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Antimicrobial resistance profiles of human *Brucella melitensis* isolates in three different microdilution broths: the first multicentre study in Bosnia and Herzegovina

Jurica Arapović[a,b,1,*], Gordan Kompes[c,1], Kanita Dedić[d], Sanela Teskeredžić[e], Maja Ostojić[a], Maja Travar[f,g], Nijaz Tihić[h], Jasminka Delić[d], Siniša Skočibušić[a,b], Minela Zekiri-Sivro[e], Antonija Verhaz[f,g], Dilista Piljić[h], Luka Laura[i], Sanja Duvnjak[c], Maja Zdelar-Tuk[c], Maja Arapović[b,i], Enisa Šabotić[d], Irena Reil[c], Jadranka Nikolić[a,b], Sead Ahmetagić[h], Željko Cvetnić[c], Boris Habrun[c], Mile Bosilkovski[j], Silvio Špičić[c,**]

[a] *University Clinical Hospital Mostar, Mostar, Bosnia and Herzegovina*
[b] *School of Medicine, University of Mostar, Mostar, Bosnia and Herzegovina*
[c] *Croatian Veterinary Institute, Zagreb, Croatia*
[d] *Cantonal Hospital 'Dr. Irfan Ljubijankic', Bihac, Bosnia and Herzegovina*
[e] *Cantonal Hospital Travnik, Travnik, Bosnia and Herzegovina*
[f] *University Clinical Centre of the Republic of Srpska, Banja Luka, The Republic of Srpska, Bosnia and Herzegovina*
[g] *Faculty of Medicine, University of Banja Luka, Banja Luka, The Republic of Srpska, Bosnia and Herzegovina*
[h] *University Clinical Center Tuzla, Tuzla, Bosnia and Herzegovina*
[i] *Veterinary Institute of Herzegovina–Neretva Canton, Mostar, Bosnia and Herzegovina*
[j] *Ss. Cyril and Methodius University in Skopje, Faculty of Medicine, University Clinic for Infectious Diseases, Skopje, Republic of North Macedonia*

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ABSTRACT

Objectives: Brucellosis is a ubiquitous emergent bacterial zoonotic disease causing significant human morbidity in Bosnia and Herzegovina. So far, a high rate of resistant *Brucella* has been found worldwide. This study prospectively analysed the rates of resistance among human *Brucella melitensis* strains isolated in Bosnia and Herzegovina.

Methods: This study included 108 *B. melitensis* isolates from 209 patients diagnosed at five medical centres in Bosnia and Herzegovina. The resistance profiles of the *B. melitensis* isolates for the 13 most commonly used antimicrobials were studied in standard *Brucella* broth (BB) and cation-adjusted Mueller-Hinton broth (CAMHB) supplemented with 4% lysed horse blood or 5% defibrinated sheep blood.

Results: Of the 209 patients, *B. melitensis* blood cultures were positive for 111 (53.1%). Among the 108 isolates investigated, 91 (84.3%) were resistant to trimethoprim-sulfamethoxazole on BB, but not on either CAMHB. Nearly all isolates (>90%) were resistant to azithromycin on BB and both CAMHBs.

Conclusion: We observed a high rate of *B. melitensis* resistance to azithromycin. The high rate of resistance to trimethoprim-sulfamethoxazole that we observed was related to BB, so an alternative broth should be used, such as the enriched CAMHBs in this study, for evaluating resistance to trimethoprim-sulfamethoxazole. Whole-genome sequencing studies are needed to understand the development of antimicrobial resistance in *B. melitensis* strains isolated from humans.

* Corresponding author. University Clinical Hospital Mostar, Kralja tvrtka b.b., Mostar 88000, Bosnia and Herzegovina.
** Corresponding author: Croatian Veterinary Institute, Savska cesta 143, 10000 Zagreb, Croatia
E-mail addresses: jurica.arapovic@mef.sum.ba (J. Arapović), spicic@veinst.hr (S. Špičić).

[1] These two authors equally contributed to this work.

