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SUSCEPTIBILITY

In Vitro Antimicrobial Susceptibility of Clinical Isolates of *Borrelia miyamotoi*

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ABSTRACT *Borrelia miyamotoi* is an emerging relapsing fever (RF) *Borrelia* species that is reported to cause human disease in regions in which Lyme borreliosis is endemic. We recently showed that *B. miyamotoi* tick isolates are resistant to amoxicillin *in vitro*; however, clinical isolates have not been studied. Therefore, our aim was to show the antimicrobial susceptibility of recently obtained clinical isolates of *B. miyamotoi*. A dilution series of various antibiotics was made in modified Kelly-Pettenkofer medium with 10% fetal calf serum. The susceptibilities of different *B. miyamotoi* clinical, *B. miyamotoi* tick, RF *Borrelia*, and *Borrelia burgdorferi sensu lato* isolates were tested by measuring MICs through colorimetric changes and by counting motile spirochetes by dark-field microscopy after 72 h of incubation. The ceftriaxone and azithromycin MIC ranges of the six *B. miyamotoi* clinical isolates tested were 0.03 to 0.06 mg/liter and 0.0016 to 0.0032 mg/liter, respectively. These values are similar to MICs for RF *Borrelia* strains and *B. miyamotoi* tick isolates. All tested RF *Borrelia* strains were susceptible to doxycycline (microscopic MIC range, 0.0625 to 0.25 mg/liter). In contrast to the MICs of the tested *B. burgdorferi sensu lato* strains and in line with our previous findings, the amoxicillin MICs (range, 8 to 32 mg/liter) of all RF *Borrelia* strains, including *B. miyamotoi* clinical isolates, were above the clinical breakpoint for resistance (≤4 mg/liter). Clinical isolates of *B. miyamotoi* are highly susceptible to doxycycline, azithromycin, and ceftriaxone *in vitro*. Interestingly, as described previously for tick isolates, amoxicillin shows poor *in vitro* activity against *B. miyamotoi* clinical isolates.

KEYWORDS hard-tick-borne relapsing fever, relapsing fever *Borrelia*, *Borrelia miyamotoi* disease, *Borrelia miyamotoi*, antibiotic susceptibility, antimicrobials

Hard-bodied *Ixodes* ticks are found across the Northern Hemisphere and are important vectors of pathogenic viruses, bacteria, and parasites (1, 2). They can lead to well-established tick-borne diseases, such as anaplasmosis, tick-borne encephalitis, and Lyme borreliosis (caused by *Borrelia burgdorferi sensu lato*), and also diseases caused by newly discovered pathogens such as *Borrelia miyamotoi* (3–8). Phylogenetically, *B. miyamotoi* clusters with relapsing fever (RF) *Borrelia* species, such as *Borrelia hermsii* and *Borrelia recurrentis*, which, unlike *B. miyamotoi*, are commonly transmitted by soft ticks or body lice (9).

Human infection with *B. miyamotoi* has recently been observed in Russia, the United States, and Japan, causing fever accompanied by nonspecific symptoms in immunocompetent patients (3, 4). Three immunocompromised patients, from the Netherlands, Germany, and the United States, were diagnosed with meningoencephalitis caused by *B. miyamotoi* (5, 6, 8). Due to lack of experimental data, *B. miyamotoi* disease (BMD) is empirically treated based on experiences with treatment of other RF *Borrelia* species

Received 7 March 2018 **Accepted** 11 April 2018

Accepted manuscript posted online 16 April 2018

Citation Koetsveld J, Manger A, Hoornstra D, Draga RO, Oei A, Kolyasnikova NM, Toporkova MG, Sarksyan DS, Wagemakers A, Platonov AE, Hovius JW. 2018. *In vitro* antimicrobial susceptibility of clinical isolates of *Borrelia miyamotoi*. Antimicrob Agents Chemother 62:e00419-18. https://doi.org/10.1128/AAC.00419-18.

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TABLE 1 MICs of all tested antibiotics for each *Borrelia* strain

Antimicrobial agent and MIC type	MIC range (mg/liter)[a]								
	PKo	HS1	HT31	Izh-4	Izh-5	Izh-14	Izh-16	Yek-1	Yek-6
Amoxicillin									
MIC_c	4	8	8	8	16	8	8	16	16
MIC_m	4	8–16[b]	16[b]	8–16[b]	16–32[b]	8[b]	8–16[b]	16[b]	16[b]
Doxycycline									
MIC_c	4	0.25	0.125	0.25	0.125	0.0625	0.125	0.125	0.125
MIC_m	4	0.25[c]	0.0625[c]	0.25[c]	0.125–0.25[c]	0.125–0.25[c]	0.25[c]	0.25[c]	0.25[c]
Ceftriaxone									
MIC_c	0.125	0.06	0.03	0.06	0.06	0.03	0.03	0.06	0.06
MIC_m	0.125	0.125	0.03	0.03–0.06	0.06–0.125	0.03–0.06	0.03	0.03–0.06	0.03–0.06
Azithromycin									
MIC_c	0.0032	0.0032	NR	0.0016	0.0016	0.0016	0.0016	0.0032	0.0032
MIC_m	0.0064	0.0064	0.0032–0.0064	0.0016	0.0016–0.0032	0.0016	0.0016–0.0032	0.0032	0.0032

