimethoprim Sulfamethoxazole	SXT-25	30-50	38,17	100	0	0	**29**	**29**
	Gentamicin	CN-10	36-60	49,11	100	0	0	**23**	**23**
	Levofloxacin	LEV-5	38-52	44,61	4/36 (11,1)	32/36 (88,9)	0	**50**	**28**
	Ciprofloxacin	CIP-5	30-54	42,92	1/36 (2,7)	35/36 (97,3)	0	**50**	**27**
	Tetracycline	TE-30	44-64	56,61	100	0	0	**42**	**42**
***Brucella abortus* (n:9)**	Ceftriaxone	CRO-30	46-58	52,67	100	0	0	**30**	**30**
	Rifampicin	RA-5	24-30	26,44	100	0	0	**20**	**20**
	Streptomycin	S-10	40-52	45,33	100	0	0	**15**	**15**
	Trimethoprim Sulfamethoxazole	SXT-25	36-48	39,67	100	0	0	**29**	**29**
	Gentamicin	CN-10	40-58	49,78	100	0	0	**23**	**23**
	Levofloxacin	LEV-5	38-52	45,33	3/9 (33,3)	6/9 (66,7)	0	**50**	**28**
	Ciprofloxacin	CIP-5	40-52	44,89	1/9 (11,1)	8/9 (88,9)	0	**50**	**27**
	Tetracycline	TE-30	50-62	56,44	100	0	0	**42**	**42**

Max: Maximum; Min: Minimum; Mm: Milimeter; R: Resistant; S: Sensitive.

minimize the risk of recurrence. Given its intracellular nature, effective treatment entails the use of long-term and combination antimicrobial agents that exhibit excellent penetration into intracellular compartments [17].

In a meta-analysis examining the epidemiology and clinical presentation of human brucellosis, fever, fatigue, and joint pain emerged as the predominant symptoms [18]. Similarly, our study identified muscle-joint pain, fatigue, and fever as the most prevalent complaints. Notably, our study focused exclusively on bacteremic patients and those diagnosed with acute brucellosis. This underscores that symptoms in bacteremic patients are more pronounced, leading to earlier diagnosis during the acute phase. Regarding diagnostic methods, the standard tube agglutination test revealed a titer of 1/1280 in 57.1% of patients, while the Coombs gel test showed this titer in 76.5%. The elevated titers in a significant number of patients likely reflect their presentation with acute brucellosis and bacteremia.

While conventional methods such as serological tests and automated culture systems are employed for diagnosing bacteremic patients, the PCR method is recommended as the definitive diagnostic approach [19]. In Türkiye, *B. melitensis* accounts for 99% of *Brucella* isolates, with *B. abortus* identified at a lower frequency [20]. Similarly, a study conducted in our country confirmed that all 80 *Brucella* isolates identified were *B. melitensis* using molecular methods [21]. In our study, we employed molecular identification for precise classification. Out of 45 strains analyzed, 36 (80%) were identified as *B. melitensis* and 9 (20%) as *B. abortus*. The relatively high prevalence of *B. abortus* among patients may be associated with the endemic nature of the region or the specific livestock breeds commonly raised there under intensive animal husbandry practices.

In our study, we assessed the susceptibility of 45 *Brucella* species isolated from blood culture to several antibiotics including gentamicin, rifampicin, ceftriaxone, levofloxacin, ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole, and streptomycin using the disc diffusion method. According to the EUCAST 2024 v.14 guideline breakpoints, we observed 100% sensitivity across most antibiotics, except for levofloxacin and ciprofloxacin. Levofloxacin showed susceptibility in 15.5% of strains, while ciprofloxacin exhibited in 4.4%. No strains were resistant. According to the guideline, strains sensitive to tetracycline were also sensitive to doxycycline.

