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RESEARCH ARTICLE

In vitro efficacy of antibiotics against different *Borrelia* isolates

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ABSTRACT

In the present study, the effectiveness of six antimicrobial agents have been tested against 24 borrelia strains isolated from *Ixodes ricinus* ticks (11 *Borrelia lusitaniae*, eight *Borrelia afzelii*, three *Borrelia garinii* and two *Borrelia valaisiana*) and one *B. lusitaniae* strain isolated from human skin. The minimum inhibitory concentration range of antimicrobial agents was as follows: amoxicillin, 0.125–2 mg/L; doxycycline, 0.125–1 mg/L, ceftriaxone, 0.016–0.063 mg/L; cefuroxime, 0.063–1 mg/L; azithromycin, 0.0017–0.11 mg/L; amikacin 32–512 mg/L. Potentially pathogenic *B. lusitaniae* and *B. valaisiana* species were more susceptible to amoxicillin and azithromycin than pathogenic *B. afzelii* and *B. garinii* ($P < 0.05$); *B. garinii*, *B. lusitaniae* and *B. valaisiana* were more susceptible to doxycycline than *B. afzelii* ($P < 0.05$) while all species showed same susceptibility to ceftriaxone and cefuroxime ($P > 0.05$). This study is the first report on *in vitro* susceptibility of isolates from Serbia to antimicrobial agents and the first report on susceptibility of larger number of isolates of potentially pathogenic species *B. lusitaniae*. We showed that antimicrobial agents *in vitro* inhibit growth of borrelia strains very effectively, indicating the potential of their equally beneficial use in the treatment of Lyme borreliosis.

KEYWORDS

Borrelia, antimicrobial agents, *in vitro* susceptibility, treatment, Lyme borreliosis

INTRODUCTION

Lyme borreliosis (LB) is a tick-borne disease that occurs in regions of the Northern Hemisphere, caused by spirochetes belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex, and transmitted to humans through a bite of ticks from *Ixodes ricinus* complex [1, 2]. In Europe, five species (*Borrelia afzelii*, *Borrelia garinii*, *Borrelia bavariensis*, *Borrelia burgdorferi* sensu stricto (s.s.) and *Borrelia spielmanii*) are known to cause LB in humans, leading to a wide spectrum of clinical manifestations, while *Borrelia lusitaniae*, *Borrelia valaisiana* and *Borrelia bissettii*, have been sporadically detected in humans, but their pathogenic potential is still unclear [1]. To date, there is only one human isolate of *B. lusitaniae* from the skin of a patient with chronic lesions in Portugal [3].

There are differences in LB incidence rates and clinical presentations across Europe due to the heterogeneous distribution of *Borrelia* species [4]; annual incidence rates range from 0.001/100,000 in Italy (2001–2005) to 111/100,000 in Germany and 188.7/100,000 in Slovenia (2014) [5], while in West Pannonian region, *B. burgdorferi* s.l. (16%) is the second most common bacterial pathogen associated with neuroinfections, following *Streptococcus pneumoniae* (20%) [6]. In Serbia the reported incidence of LB is in the range of 6.83–13.32/100,00 inhabitants (2013–2017) [7]. Despite the evidence that different *Borrelia* species are involved

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in distinct clinical manifestations of LB, in the early stage of the disease they usually all cause a skin lesion–erythema migrans. In ongoing infection, *B. afzelii* usually remains localized in the skin, *B. garinii* and *B. bavariensis* are usually associated with nervous system disorders, while *B. burgdorferi* s.s. is commonly associated with development of the arthritis (more common in North America than in Europe) [1, 2, 8]. The most common clinical manifestation of LB in Serbia is erythema migrans, found in 93.21% cases, followed by neurological, rheumatological and cardiac manifestations in 2.80, 2.46 and 1.10% cases, respectively [9]. While, *B. afzelii*, *B. garinii* and *B. valaisiana* are the most frequently detected species in ticks across Europe [10], studies on the presence of *B. burgdorferi* s.l. in ticks from Serbia revealed high diversity on species and subspecies level and pointed to the domination of *B. lusitaniae*, followed by *B. afzelii, B. garinii*, *B. bavariensis, B. valaisiana* and *B. burgdorferi* s.s. [11, 12]. However, information on exact *Borrelia* species that cause LB in Serbia are lacking.