[a]MIC ranges are the results of tests conducted in quadruplicate. PKo, *B. afzelii* (*B. burgdorferi sensu lato* control); HS1, *B. hermsii* (RF *Borrelia* control); HT31, *B. miyamotoi* laboratory strain (*B. miyamotoi* control); Izh-4, Izh-5, Izh-14, Izh-16, Yek-1, and Yek-6 (*B. miyamotoi* clinical isolates). NR, not reached.
[b]Significantly ($P < 0.05$) higher from the amoxicillin MIC_m found for PKo.
[c]Significantly ($P < 0.05$) lower from the doxycycline MIC_m found for PKo.

and guidelines for treatment of Lyme borreliosis, and only minor treatment failures have been reported (3–6, 8, 10). Of interest, a recent case of BMD in the United States was described as showing full recovery without antibiotics (11).

We recently described the *in vitro* susceptibility of two tick isolates of *B. miyamotoi* (12). We found that *B. miyamotoi* tick isolates were susceptible to azithromycin, doxycycline, and ceftriaxone but, unlike the tested *B. burgdorferi sensu lato* strains, were resistant to amoxicillin *in vitro* (12). Our aim was to improve the clinical importance of these findings by using six recently obtained clinical isolates of *B. miyamotoi* (13). We used two well-established and reproducible methods for determination of antimicrobial susceptibility (12, 14, 15), by measuring the MICs of these isolates for the antibiotics most commonly used in the treatment of Lyme and RF borreliosis (10). This study offers further insights in options for antimicrobial therapy in BMD.

RESULTS

Antimicrobial susceptibility was determined as the colorimetric MIC (MIC_c) and microscopic MIC (MIC_m) of each *Borrelia* strain for all tested antibiotics (Table 1). MIC_c values were equal to or within 2 dilutions (2-fold) of the MIC_m values. Clinical isolates of *B. miyamotoi* showed similar susceptibilities to ceftriaxone and azithromycin, compared to *B. burgdorferi sensu lato*, RF *Borrelia*, and *B. miyamotoi* control strains (Table 1). Susceptibilities to doxycycline were similar (within 2 dilutions [2-fold]) for RF *Borrelia* strains, *B. miyamotoi* tick isolates, and *B. miyamotoi* clinical isolates (MIC_m range, 0.0625 to 0.25 mg/liter). However, the MIC_m values of all tested RF *Borrelia* strains were significantly lower ($P = 0.029$) than those of the *B. burgdorferi sensu lato* control (PKo) (Table 1). Interestingly, the amoxicillin MIC_m values of both *B. miyamotoi* tick isolates, all *B. miyamotoi* clinical isolates, and the *B. hermsii* control strain were significantly higher than the clinical breakpoint for resistance (≤4 mg/liter, according to the Clinical and Laboratory Standards Institute [CLSI]) (15, 16). In line with our previous findings, the MIC_m value of *Borrelia afzelii* strain PKo was 4 mg/liter, thus significantly lower ($P = 0.029$) than the MIC_m values of all tested RF *Borrelia* strains (Table 1). This difference was also observed in colorimetric assays testing the antibiotic susceptibility of *B. burgdorferi* strain N40 and *B. afzelii* strain PKo versus *B. hermsii* strain HS-1 and two *B. miyamotoi* clinical isolates (Fig. 1). In line with these findings, the MIC_m value of *B. burgdorferi* strain N40 was 1 mg/ml, significantly lower ($P = 0.029$) than those of all tested RF *Borrelia* strains (data not shown).