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1. Introduction

Brucellosis is a serious emergent zoonotic disease caused by the bacterial genus *Brucella. Brucella melitensis* is the most widespread species of the genus, although other *Brucella* species also have zoonotic potential. The major reservoir for *B. melitensis* is domestic animals, and humans can become infected under certain circumstances through direct or indirect contact with animals or their products [1]. More than 500,000 new yearly cases of human brucellosis have been estimated worldwide, with the disease ranking among the most widespread bacterial zoonoses and as a major global public health priority [2]. No vaccines for humans have been approved, and standard treatments are often ineffective, with a high risk of disease recurrence. Thus, it is important to increase our knowledge about diagnosis and treatment of *Brucella* infections in humans, especially in endemic regions [3].

In recent years, the epidemiology of human brucellosis has changed globally, and new foci of the disease are continuously emerging because of poor control and reporting of the disease [2]. After the war in Bosnia and Herzegovina, brucellosis grew into an emerging veterinary and public health problem, especially in areas where imported cattle were donated to refugees and displaced persons [4,5]. In fact, brucellosis has become an endemic zoonotic disease in Bosnia and Herzegovina, where it is a significant public health problem not only for the country but also for the region [5].

The "gold standard" for diagnosis of brucellosis is the isolation of *Brucella* from blood, bone marrow or other tissues, a technique offering high specificity and sensitivity [6]. However, the success rate of bacterial cultures from hospitalised patients in previous epidemics in Bosnia and Herzegovina averaged only 30% [7]. In principle, this rate could be as high as 85% [5].

In 2009, Bosnia and Herzegovina conducted a preventive vaccination campaign of small ruminants against *B. melitensis*-related abortion. Rev.1 vaccines composed of live *B. melitensis* attenuated strain were administered by the conjunctival route at standard doses [8]. The government continued the campaign in 2012, when the lowest rate of human morbidity was recorded, with an incidence of 3.5 cases/100,000 people. Nevertheless, human brucellosis morbidity has gradually increased in recent years, primarily due to the insufficient vaccination of ruminants [5].

Brucellosis is most often treated with the antibiotic combination doxycycline and rifampin (RIF) in combination of aminoglycosides, whereas pregnant women or young patients under 8 years old require the second line of antibrucellar treatment such as trimethoprim-sulfamethoxazole (T/S) or RIF [9]. However, studies of antimicrobial resistance among *B. melitensis* strains isolated from young patients in Bosnia and Herzegovina identified some strains resistant to T/S [10]. In addition, growing worldwide resistance to T/S is feared because the antibiotics are given to many around the world [11]. However, in Bosnia and Herzegovina, azithromycin was also used as an alternative when treating brucellosis in children in some circumstances, raising concerns about the emergence of resistance [10].

Here we present the first prospective study of antimicrobial resistance among *B. melitensis* isolates from humans in southeastern Europe, specifically from the endemic region of Bosnia and Herzegovina. Our results may provide a valuable baseline for monitoring the emergence and spread of resistance of commonly used antimicrobials against *Brucella*.

2. Patients and methods

2.1. Patients

This study included 209 patients diagnosed with brucellosis between January and December 2018 in five medical centres in Bosnia and Herzegovina (Banja Luka, Bihać, Mostar, Travnik and Tuzla). Of the 209 patients with serologically confirmed brucellosis, 111 were definitively diagnosed based on positive *Brucellae* spp. blood culture, and 108 were further microbiologically analysed.

2.2. Bacterial detection and molecular analysis

For all patients, at least four independent blood culture samples were obtained, two on aerobic medium and two on anaerobic medium (BD BACTEC™ Plus Aerobic/Anaerobic medium, BD, USA). Isolates were stored either in SkimMilk® medium (Sigma Aldrich, Germany) or in 1:1 vol/vol media BHI (Brain Hearth Infusion) and 50% glycerol, and then stored at –20°C for one to three months. Isolates were identified using 'Bruce-ladder' multiplex PCR as described [5].

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed for 13 antimicrobials using the microdilution method in three broths: *Brucella* broth with pH adjusted to 7.1 ± 0.1 (hereafter BB; *Brucella* medium base OXOID, Horse Serum, Oxoid) following the guidelines of the Clinical and Laboratory Standards Institute [12]; cation-adjusted Mueller-Hinton broth with pH adjusted to 7.2 ± 0.1 (hereafter 'CAMHB'; TREK Diagnostic Systems Ltd., Lenexa, KS, USA) supplemented with 4% lysed horse blood (LHB; Thermo Scientific); and CAMHB supplemented with 5% of defibrinated sheep blood (DSB; Biognost, Zagreb, Croatia). These broths were used to test sensitivity of isolates in a custom-made plate system (Sensititre, TREK Diagnostic Systems, East Grinstead, UK).