The choice of antibiotics, dosage and duration of antibiotic therapy depend on clinical manifestations of the disease, age of the patient, possible existing allergic reactions to the drug, and general health status of the patient [1, 8]. Although the most used antimicrobial agents in the treatment of LB are amoxicillin, phenoxymethylpenicillin, penicillin G, doxycycline, azithromycin, erythromycin, ceftriaxone, and cefuroxime, there are various, partly contradicting recommendations about the choice of antimicrobial agents, dose and length of treatment in the therapy of different clinical manifestation of LB [8, 13, 14]. *Borrelia* species does not usually possess resistance mechanisms but a small number of studies have demonstrated acquired resistance to antimicrobial agents (aminoglycosides, fluoroquinolones) in both laboratory and clinical settings [15]. Number of *in vitro* studies have shown that analysed *B. burgdorferi* s.l. strains are susceptible to different antimicrobial agents, including amoxicillin, erythromycin, clarithromycin, azithromycin, ceftriaxone, cefuroxime, cefixime, cefotaxime, tigecycline, doxycycline, penicillin G, etc. [16–21], but results are often inconsistent concerning the determination of minimum inhibitory concentrations (MICs) for antimicrobial agents due to lack of standardized methodology. Some *in vitro* studies showed interspecies [16–18], while others [22, 23] showed intraspecies differences in susceptibility of analysed borrelia strains to antimicrobial agents.

Knowledge on diversity of local borrelia strains contributes to an estimation of their pathogenic potential and risk of the disease in a particular geographical area. Considering the variations in geographic distribution and clinical manifestation of LB for each species, we found it useful to look for differences in the antibiotic response of larger number of different borrelia strains. The aim of our study was to compare the *in vitro* susceptibility of borrelia strains isolated from *I. ricinus* ticks, belonging to four different species, potentially pathogenic – *B. lusitaniae* and *B. valaisiana* and species with proven pathogenic potential – *B. afzelii* and *B. garinii,* to antimicrobial agents usually used for the treatment of LB, evaluate inter– and intraspecies differences in susceptibility to antimicrobial agents of analysed *Borrelia* isolates, and evaluate the efficacy of antimicrobial agents concerning inhibition of *Borrelia* growth.

MATERIALS AND METHODS

This study was conducted under aseptic conditions provided by a laminar flow box to reduce the risk of contamination. The susceptibility of *B. burgdorferi* s.l. strains to five different classes of the antimicrobial agent were evaluated by measurement of their MICs.

Borrelia strains

The twenty-five borrelia strains were included in the study – 24 isolated from *I. ricinus* ticks and one strain isolated from human skin. Twenty-one borrelia strains (eight *B. afzelii*, eight *B. lusitaniae*, three *B. garinii*, and two *B. valaisiana*) were selected from the collection of strains of the Institute for Medical Research, University of Belgrade, Serbia. These strains were isolated from *I. ricinus* ticks collected from different localities in Serbia [12]. Stock cultures of these few passage borrelia strains had been stored at –80 °C, and subcultured for the study.

Four external *B. lusitaniae* strains were included in the study, three strains isolated from *I. ricinus* ticks–two strains from Spain (Heavy and Listu) [24, 25] and one strain (PotiB2) from Portugal [26], while one strain from Portugal was isolated from human skin (PoHL–1) [3]. Strains from Spain were provided by the Group of Dr. Pedro Anda from Madrid, and strains from Portugal were provided by Group of Dr. Margarida Collares–Pereira from Lisbon. All tested isolates are listed in Supplementary Material (Table 1).

Antimicrobial agents

Six antimicrobial agents, consisting of three β-lactam agents (amoxicillin, ceftriaxone, and cefuroxime), one macrolide (azithromycin), one tetracycline (doxycycline) and one aminoglycoside (amikacin) were tested in this study. All antimicrobial agents were obtained as standard powders from the Faculty of Pharmacy, University of Belgrade, and prepared according to the recommendations of the Faculty of Pharmacy. Briefly, the powders were dissolved in suitable solvents (sterile water and/or 0.1M phosphate buffer) to obtain stock solutions and two–fold serial dilutions were made. All dilutions of antimicrobial agents were filtered through a 0.22 µm pore size sterile filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany), divided into 2 mL microtubes and stored at –20 °C for maximum 5 days or used immediately.