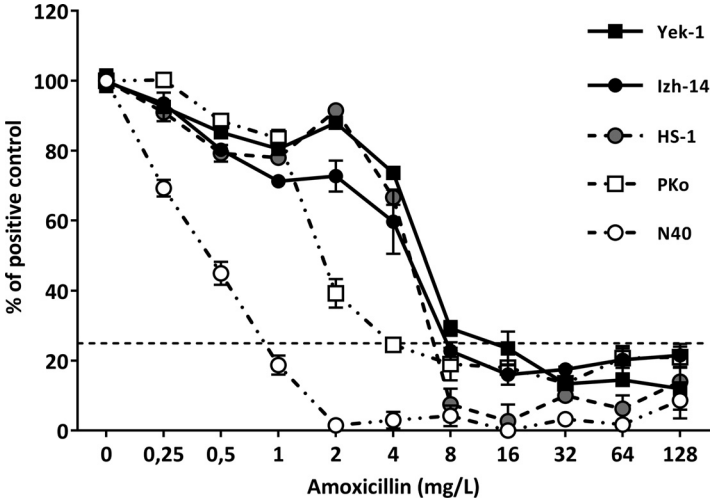

FIG 1 Decrease in absorbance after incubation with amoxicillin. Absorbance was measured at 562 (corrected for absorbance at 630 nm), and the decrease in absorbance was measured after 72 h of incubation with amoxicillin and compared with the decrease in absorbance of the positive-control sample ($[E_{t0} - E_{t72}]$ of <25% of $[E_{POS,t0} - E_{POS,t72}]$). Shown are representative results of two individual experiments with five representative *Borrelia* strains, i.e., PKo (*B. afzelii*), N40 (*B. burgdorferi sensu stricto*), HS1 (*B. hermsii* from the United States), and Izh-14 and Yek-1 (*B. miyamotoi* clinical isolates). Symbols and error bars represent the mean ± standard error of the mean of quadruplicate results. If no error bars are visible, then the error bars were smaller than the symbol.

DISCUSSION

BMD is an emerging infectious disease and, since cultivation is cumbersome and has only recently been described (17), experimental studies on *B. miyamotoi* are scarce. We combined previous experience with cultivation and antimicrobial susceptibility testing of *Borrelia* with a unique set of recently isolated *B. miyamotoi* clinical strains (12, 13, 17). By measuring acidification as a result of *Borrelia* growth, using colorimetry, we were able to simultaneously test MIC_c values for several clinical isolates and control strains in single experiments and thus reliably compare results. Colorimetry is a reproducible method to approximate the antimicrobial susceptibility of *Borrelia* spirochetes (12). We subsequently confirmed susceptibilities by enumerating motile spirochetes near the MIC_c by dark-field microscopy (MIC_m).

We were able to confirm our previous findings on the antibiotic susceptibility of *B. miyamotoi* tick isolates and demonstrated *in vitro* susceptibility of *B. miyamotoi* clinical isolates to azithromycin, doxycycline, and ceftriaxone. Importantly, *in vitro* resistance to amoxicillin was observed for *B. miyamotoi* clinical isolates, confirming the previously observed resistance of tick isolates (12). It is known that the efficacy of β-lactam antibiotics, including amoxicillin, is temperature dependent (18). Therefore, the fact that the amoxicillin MIC_m found for RF *Borrelia* is over the clinical breakpoint for resistance could be partially attributed to experimental conditions. The experimental conditions, however, cannot explain the relative difference in susceptibilities between RF *Borrelia* and the tested *B. burgdorferi sensu lato* strains.

Current treatment of BMD is based mainly on guidelines for treatment of Lyme borreliosis. Supported by successful treatment described in the literature (3, 5, 7, 19), our results confirm doxycycline, ceftriaxone, and azithromycin as treatment options for BMD. The doxycycline MIC_c values we report for *B. burgdorferi sensu lato* and RF *Borrelia* are comparable to MICs found in the literature (12). Interestingly, the significantly lower doxycycline MICs of *B. miyamotoi* clinical isolates, compared to *B. burgdorferi sensu lato* isolates (Table 1), might suggest that the therapeutic dose of doxycycline for BMD could be decreased, to avoid side effects; however, this would require additional *in vivo* studies and clinical trials.

The low *in vitro* activity of amoxicillin against clinical isolates of *B. miyamotoi*, which

TABLE 2 Detailed information on tested antimicrobial agents

Antimicrobial	Supplier[a]	Product no.	Dilution range (mg/liter)	Breakpoint (mg/liter)[b]	Stock concn (mg/ml) (solvent)[c]
Amoxicillin	Centrafarm	14029596	0.25–128	≤4	8.89 (PBS)
Doxycycline	Sigma	D3447	0.016–8	≤4	44.4 (DMSO)
Ceftriaxone	Sigma	PHR1382	0.016–8	≤8	8.89 (PBS)
Azithromycin	Sigma	PHR1088	0.0004–0.2048	≤2	5.56 (DMSO)

[a]The suppliers were Centrafarm (Etten-Leur, The Netherlands) and Sigma-Aldrich (St. Louis, MO, USA).
[b]Breakpoints are according CLSI guidelines (16).
[c]PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide.

is in line with our previous findings (12), might suggest that amoxicillin is not a preferred antibiotic for the treatment of BMD. While *in vitro* results by themselves do not allow us to draw conclusions regarding the clinical efficacy of the tested antimicrobial agents, they reveal the need for further investigation in animal models and clinical settings.