The following antimicrobials were applied: gentamicin (GEN; 0.12–16 mg/L), tetracycline (TET; 0.03–4 mg/L), doxycycline (DOX; 0.03–4 mg/L), ciprofloxacin (CIP; 0.03–4 mg/L), levofloxacin (LEVO; 0.03–4 mg/L), trimethoprim-sulfamethoxazole mixed in a 1:19 (m/m) ratio (T/S; 0.06/1.19–8/152 mg/L), rifampin (RIF; 0.12–4 mg/L), ceftriaxone (AXO; 0.25–8 mg/L), amikacin (AMI; 0.25–8 mg/L), streptomycin (STR; 0.5–32 mg/L), chloramphenicol (CHL; 0.5–32 mg/L), tigecycline (TIG; 0.015–0.5 mg/L) and azithromycin (AZI; 0.12–16 mg/L).

Before susceptibility testing, isolates were revived on BB for 48 hours under aerobic conditions at 35°C. The procedure followed Guideline M45 of the Clinical and Laboratory Standards Institute [12]. Briefly, a colony suspension equivalent to 0.5 MacFarland was prepared in CAMHB. Then 10 µL of the suspension was transferred into 11 mL of each of the three broths, from which 100 μL was inoculated into each well of a 96-well plate. Microplates were incubated at 37°C for 48 hours.

Minimum inhibitory concentrations (MIC) breakpoints were calculated for GEN, TET, DOX, T/S and STR as recommended in Guideline M45. MIC breakpoints have not been established for *Brucella* spp. against LEV, RIF, AXO, CHL, CIP or AZI, so for these antibiotics we applied Guideline M100 of the Clinical and Laboratory Standards Institute for the slow-growing bacterium *Haemophilus influenzae* [12].

As AMI and TIG do not have a defined breakpoint, it was determined by its MIC 50, MIC 90 and MIC range. MIC_{50} and MIC_{90} levels were defined as the lowest concentration of the antibiotic at which 50% and 90% of the isolates were inhibited, respectively.

Reference strains *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Streptococcus pneumoniae* ATCC 49619 were used to ensure that the results were within acceptable limits of quality control for susceptibility testing and to establish which broth would be the best for antimicrobial susceptibility testing of *B. melitensis* by the broth-microdilution method.

Vaccine strains *B. melitensis* Rev.1 biovar (bv.) 1, *B. melitensis* 16M bv. 1 and *B. abortus* S99 bv.1 were also included in this study.