The ranges of antibiotic concentrations were as follows: amoxicillin 0.125–64 mg/L; ceftriaxone 0.016–8 mg/L; cefuroxime 0.063–32 mg/L; doxycycline 0.125–64 mg/L; azithromycin 0.0017–0.88 mg/L; and amikacin 4–2048 mg/L. The ranges were chosen according to previously published

Table 1. Individual minimum inhibitory concentrations (MICs) of six antimicrobial agents (MIC in mg/L) against various *Borrelia burgdorferi* sensu lato isolates determined by broth microdilution method

Strain number	Isolate (name for this study)	Amoxicillin	Doxycycline	Ceftriaxone	Cefuroxime	Azithromycin	Amikacin
167_11b RS	*B. lusitaniae* (Bl1)	0.125	0.125	0.016	0.063	0.0138 (0.0069–0.0138)	128 (64–128)
221_10c RS	*B. lusitaniae* (Bl2)	1 (0.5–1)	0.125	0.032	1 (0.5–1)	0.055 (0.0275–0.055)	256 (128–256)
76_12a RS	*B. lusitaniae* (Bl3)	0.125	0.25 (0.125–0.25)	0.016	0.063	0.0017	128 (64–128)
77_12b RS	*B. lusitaniae* (Bl4)	0.125	0.25 (0.125–0.25)	0.016	0.063	0.0017	128
167_11c RS	*B. lusitaniae* (Bl5)	0.125	0.25 (0.125–0.25)	0.032 (0.016–0.032)	0.063	0.0017	256 (128–256)
226_10d RS	*B. lusitaniae* (Bl6)	0.125	0.25 (0.125–0.25)	0.016	0.063	0.0017	256 (128–256)
162_11b RS	*B. lusitaniae* (Bl7)	0.125	0.25 (0.125–0.25)	0.016	0.063	0.0017	128
222_10d RS	*B. lusitaniae* (Bl8)	0.125	0.125	0.016	0.063	0.0035 (0.0017–0.0035)	256 (128–256)
LISTU	*B. lusitaniae* (Bl9)	0.125	0.125	0.016	0.063	0.0017	256 (128–256)
HEAVY	*B. lusitaniae* (Bl10)	0.25 (0.125–0.25)	0.25 (0.125–0.25)	0.032	0.063	0.0035 (0.0017–0.0035)	128
PotiB2	*B. lusitaniae* (Bl11)	0.125	0.25 (0.125–0.25)	0.016	0.063	0.0017	256 (128–256)
PoHL1	*B. lusitaniae* (Bl12)	0.25 (0.125–0.25)	0.25 (0.125–0.25)	0.032 (0.016–0.032)	0.063	0.0017	128
	MIC range	0.125–1	0.125–0.25	0.016–0.032	0.063–1	0.0017–0.055	64–256
MIC	**MIC_{50}**	**0.125**	**0.125**	**0.016**	**0.063**	**0.0017**	**128**
	MIC_{90}	0.25	0.25	0.032	0.063	0.0069	256
32_12b RS	*B. afzelii* (Ba1)	1 (0.5–1)	1 (0.5–1)	0.016	0.063	0.0138 (0.0069–0.0138)	128 (64–128)
230_13c RS	*B. afzelii* (Ba2)	2 (1–2)	1	0.016	0.063	0.11 (0.055–0.11)	128
164_11a RS	*B. afzelii* (Ba3)	2 (1–2)	1	0.016	0.063	0.0017	256
168_11c RS	*B. afzelii* (Ba4)	2 (1–2)	0.125	0.016	0.125 (0.063–0.125)	0.0275 (0.0138–0.0275)	512
235_13cd RS	*B. afzelii* (Ba5)	0.5 (0.125–0.5)	1	0.016	0.5(0.125–0.5)	0.0138 (0.0069–0.0138)	32
232_13b RS	*B. afzelii* (Ba6)	0.125	0.125	0.016	0.125 (0.063–0.125)	0.0017	128 (32–128)
168_11g RS	*B. afzelii* (Ba7)	2 (1–2)	0.25 (0.125–0.25)	0.032 (0.016–0.032)	0.125 (0.063–0.125)	0.0035 (0.0017–0.0035)	32
163_11i RS	*B. afzelii* (Ba8)	0.125	0.5 (0.125–0.5)	0.016	0.5 (0.125–0.5)	0.0138 (0.0069–0.0138)	256 (128–256)
	MIC range	0.125–2	0.125–1	0.016–0.032	0.063–0.5	0.0017–0.11	32–512
MIC	**MIC_{50}**	**1**	**0.5**	**0.016**	**0.063**	**0.0138**	**128**
	MIC_{90}	2	1	0.016	0.5	0.0275	256
226_10a RS	*B. garinii* (Bg1)	0.5 (0.25–0.5)	0.25 (0.125–0.25)	0.032 (0.016–0.032)	0.063	0.0017	256 (128–256)
160_13e RS	*B. garinii* (Bg2)	2	0.125	0.032 (0.016–0.032)	0.063	0.0275 (0.0138–0.0275)	128
164_11g RS	*B. garinii* (Bg3)	2 (1–2)	0.125	0.016	0.125 (0.063–0.125)	0.0275	64
	MIC range	0.25–2	0.125–0.25	0.016–0.032	0.063–0.125	0.0017–0.0275	64–256
MIC	**MIC_{50}**	**1**	**0.125**	**0.016**	**0.063**	**0.0275**	**128**
	MIC_{90}	2	0.25	0.032	0.063	0.0275	256
224_10b RS	*B. valaisiana* (Bv1)	0.5 (0.25–0.5)	0.5 (0.125–0.5)	0.016	0.063	0.0017	128