MATERIALS AND METHODS

***Borrelia* strains.** The *Borrelia* control strains included were PKo (*B. afzelii*; a skin isolate from Germany), N40 (*B. burgdorferi sensu stricto*; a tick isolate from the United States), HT31 (*B. miyamotoi*; a tick isolate from Japan), and HS1 (*B. hermsii*; a tick isolate from the United States). The *B. miyamotoi* clinical isolates used in this study were obtained through cultivation of pelleted plasma from patients with PCR-proven *B. miyamotoi* disease, in Izhevsk, Russia, and Yekaterinburg, Russia. Isolation of these strains was described previously (13), and they were designated Izh-4, Izh-5, Izh-14, Izh-16, Yek-1, and Yek-6.

***Borrelia* cultures.** *Borrelia* glycerol stocks (<5 passages) were thawed from −80°C and cultured at 33°C in modified Kelly-Pettenkofer (MKP) medium (for *B. burgdorferi sensu lato*) or MKP medium with the addition of 10% fetal calf serum (MKP-F medium) (for RF *Borrelia*), in a regular incubator (Memmert, Schwabach, Germany), as described previously (17). Spirochete concentrations were determined using a Petroff-Hausser counting chamber and dark-field microscopy.

Antimicrobial agents. The antibiotics tested were the antibiotics suggested for the treatment of Lyme borreliosis, i.e., doxycycline, amoxicillin, azithromycin, and ceftriaxone (10). Detailed information on each antibiotic is shown in Table 2.

Experimental design. The experimental design was described previously (12). Spirochetes were cultured for 4 days to reach the mid-log phase of growth. For all *Borrelia* isolates (including *B. burgdorferi sensu lato*), MKP-F medium containing phenol red (25 mg/liter) was preincubated for 96 h at 33°C in 50-ml Falcon tubes with 30% air, to circumvent nonspecific color shifts (12). Medium containing spirochetes was centrifuged at 4,500 × *g* for 45 min at room temperature, and spirochetes were suspended in the preincubated MKP-F medium. Spirochetes were enumerated and adjusted to 5×10^7 spirochetes/ml. A 10-step, 2-fold dilution series of each antimicrobial agent was made in MKP-F medium containing phenol red; 180 μl of each antibiotic concentration was added to 96-well, flat-bottom, polystyrene microtiter trays (product no. M0687; Greiner), in quadruplicate, and 20 μl of spirochete suspension was added to each well for a final concentration of 5×10^6 spirochetes/ml. The microtiter plates were sealed with adhesive plastic and incubated at 33°C for 72 h.

MIC measurements. Colorimetric changes through acidification of MKP-F medium by expansion of viable *Borrelia* spirochetes were used to determine antimicrobial susceptibility, as described previously (12). In short, absorbance values at 562 nm (corrected for absorbance at 630 nm) were measured at 0 and 72 h using a commercially available enzyme-linked immunosorbent assay (ELISA) reader (PowerWave 200; BioTek Instruments). Colorimetric changes were calculated by comparing the absorbance at 72 h (E_{t72}) and the initial absorbance (E_{t0}) for each well ($E_{t0} - E_{t72}$), corrected for the change in absorbance of the negative-control sample (MKP-F medium without *Borrelia*). The colorimetric MIC (MIC_c) was calculated by comparing the decrease in absorbance to the decrease in absorbance of the positive-control sample (no antibiotics). MIC_c was defined as the lowest concentration of antibiotic at which the average decrease in absorbance (of four replicates) was <25% of the decrease in absorbance found for the positive-control sample ($[E_{t0} - E_{t72}]$ of <25% of $[E_{POS,t0} - E_{POS,t72}]$). Findings were confirmed by dark-field microscopy; well contents were resuspended, and 5 μl of each well sample at the MIC_c was transferred to a microscopy slide and covered by a coverslip. The microscopic MIC (MIC_m) was defined as the lowest concentration of antimicrobial agent at which no motile spirochetes were observed by dark-field microscopy, as described previously (12). In cases in which motile spirochetes were observed, one antibiotic concentration above the MIC_c was also tested, until no motile spirochetes were observed in any of the four replicate wells. The lower detection limit using this method was 4×10^4 spirochetes/ml (12).

Statistical analyses. Data analyses were performed using PASW Statistics 19.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7.0.2 (GraphPad Software Inc., La Jolla, CA, USA). Nonparametric Mann-Whitney tests were used to calculate the significance between MIC_m results from different tested strains.

ACKNOWLEDGMENTS

We thank Barbara Johnson and Volker Fingerle for providing *B. miyamotoi* strain HT31.

The Ticking on Pandora's Box project by J.W.H. and D.H. is funded by ZonMW (project 50-52200-98-313). This study was also supported by a grant from the Russian Science Foundation (project 15-15-00072) to N.M.K., D.S.S., M.G.T., and A.E.P.

We have no conflicts of interest to disclose.

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