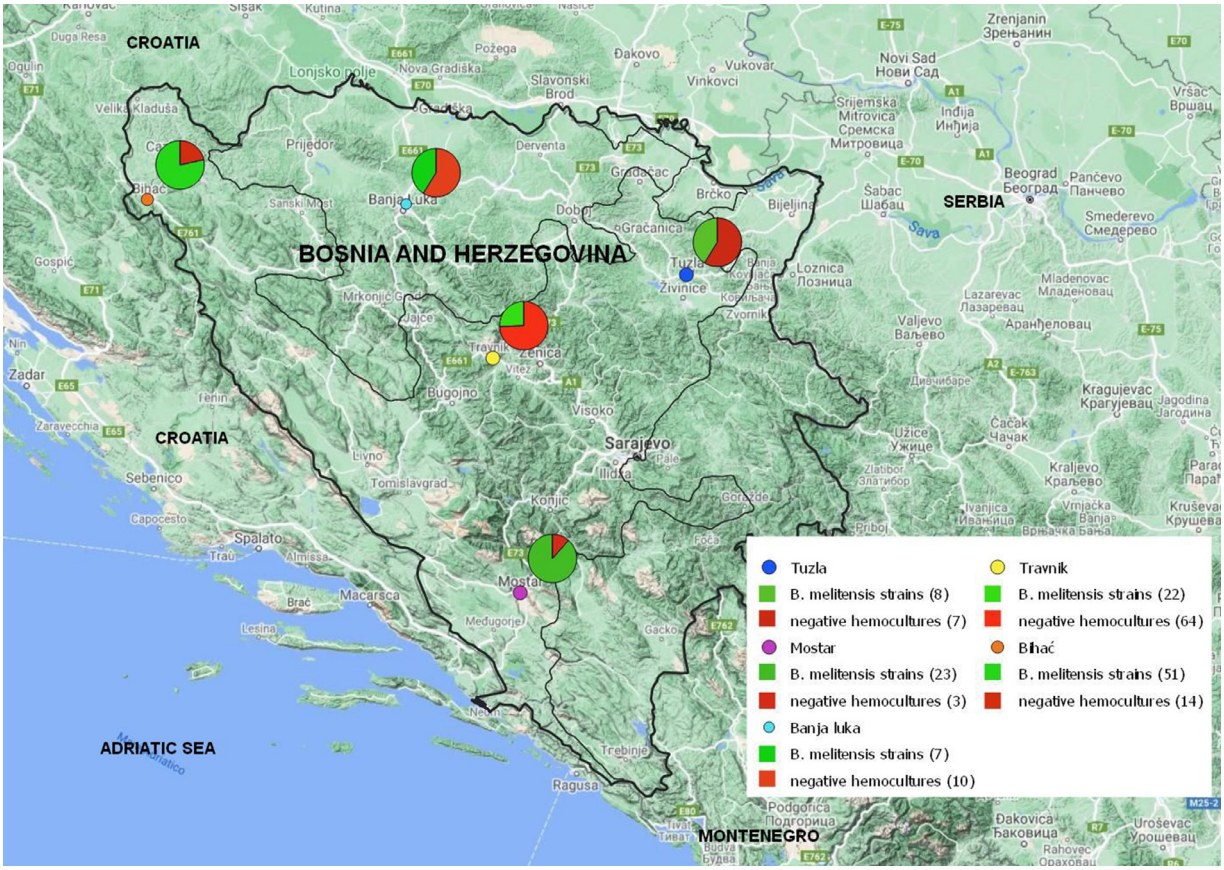

Fig. 1. Geographic distribution of *Brucella melitensis* isolates from blood cultures collected at five medical centres in Bosnia and Herzegovina. Numbers of isolates are indicated in brackets.

Vaccine strain *B. melitensis* Rev.1 has been used for more than a decade for vaccination of small ruminants in Bosnia and Herzegovina, and also it can be found as a human pathogen in some circumstances [13]. In addition, standard strains *B. melitensis* 16 M and *B. abortus* S99 were used for consistent result evaluation of *B. melitensis* strains. Strains belong to the archive of NRL for brucellosis, Croatian Veterinary Institute Zagreb.

2.4. Ethics statement

All procedures were performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. As this was a prospective study conducted at five medical centres, the protocol was approved by the Council for Ethics of the Federal Ministry for Health of the Government of the Federation of Bosnia and Herzegovina (approval no. 03-37-3103/18).

3. Results

Blood cultures were positive for *B. melitensis* in 111 of 209 diagnosed patients, corresponding to an efficiency of 53.1%, and the 108 isolates were microbiologically analysed for antimicrobial resistance. The geographic distribution of *B. melitensis* isolates is shown in Fig. 1.

MICs for the reference strains tested in different broths are shown in Table 1. We obtained the same MICs for *E. coli* ATCC 25922, *E. faecalis* ATCC 29212 and *S. pneumoniae* ATCC 49619 in the two enriched CAMHBs. The MICs in all three broths were within the ranges recommended by the Clinical and Laboratory Standards Institute [12], except for some MICs against T/S in BB: for *E. coli* ATCC 25922 and *S. pneumoniae* ATCC 49619, the 3 $\log_2$ dilution and *E. faecalis* ATCC 29212, the 4 $\log_2$ dilution values were higher than the maximal recommended MICs.

MIC ranges for the *B. melitensis* isolates as well as MIC_{50} and MIC_{90} values in the three broths are shown in Table 2 and more precisely in Supplementary Table S1. Susceptibility to most antibiotics was similar across the different broths. In all three broths, all strains were susceptible to GEN, TET, DOX, LEVO, AXO, STR and CHL, but one (0.9%) was not susceptible to CIP. In BB and CAMHB + 5% DSB, one strain (0.9%) was intermediate-resistant to RIF; in CAMHB + 4% LHB, two strains (1.8%) were intermediate-resistant. Nearly all isolates were resistant to AZI in BB (102, 94.4%) or in enriched CAMHBs (91%–92%).

In contrast, the different broths gave strikingly divergent results in the case of T/S. Most isolates (91, 84.3%) were resistant to T/S in BB, whereas all isolates were susceptible to those antibiotics in enriched CAMHBs.