(continued)

Table 1. Continued

Strain number	Isolate (name for this study)	Amoxicillin	Doxycycline	Ceftriaxone	Cefuroxime	Azithromycin	Amikacin
164_12b RS	*B. valaisiana* (Bv2)	0.125	0.125	0.063 (0.016–0.063)	0.25 (0.063–0.25)	0.0035 (0.0017–0.0035)	256
	MIC range[b]	0.125–0.5	0.125–0.5	0.016–0.063	0.063–0.25	0.0017–0.0035	128–256
MIC[a]	**MIC_{50}**	**0.125**	**0.125**	**0.016**	**0.063**	**0.0017**	**128**
	MIC_{90}	0.5	0.5	0.016	0.063	0.0017	256
All *Borrelia* isolates							
	MIC range	0.125–2	0.125–1	0.016–0.063	0.063–1	0.0017–0.11	32–512
	MIC_{50}	**0.5**	**0.25**	**0.016**	**0.063**	**0.0035**	**128**
	MIC_{90}	2	1	0.063	0.5	0.0275	256
	Breakpoint*	≤4	≤4	≤8	≤8	≤2	≤16
	Literature**	0.03–4	0.06–4	<0.01–4	0.03–>4	0.0004–0.03	32–>128
E. coli ATCC 25922							
S. aureus	Median MIC	2	1	0.03	2	4	2
ATCC 29213	MIC range***	2–8	0.5–2	0.03–0.12	2–8	2–8	0.5–4

Note: *Breakpoint value indicating susceptible strains according to Jorgensen and Turnidge [29]. **MIC range adopted from the literature [16-19, 22, 27]. ***MIC range according to Clinical and Laboratory Standards Institute [28]. The bold font represents the MIC_{50}.

data [17–20, 27]. Amikacin previously demonstrated inactivity against *Borrelia* species [19, 20, 27] and served as a control compound.

Cultivation and counting of *Borrelia* cells

Borrelia stock cultures were thawed at room temperature, transferred into 6.5 mL sterile glass tubes (Sigma–Aldrich, Steinheim, Germany) with screw caps, containing Barbour–Stoenner–Kelly–H (BSK–H) medium (Sigma–Aldrich, St. Louis, MO, USA) and incubated at 33 °C. After incubation of 5–10 days, the number of *Borrelia* cells/mL were determined by dark-field microscopy using a Neubauer counting chamber (Brand GmbH & Co. KG, Wertheim, Germany). Individual cultures (200 µL, a final density of 10^5cells/mL) were transferred into 96–well U–shaped microtiter plates (Thermo Fisher Scientific, Rochester, NY, USA) for performing an antimicrobial susceptibility test [19].

Susceptibility testing

The *in vitro* susceptibility of borrelia strains to antimicrobial agents was evaluated by determining of MICs. The broth microdilution method by dark-field microscopy (microscopic method) was used in the determination of MIC as described previously [19]. Briefly, the first column of 96–well U–shaped microtiter plate served as the negative control and contained 200 µL of BSK–H medium. In the wells of all other columns, 200 µL of individual culture was added at a final density of 10^5 *Borrelia* cells/mL. The second column served as the positive control and contained no antimicrobial agent. From the third column onward, aliquots of 10 µL of antimicrobial agents were added in decreasing concentrations.