Before the publication of the EUCAST 2024 v.14 guideline, there was no standardized antimicrobial breakpoint guidance specifically for *Brucella* species. In the literature, studies often investigate minimum inhibitory concentration (MIC) values using methods such as the E-test. In a study by Liu *et al*., *Brucella* strains isolated from human serum samples were evaluated using the E-test method and interpreted based on CLSI threshold values. Results indicated 1% resistance to rifampin, 7% resistance to trimethoprim-sulfamethoxazole, and 100% sensitivity to other antimicrobial agents, including quinolones [22]. In another study conducted by Asadi *et al.,* employing similar methodologies, rifampin resistance was observed in 35.1% of cases, while trimethoprim-sulfamethoxazole resistance was noted in 3.5%. All other antimicrobial agents, including quinolones, demonstrated 100% sensitivity [4]. In a meta-analysis conducted by Shahrabi *et al*., it was observed that initial rates of resistance to tetracycline and doxycycline were notably low at 1.7%. However, the study revealed a significant upward trend in resistance levels over time (tetracycline: r = 0.077, p = 0.012; doxycycline: r = 0.059, p = 0.026) [23]. İlhan *et al.* conducted a study where strains isolated from sheep were assessed using the Kirby-Bauer disk diffusion method and evaluated based on CLSI threshold values. The findings indicated a sensitivity of 92.6% and a resistance rate of 7.3% to ciprofloxacin [24]. In a separate investigation carried out by Khan *et al*. involving animals, the prevalence of ciprofloxacin resistance was reported to be 76.1% using the E-test method [25]. In light of all these studies, variations in findings regarding antimicrobial resistance can be attributed to the endemic nature of the disease and regional disparities. Ghimpeteanu *et al.* demonstrated that prolonged and escalating use of antibiotics in livestock has resulted in a rise in resistance to antimicrobial agents [26]. The higher incidence of quinolone resistance in animals compared to humans underscores concerns regarding potential future increases in quinolone resistance. We advocate for integrating animal treatment into a unified health policy as crucial as human treatment. Studies indicating resistance to certain antimicrobial agents and treatment ineffectiveness emphasize the need for vigilant monitoring of *Brucella* isolates for drug resistance in the years ahead.

Primary treatment recommendations for human brucellosis consist of regimens that include doxycycline, streptomycin, and rifampicin [17]. According to a meta-analysis conducted by Skalsky *et al.*, treatment regimens combining rifampicin with

quinolones were found to be less effective compared to combinations of rifampicin or streptomycin with doxycycline [27]. In a randomized controlled study conducted by Keramat *et al*., comparisons were made between combinations of doxycycline and rifampicin versus either doxycycline or rifampicin combined with ciprofloxacin. The findings indicated that combinations including quinolones were less effective in treatment [28]. Another study highlighted that the addition of levofloxacin to the rifampicin and doxycycline regimen could prevent relapse [29]. Quinolones are not in the first line of treatment protocols because of reduced antimicrobial effectiveness under acidic conditions, absence of synergistic effects with other antimicrobial agents, and documented resistance observed in *B. melitensis* strains during therapy [30]. In our study, we observed a low sensitivity among strains to quinolones, whereas increased exposure was more prevalent. The apprehension regarding heightened side effects associated with quinolones, particularly with black box warnings, when doses are escalated for strains sensitive to higher doses, complicates the selection of quinolone-based regimens as the primary treatment choice.

In our study, the antimicrobial susceptibility profiles of *B. melitensis* and *B. abortus* strains exhibited similarity, indicating that treatment strategies need not be differentiated between these strains.

The limitations of our study include its single-center design and limited strain numbers. Therefore, there is a need for multicenter studies with a larger sample size and long-term patient follow-up to assess clinical outcomes post-treatment. Given the potential evolution of Brucellosis resistance patterns over time, it is crucial to support studies that periodically evaluate resistance patterns, particularly in endemic regions.

Our study utilized EUCAST 2024 v.14 breakpoints to assess *Brucella* spp. This is the first study conducted in Türkiye to evaluate antibiotic sensitivity in vitro using the disk diffusion method on strains.

Conclusions

Our study predominantly identifies increased exposure patterns for ciprofloxacin and levofloxacin, suggesting that quinolone resistance could pose future challenges in treating brucellosis. Monitoring antibiotic resistance in *Brucella* isolates is crucial for disease management. Regular surveillance of resistance distribution and prevalence among isolates in endemic countries is essential. This data could inform updates to treatment guidelines for brucellosis and aid in disease control efforts.

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Conflict of interests

No conflict of interests is declared.

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