In all three broths, the lowest MIC_{50} and MIC_{90} values (0.03–0.5 mg/L) were obtained for GEN, TET, DOX, CIP, LEVO and TIG, while very low MIC_{50} and MIC_{90} values (1–2 mg/L) were obtained for RIF, AMI, STR and CHL. In all three broths, the highest MIC_{50} and MIC_{90} values (8 mg/L) were for AZI, while MIC_{50} and MIC_{90} for T/S were high in BB but extremely low in enriched CAMHBs (0.25–0.5 mg/L).

As a control for our susceptibility profiling, we determined MICs of the antimicrobials against the *B. melitensis* vaccine strains Rev.1 and 16M in the three broths, and against *B. abortus* S99 in BB only, since bacterial growth in other two enriched broths was considerably less pronounced (Table 3). For all antimicrobials except T/S, MICs against the two *B. melitensis* vaccine strains were similar regardless of the broth. In contrast, the MIC for T/S was 2–4 $\log_2$ dilutions higher in BB than enriched CAMHBs.

Table 1
Minimum inhibitory concentrations (MIC) of *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212 and *Streptococcus pneumoniae* ATCC 49619 in different broths.

Reference strain	Broth	Antibiotic/MIC (mg/L) GEN	TET	DOX	CIP	LEVO	T/S	RIF	AXO	AMI	STR	CHL	TGC	AZI
ATCC	CAMHB	0.5	0.5	1	≤0.03	≤0.03	≤0.06	4	≤0.25	1	4*	2	0.06	4^{ND}
25922	CAMHB + 4% LHB	0.5	0.5	1	≤0.03	≤0.03	≤0.06	4	≤0.25	1	4*	4	0.06	4^{ND}
	CAMHB + 5% DSB	0.25	0.5	1	≤0.03	≤0.03	≤0.06	4	≤0.25	1	4*	2	0.06	2^{ND}
	BB	1	0.5	1	≤0.03	≤0.03	8^{+3}	>4	≤0.25	4	8	4	0.25	4^{ND}
ATCC	CAMHB	4	>4	4	0.5	0.5	≤0.06	1	$>8^{ND}$	>8	32^{ND}	4	0.03	2
29212	CAMHB + 4% LHB	4	>4	2	0.5	0.5	≤0.06	0.5	8^{ND}	>8	16^{ND}	4	0.12	1
	CAMHB + 5% DSB	4	>4	2	0.5	0.5	≤0.06	0.5	4^{ND}	>8	8^{ND}	4	0.03	1
	BB	16	>4	4	0.5	0.25	4^{+4}	1	8^{ND}	>8	$>32^{ND}$	4	0.12	4
ATCC	CAMHB + 4% LHB	16^{ND}	0.12	≤0.03	0.5^{ND}	0.5	0.25	≤0.12	≤0.25	$>8^{ND}$	$>32^{ND}$	4	0.06	≤0.12
49619	CAMHB + 5% DSB	16^{ND}	0.12	≤0.03	0.5^{ND}	0.5	0.25	≤0.12	≤0.25	$>8^{ND}$	$>32^{ND}$	4	0.03	≤0.12
	BB	$>16^{ND}$	0.25	≤0.03	1	1	8^{+3}	≤0.12	≤0.25	$>8^{ND}$	>32	4	0.06	≤0.12

NOTE: +3 = three $\log_2$ dilutions higher than maximum MIC value range according to CLSI; +4 = four $\log_2$ dilutions higher than maximum MIC value range according to CLSI.
GEN, gentamicin; TET, tetracycline; DOX, doxycycline; CIP, ciprofloxacin; LEVO, levofloxacin; T/S, trimethoprim-sulfamethoxazole; RIF, rifampin; AXO, ceftriaxone; AMI, amikacin; STR, streptomycin; CHL, chloramphenicol; TGC, tigecycline; AZI, azithromycin; ND, not defined with CLSI.
* Sensititre development range (4–16 mg/L).

Table 2
Minimum inhibitory concentrations MIC_{50}, MIC_{90} and MIC ranges for antimicrobials against *Brucella melitensis* isolates in the three broths.

		MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC ranges (mg/L)	S	I	R	Breakpoint (mg/L) S	I	R
GEN	BB	0.25	0.5	0.25–0.5	108	–	–	≤4[a]		
	CAMHB + 4% LHB	0.25	0.25	≤0.12–0.25						
	CAMHB + 5% DSB	0.25	0.25	≤0.12–0.25						
TET	BB	0.12	0.25	0.12–0.25	108	–	–	≤1[a]		
	CAMHB + 4% LHB	0.06	0.12	0.06–0.25						
	CAMHB + 5% DSB	0.12	0.12	0.06–0.25						
DOX	BB	0.12	0.12	≤0.03–0.25	108	–	–	≤1[a]		
	CAMHB + 4% LHB	0.06	0.12	≤0.03–0.12						
	CAMHB + 5% DSB	0.12	0.12	≤0.03–0.12						
CIP	BB	0.5	0.5	0.5 – 4	107	–	1	≤1[b]		
	CAMHB + 4% LHB	0.25	0.5	0.25–2						
	CAMHB + 5% DSB	0.5	0.5	0.25–4						
LEVO	BB	0.5	0.5	0.5–2	108	–	–	≤2[b]		
	CAMHB + 4% LHB	0.25	0.5	0.25–1						
	CAMHB + 5% DSB	0.5	0.5	0.25–1						
T/S	BB	4	8	1 to >8	17	–	91	≤2/38[a]		
	CAMHB + 4% LHB	0.25	0.5	≤0.06 to 0.5	108	–	–			
	CAMHB + 5% DSB	0.25	0.5	≤0.06 to 1	108	–	–			
RIF	BB	1	1	0.25–2	107	1	–	≤1	2	≥ 4[b]
	CAMHB + 4% LHB	1	1	0.5–2	106	2	–			
	CAMHB + 5% DSB	1	1	0.25–2	107	1	–			
AXO	BB	1	1	0.5–2	108	–	–	≤2[b]		
	CAMHB + 4% LHB	0.5	0.5	≤0.25–0.5						
	CAMHB + 5% DSB	0.5	0.5	≤0.25–0.5						
AMI	BB	2	2	1–2	–	–	–	ND		
	CAMHB + 4% LHB	1	1	0.5–1						
	CAMHB + 5% DSB	1	1	0.5–1						
STR	BB	2	2	1–2	108	–	–	≤8[a]		
	CAMHB + 4% LHB	1	2	1–2						
	CAMHB + 5% DSB	1	2	1–2						
CHL	BB	1	1	≤0.5–2	108	–	–	≤2	4	≥ 8[b]
	CAMHB + 4% LHB	1	1	≤0.5–1						
	CAMHB + 5% DSB	1	1	≤0.5–1						
TIG	BB	0.06	0.12	0.03–0.25	–	–	–	ND		
	CAMHB + 4% LHB	0.03	0.06	0.03–0.06						
	CAMHB + 5% DSB	0.12	0.25	0.06–0.25						
AZI	BB	8	8	4–8	6	–	102	≤4[b]		
	CAMHB + 4% LHB	8	8	2–8	10	–	98			
	CAMHB + 5% DSB	8	8	4–8	9	–	99			

BB, *Brucella* broth; CAMHB + 4% LHB, cation-adjusted Mueller-Hinton broth + 4% lysed horse blood; CAMHB + 5% DSB, cation-adjusted Mueller-Hinton broth + 5% defibrinated sheep blood; ND, not defined; S, sensitive; I, intermediate; R, resistant.
[a] CLSI M45.
[b] CLSI M100 (slow-growing bacteria).

Table 3
Minimum inhibitory concentrations (MICs) for antimicrobials against the *Brucella melitensis* vaccine strains Rev.1 and 16M biovar1 or against *B. abortus* S99 biovar1.

		GEN	TET	DOX	CIP	LEVO	T/S	RIF	AXO	AMI	STR	CHL	TGC	AZI
B. melitensis Rev.1	BB	0.25	≤0.03	≤0.03	0.25	0.25	1	0.5	0.5	1	4	1	0.03	1
	CAMHB + 4% LHB	≤0.12	≤0.03	≤0.03	0.25	0.25	≤0.06	1	≤0.25	0.5	4	≤0.5	0.03	1
	CAMHB + 5% DSB	≤0.12	0.06	≤0.03	0.25	0.25	≤0.06	1	≤0.25	0.5	4	≤0.5	0.06	1
B. melitensis 16M biovar1	BB	0.5	0.12	0.06	0.25	0.25	1	1	1	2	2	1	0.06	1
	CAMHB + 4% LHB	0.25	0.12	0.06	0.25	0.25	0.12	1	0.5	1	2	1	0.03	1
	CAMHB + 5% DSB	0.25	0.12	0.12	0.25	0.5	0.25	1	0.5	1	2	1	0.06	1
B. abortus S99 biovar1	BB	0.5	0.25	0.25	0.5	0.5	0.5	2	≤0.25	2	2	2	0.25	4