BD GasPak^{TM}EZ container system (Becton, Dickinson and Company, Sparks, MD, USA) was used to generate an anaerobic environment. Plates were sealed with adhesive plastic, placed in an incubation container containing anaerobe sachets with indicator and incubated at 33 °C for 72 h. After incubation, 5 µL of culture from each well was examined by dark-field microscopy. The MIC was defined as the lowest concentration of antimicrobial agents at which no motile or only very slightly motile spirochetes were observed and their numbers reduced. The MIC was determined in quadruplicate in two or three experiments for each strain to give a MIC range, and the highest value being interpreted as the MIC for a particular borrelia strain [19, 20].

For quality control and to investigate possible antibiotic–BSK–H medium interaction, MICs for *Escherichia coli* reference strain ATCC 25922 and MICs of azithromycin for *Staphylococcus aureus* reference strain ATCC 29213 (data for MICs of azithromycin for *E. coli* ATCC 25922 are not available), were determined in quadruplicate in two or three experiments under the same conditions after 24 h of incubation in accordance with the Clinical and Laboratory Standards Institute (CLSI) [28]. European Committee on Antimicrobial Susceptibility Testing (EUCAST) and CLSI do not define breakpoints for antimicrobial agents against *B. burgdorferi* s.l. strains. In the present study, breakpoints for antimicrobial agents was interpreted according to breakpoints for commonly prescribed antimicrobial agents against other bacteria species as described by Jorgensen and Turnidge [29].

Statistical analysis

Statistical tests were performed and analysed using SigmaPlot 11.0 (Systat. Software Inc., Richmond, CA). Kruskal-Wallis and Mann-Whitney tests (both compare differences in the median MIC values among species and strains) were used to assess possible differences in susceptibility to antimicrobial agents between different *Borrelia* isolates. *P* values less than 0.05 were considered statistically significant to statistical analyses.

RESULTS

The *in vitro* susceptibility of 25 isolates of *B. burgdorferi* s.l. (12 *B. lusitaniae*, eight *B. afzelii*, three *B. garinii* and two *B. valaisiana*) to six antimicrobial agents was tested. All antimicrobial agents, except amikacin used as control compound, inhibited growth of all *Borrelia* isolates very effectively. Obtained MIC values indicated that all isolates were susceptible to all the tested antimicrobial agents with except to amikacin according to breakpoints described by Jorgensen and Turnidge [29]. Table 1 shows the MICs, MIC ranges, $MIC_{50\%}$ (equivalent to the median MIC value) and $MIC_{90\%}$ values of each antimicrobial agents tested against *Borrelia* isolates. Table 2 shows the median MIC of each antimicrobial agent tested against individual *Borrelia* isolates.

Kruskal-Wallis test showed that there were no statistically significant interspecies differences concerning *in vitro* susceptibilities of analysed strains to ceftriaxone ($P = 0.117$) and cefuroxime ($P = 0.076$), while statistically significant interspecies differences were found for amoxicillin ($P < 0.001$), doxycycline ($P = 0.001$), and azithromycin ($P = 0.001$). Mann-Whitney test showed that MICs of amoxicillin and azithromycin were statistically significant lower in *B. lusitaniae* and *B. valaisiana* than in *B. afzelii* and *B. garinii*, respectively ($P < 0.05$), while decrease of MICs of doxycycline in *B. garinii*, *B. lusitaniae* and *B. valaisiana* isolates were statistically significant compared to *B. afzelii* ($P < 0.05$).

Small, but statistically significant intraspecies differences in the MICs of antimicrobial agents ($P < 0.05$) were recorded. Median MICs of amoxicillin were lower for 2/8 *B. afzelii* (Ba6 and Ba8), and 1/3 *B. garinii* (Bg1) isolates and higher for 1/12 *B. lusitaniae* (Bl2) isolate; median MICs of doxycycline were lower for 3/8 *B. afzelii* (Ba4, Ba6, Ba7) isolates; median MICs of azithromycin were lower for 1/3 *B. garinii* (Bg1) isolate and higher for 1/8 *B. afzelii* (Ba2) and 1/12 (Bl2) *B. lusitaniae* isolates; also median MICs of azithromycin for Ba3 and Ba6 were lower than MICs for Ba4, Ba5 and Ba8 isolates; median MIC of cefuroxime was higher for 1/12 *B. lusitaniae* (Bl2) isolate. There were no statistically significant differences between two *B. valaisiana* isolates (Bv1 and Bv2) with respect to *in vitro* susceptibilities to tested antimicrobial agents.