GEN, gentamicin; TET, tetracycline; DOX, doxycycline; CIP, ciprofloxacin; LEVO, levofloxacin; T/S, trimethoprim-sulfamethoxazole; RIF, rifampin; AXO, ceftriaxone; AMI, amikacin; STR, streptomycin; CHL, chloramphenicol; TGC, tigecycline; AZI, azithromycin.
BB, *Brucella* broth; CAMHB + 4% LHB, cation-adjusted Mueller-Hinton broth + 4% lysed horse blood; CAMHB + 5% DSB, cation-adjusted Mueller-Hinton broth + 5% defibrinated sheep blood.

4. Discussion

Our study prospectively aimed to determine the rate of antimicrobial resistance of mainly used antimicrobials in the treatment of human brucellosis. We found that a high proportion of *B. melitensis* isolates are sensitive to commonly used antimicrobials such as tetracyclines, aminoglycosides, rifampicin and quinolones. Our work suggests a high prevalence of *B. melitensis* resistance to AZI and T/S in Bosnia and Herzegovina, which has implications for treating brucellosis infections in southeastern Europe. Our results also demonstrate that the ability to detect T/S resistance depends on the broth used in the microdilution method, which may be a useful insight for epidemiological programmes around the world.

In our hands, the overall efficiency of blood culture isolation was 53.1%, which is higher than in previous work retrospectively observed in Bosnia and Herzegovina [7]. Nevertheless, efficiency ranged from 25.6% to 88.5% across the five medical centres in our study, implicating that some other affecting factors such as antimicrobial therapy given prior to taking of blood cultures could be a reason for that.

Brucella spp. antimicrobial susceptibility testing is not routinely performed since these bacteria are considered highly infectious and hazardous [14]. Various techniques have been reported for *Brucella* spp. testing, including broth microdilution, agar dilution, and E–testing. Previous work has suggested that the test format can affect MICs [15,16], and here we extend that literature by showing that within the microdilution format, the choice of broth can substantially affect MICs at least for certain antibiotics, in our case T/S.

The Clinical and Laboratory Standards Institute recommends the microdilution format in BB when determining MICs against *Brucella* strains [12]. In addition to this broth, we chose to use enriched CAMHB because it is known to contain low concentrations of thymine and thymidine due to high activity of thymidine phosphorylase, whereas the levels of these components in BB are unknown. We prepared CAMHB supplemented with 5% LHB according to the Institute guidelines, but we also prepared it with 5% DSB because of the low concentration of thymidine and thymine [17].

Across all three broths, we found TET (MIC_{50}, 0.12 mg/L; MIC_{90} 0.25 mg/L) and DOX (MIC_{50} and MIC_{90}, 0.12 mg/L) to be the most effective agents against the *B. melitensis* isolates. This corroborates the fact that the most frequently used antibiotics for treating brucellosis are TET, aminoglycosides, RIF, AXO and quinolones. The consistent MICs across broths is consistent with previous studies of *Brucella* spp. susceptibility based on broth microdilution [18] and E–testing [19].

We observed lower MIC_{50} and MIC_{90} values for TIG than for TET and DOX, similar to a previous report [20], when we performed the testing in BB. The MICs for TIG differed by as much as two $\log_2$ dilutions between the two CAMHBs.

We measured similar MIC_{50} and MIC_{90} values for GEN in all three broths, and the values agree with those reported previously [18,21]. However, they are two-fold lower than in one previous study [22]. MIC_{50} and MIC_{90} values for AMI were 2 mg/L, with an MIC range of 1–2 mg/L, consistent with one previous study [18], and yet much lower than in another study [23].