Median MIC of each antibiotic for *E. coli* and median MIC of azithromycin for *S. aureus* were in the ranges published by CLSI [28] (Table 1), indicating exclusion antibiotic– BSK–H medium interaction.

DISCUSSION

In this study, we tested *in vitro* susceptibility of 21 *B. burgdorferi* s.l. strains (eight *B. lusitaniae*, eight *B.afzelii*, three *B. garinii*, two *B. valaisiana*) isolated from *I. ricinus* ticks from Serbia to the antimicrobial agents usually used in the treatment of LB. According to previous studies *B. lusitaniae*

Table 2. Median minimum inhibitory concentrations (MIC) of each antimicrobial agent (MIC in mg/L) tested against individual *Borrelia* isolates

Isolate	Isolate (name for this study)	Amoxicillin	Doxycycline	Ceftriaxone	Cefuroxime	Azithromycin
167_11b RS	Bl1	0.125	0.125	0.016	0.063	0.0069
221_10c RS	Bl2	0.5	0.125	0.032	0.5	0.055
76_12a RS	Bl3	0.125	0.25	0.016	0.063	0.0017
77_12b RS	Bl4	0.125	0.25	0.016	0.063	0.0017
167_11c RS	Bl5	0.125	0.25	0.032	0.063	0.0017
226_10d RS	Bl6	0.125	0.25	0.016	0.063	0.0017
162_11b RS	Bl7	0.125	0.25	0.016	0.063	0.0017
222_10d RS	Bl8	0.125	0.125	0.016	0.063	0.0035
LISTU	Bl9	0.125	0.125	0.016	0.063	0.0017
HEAVY	Bl10	0.125	0.25	0.032	0.063	0.0035
PotiB2	Bl11	0.125	0.125	0.016	0.063	0.0017
PoHL1	Bl12	0.125	0.125	0.016	0.063	0.0017
32_12b RS	Ba1	1	1	0.016	0.063	0.0069
230_13c RS	Ba2	2	1	0.016	0.063	0.055
164_11a RS	Ba3	2	1	0.016	0.063	0.0017
168_11c RS	Ba4	2	0.125	0.016	0.125	0.0138
235_13cd RS	Ba5	0.5	1	0.016	0.125	0.0138
232_13b RS	Ba6	0.125	0.125	0.016	0.125	0.0017
168_11g RS	Ba7	2	0.125	0.032	0.125	0.0035
163_11i RS	Ba8	0.125	0.5	0.016	0.125	0.0138
226_10a RS	Bg1	0.5	0.25	0.032	0.063	0.0017
160_13e RS	Bg2	2	0.125	0.032	0.063	0.0275
164_11g RS	Bg3	2	0.125	0.016	0.125	0.0275
224_10b RS	Bv1	0.25	0.25	0.016	0.063	0.0017
164_12b RS	Bv2	0.125	0.125	0.016	0.063	0.0035

Note: Borrelia lusitaniae–Bl, *Borrelia afzelii*–Ba, *Borrelia garinii*–Bg, *Borrelia valaisiana*–Bv.

was determined as the most dominant species in *I. ricinus* ticks in Serbia [11, 12] and taking in mind still unclear pathogenic potential of this species [1] and lack of *in vitro* antimicrobial testing studies for this species, four additional external *B. lusitaniae* strains were included (two isolated from *I. ricinus* from Spain, one from *I. ricinus* from Portugal, and one from human skin from Portugal) to get comprehensive insight in susceptibility of diverse strains to antimicrobial agents.

As with other bacterial diseases, one of the prerequisites for a successful clinical treatment of LB is *in vitro* susceptibility of the pathogen to antimicrobial agents used for therapy. Previous reports on the *in vitro* susceptibility of different *Borrelia* species to antimicrobial agents [16–20, 23, 27, 30–32] are based on the analysis of the limited number of strains and different test conditions. In this study, we analysed an extensive number of different *B. burgdorferi* s.l. isolates under uniform test conditions to six antimicrobial agents.

The broth microdilution method is considered as the gold standard for MIC determination [33]. Published *in vitro* susceptibility data on MICs are often difficult to compare because there are many differences in test conditions and the determination of MICs. These include broth microdilution (colorimetric and microscopic) and macrodilution methods for determination of MICs using various media, inoculum concentrations, different incubation period and endpoint determination, various numbers of isolates (usually small number), origin of isolates (strains isolated from different human materials and ticks), etc. [16–20, 30]. A possible interaction between test medium and antimicrobial agents, preparation and storage of antimicrobial agents and chemical instability of some antimicrobial agents during incubation period may affect *in vitro* testing of *B. burgdorferi* s.l. to antimicrobial agents [30, 33, 34]. All the above mentioned and differences in study design have led to different definitions of MIC and a wide MIC range of antimicrobial agents.

Following uniform test conditions for extensive number of different borrelia strains and criteria determined for this study (the density of the inoculum 10^5 cells per mL, BSK–H medium and cultivation time of 3 days for MIC) and using the microscopic method, the MICs indicated that all tested strains were susceptible to all antimicrobial agents commonly used for the treatment of LB (except to amikacin) according to breakpoints for commonly prescribed antimicrobial agents against other bacteria species described by Jorgensen and Turnidge [29] (Table 1). The results showed that *B. afzelii*, *B. garinii*, *B. lusitaniae* and *B. valaisiana* species have a different reaction to antimicrobial agents usually used in the treatment of LB (Table 1) and that slight but significant different reaction to given antimicrobial agents (based on median MIC values) also exist within one species (Table 2).

The MICs of amoxicillin, doxycycline, ceftriaxone, cefuroxime and azithromycin for eight *B. afzelii*, three *B. garinii* and two *B. valaisiana* isolates (Table 1) were mostly in agreement with those obtained by other authors using colorimetric and microscopic method, similar final inocula, and incubation period [16–19, 21, 22]. However, the MIC results of our study contradict findings of Hunfeld and colleagues [16]. The authors used colorimetric methods for determination of MICs and described susceptibilities of *B. garinii* human isolates to amoxicillin and penicillin were higher than those of *B. afzelii* human isolates and *B. valaisiana* tick isolate. Based on our results, we found no statistically significant differences between MICs of amoxicillin and azithromycin for *B. afzelii* and *B. garinii* isolates, respectively ($P > 0.05$), while the MICs of *B. valaisiana* and *B. lusitaniae* were lower than MICs for the other two species ($P < 0.05$) (Table 1), indicating that species with proven pathogenicity are less susceptible to antimicrobial agents than potentially pathogenic species.

Macrolides such as azithromycin are considered less effective in the treatment of erythema migrans than doxycycline and beta-lactams (amoxicillin and cefuroxime) and are consequently used as second-line drugs [1]. Our observation on equal *in vitro* efficiency of azithromycin and amoxicillin to *B. afzelii* and *B. garinii*, the major pathogenic species in Europe, agrees with the clinical study by Arnež and Ružić–Sabljić [35] on equal efficiency of these two antimicrobial agents in therapy solitary erythema migrans in children.

In our study the MICs of doxycycline for *B. afzelii* were higher ($P < 0.05$) than those for *B. garinii*, *B. lusitaniae* and *B. valaisiana* ($P < 0.05$). Sicklinger and colleagues [18] described higher susceptibility of *B. garinii* to azithromycin and no statistically differences to doxycycline for *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s., while Preac–Mursic and colleagues [23] tested clinical isolates and also found susceptibility of *B. garinii* to amoxicillin, doxycycline, ceftriaxone and azithromycin that were higher than those of *B. afzelii*. Our study suggested that analysed *B. afzelii* and *B. garinii* tick isolates were equally susceptible to all tested antimicrobial agents except to doxycycline (*B. garinii* isolates were more susceptible than *B. afzelii* isolates).

The only data available for *in vitro* susceptibility of *B. lusitaniae* isolates to antimicrobial agents are based on the colorimetric analysis of two strains isolated from ticks [32]. The MIC range of 0.016–0.032 mg/L of ceftriaxone and 0.125–0.25 mg/L of doxycycline obtained in our study for 12 *B. lusitaniae* strains were close to previously reported values, 0.03–0.06 mg/L of ceftriaxone and 0.125–0.5 mg/L of doxycycline [32]. These two drugs were the only antimicrobial agents tested by Ates and colleagues [32] so MICs for amoxicillin, cefuroxime, azithromycin and amikacin obtained in our study are firstly reported and therefore couldn't be compared.

We found no statistically significant differences between MICs of ceftriaxone and cefuroxime for *B. afzelii*, *B. garinii*, *B. lusitaniae* and *B. valaisiana* isolates, respectively ($P > 0.05$) (Table 1) and our results for *B. afzelii*, *B. garinii*, and *B. valaisiana* are in accordance with those of Hunfeld and colleagues [31].