We also determined MICs for the fluoroquinolones CIP and LEV, as well as for RIF, based on the Clinical and Laboratory Standards Institute guidelines for slow-growing bacteria [12]. In all three broths, all *B. melitensis* isolates were susceptible to LEV, while all but one was resistant to CIP. These results are similar to previous studies using broth microdilution and the E–test method [21,24,25], but they were much lower than those reported in other work by E–test provided [20]. We found a similarly high prevalence of susceptibility to RIF, consistent with previous results [18].

We measured MIC_{50} and MIC_{90} values of 1 mg/L for AXO in all three broths. This value is consistent with earlier studies [26], although it is lower than more recent work [22].

We found nearly all *B. melitensis* isolates to be resistant to AZI in all three broths; we determined MICs according to criteria for slow-growing bacteria [12]. Although published MICs for this antimicrobial against *Brucella* can vary, probably because of differences in testing format and geographical origin of isolates [15,25,27], in our study MIC_{50} and MIC_{90} values of 8 mg/L in all three broths are consistent with earlier work [28,29]. Non-critical uses of AZI in the clinic may help explain the high rate of resistance among *B. melitensis* isolates.

We found all 108 *B. melitensis* isolates to be sensitive to CHL, with a MIC range ≤0.5–2 mg/L and $MIC_{50/90}$ values of 1 mg/L in all three broths. These values are much lower than those previously reported for *B. abortus* [24], suggesting that results for CHL must therefore be restricted to *B. melitensis* only. Nevertheless, CHL is not recommended for treatment of human brucellosis because of the risk of serious adverse effect.

Previous studies identified T/S as the most effective antibiotic against *Brucella spp*. based on MIC_{50} and MIC_{90} values [19,20], and yet various studies have highlighted disturbing levels of resistance in natural *B. melitensis* isolates. Rates have varied widely: for example, 2% in Turkey [26], 29% in Saudi Arabia based on the broth dilution method [30], 37.5% in India [31], 62% in Saudi Arabia based on the disc-diffusion method [32], or even 100% in China based on E–testing [23]. Here, we measured a resistance rate of 84.3% in BB, but this fell to 0% in CAMHBs. These results are in accordance with earlier published studies provided antimicrobial resistance tests on modified broths [18,25].

Our observations confirm that, in contrast to the current guidelines from the Clinical and Laboratory Standards Institute [12], media with low or no thymine and thymidine should be used when testing isolates for susceptibility to T/S and other sulphonamide drugs [33]. Thymine and thymidine in the medium can weaken the efficacy of T/S and other sulphonamides, making susceptible or-

ganisms appear resistant. In fact, in our hands, using BB increased MICs for T/S by up to 5 $\log_2$ dilutions. To prevent antagonism of the dihydrofolate reductase by thymidine and thymine, a solution is to use lysed horse blood, which is rich in the enzyme thymidine phosphorylase, which converts thymidine to thymine [17].

Our study presents several limitations. First, overall efficiency of blood culture isolation across the five medical centres in our study has unacceptable variety, and standardized protocol prior taking of blood cultures could be helpful; second, therapy of brucellosis is not standardised to the whole country; third, the overall consumption of antibiotics at the level of the whole country and individual administrative units-cantons is not known; and, finally, future collaboration should include other medical institutions, thus forming an institutional network for rapid and uniform diagnosis and treatment of human brucellosis throughout Bosnia and Herzegovina.

5. Conclusion

Our study is the first evaluation of antibiotic resistance among *B. melitensis* strains in brucellosis patients in southeastern Europe and specifically in an endemic part of that region. Our results suggest that, at least in Bosnia and Herzegovina, circulating strains of *B. melitensis* generally show low rates of resistance to several antimicrobials commonly used to treat patients with brucellosis. However, the strains show high prevalence of resistance to AZI and T/S on BB. The high rate of resistance to T/S in BB is abolished when an alternative broth is used, such as the enriched CAMHBs in this study. Thus, resistance of *B. melitensis* to antimicrobials should be taken in account in the case of T/S. Our work may provide an important reference for monitoring antimicrobial resistance of *B. melitensis* in Bosnia and Herzegovina, a country where the disease has been prevalent in humans and animals over the last two decades. Such monitoring is likely to become more important in the wake of generally increased antimicrobial use during the coronavirus disease 2019 pandemic.

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Competing interests

No conflict of interest.

Ethical approval

The protocol was approved by the Council for Ethics of the Federal Minstry for Health of the Government of the Federation of Bosnia and Herzegovina (approval no. 03-37-3103/18).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.02.005.

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