Findings on the existence of the intraspecies differences in antimicrobial agent's susceptibility are in accordance with

previously published data [22, 23]. We observed intraspecies differences in susceptibility for tested borrelia strains (Table 2), except for the median MICs of ceftriaxone for *B. afzelii*, *B. garinii* and *B. lusitaniae* isolates, respectively ($P > 0.05$), cefuroxime for *B. afzelii* and *B. garinii* ($P > 0.05$), and doxycycline for *B. garinii* and *B. lusitaniae* ($P > 0.05$). There was no difference in susceptibility among two tested *B. valaisiana* isolates to antimicrobial agents. The greatest variations in the median MICs were of azithromycin for *B. afzelii* isolates (Table 2) while the MIC range of azithromycin (0.055–0.11 mg/L) for *B. afzelii* isolate (Ba2) (Table 1) was higher than MICs previously published (a total MIC range of 0.0004–0.03 mg/L) [16, 19, 22] indicating that this isolate may be less susceptible to azithromycin than previously tested European *B. afzelii* human isolates. The MIC range of azithromycin for Ba2 isolate is close to the MIC range (0.027–0.22 mg/L) of azithromycin for *B. burgdorferi* s.s. human isolates [20], the species considered more aggressive, more virulent, with hematogenous dissemination more frequent than *B. afzelii* or *B. garinii* [36].

There was no difference in susceptibility among tested *B. lusitainae* isolates to antimicrobial agents, except for Bl2 which was less susceptible to amoxicillin, cefuroxime, and azithromycin (higher median MICs values) than other *B. lusitanie* strains (Table 2). MIC range (0.5–1 mg/L of amoxicillin for Bl2 was close to MIC range (0.5–4 mg/L) for *B. afzelii* isolated from human samples [16, 17, 19] and higher than MIC range (0.05–0.4 mg/L) for *B. garinii* isolated also from human samples [16, 17]. MIC range (0.0275–0.055 mg/L) of azithromycin for Bl2 was close to the MIC range (0.027–0.22 mg/L) for *B. burgdorferi* s.s. isolated from human samples [20]; primarily was close to MIC range (0.022–0.11 mg/L) for *B. burgdorferi* s.s. isolated from the skin and to MIC range (0.055–0.11 mg/L) for *B. burgdorferi* s.s. isolated from cerebrospinal fluid [20]. The MIC range (0.5–1 mg/L) of cefuroxime for Bl2 was higher than MIC ranges of cefuroxime for other *Borrelia* isolates tested in this study (Table 1). With such interestingly high MIC values of amoxicillin, cefuroxime and azithromycin for Bl2 isolates (Tables 1 and 2) we can't rule out possibility that some strains of potentially pathogenic species *B. lusitaniae* circulating in this region perhaps have similar pathogenic potential and similar susceptibility to antimicrobial agents as human pathogenic *B. burgdorferi* s.s. strains previously described by Veinović and colleagues [20], and that these antimicrobial agents are less effective against some local strains of potentially pathogenic species *B. lusitaniae*.

CONCLUSIONS

According to the MIC findings of our study, all tick isolates of *B. burgdorferi* s.l. from Serbia, Spain and Portugal and one *B. lusitaniae* isolate from human skin from Portugal, were susceptible to antimicrobial agents usually used for the treatment of patients with LB. We have shown the existence of interspecies and intraspecies differences in susceptibility to antimicrobial agents *in vitro*. The existence of differences in the MICs of some antimicrobial agents could be attributed to natural characteristics of borrelia strains and associated with a high diversity of borrelia strains in ticks in Serbia. With high MICs of amoxicillin, cefuroxime and azithromycin for one local *B. lusitaniae* strain, we have noticed less efficacy of these antimicrobial agents for this potentially pathogenic species than for other *B. lusitaniae* strains. This study is the first report on *in vitro* susceptibility of local isolates of *Borrelia* from Serbia to antimicrobial agents and the first report on *in vitro* susceptibility of a larger number of isolates of potentially pathogenic species *B. lusitaniae*. Based on breakpoints for antimicrobial agents against other bacteria species we showed that antimicrobial agents usually used in the treatment of LB are effective against local borrelia strains isolated from ticks, indicating potential of their equally beneficial use in the clinical practice. However further work based on human isolates is needed to confirm our results.

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