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In Vitro Susceptibility of the Relapsing-Fever Spirochete *Borrelia miyamotoi* to Antimicrobial Agents

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ABSTRACT Hard-tick-borne relapsing fever (HTBRF) is an emerging infectious disease throughout the temperate zone caused by the relapsing-fever spirochete *Borrelia miyamotoi*. Antibiotic treatment of HTBRF is empirically based on the treatment of Lyme borreliosis; however, the antibiotic susceptibility of *B. miyamotoi* has not been studied to date. Thus, we set out to determine the *in vitro* antimicrobial susceptibility of *B. miyamotoi*. A microdilution method with 96-well microtiter plates was used to determine the antibiotic susceptibilities of two *B. miyamotoi* strains isolated on two different continents (Asia and North America), two *Borrelia burgdorferi sensu lato* strains, and one *Borrelia hermsii* isolate for purposes of comparison. The MIC and minimal bactericidal concentration (MBC) were determined by both microscopy and colorimetric assays. We were able to show that relative to the *B. burgdorferi sensu lato* isolates, both *B. miyamotoi* strains and *B. hermsii* demonstrated greater susceptibility to doxycycline and azithromycin, equal susceptibility to ceftriaxone, and resistance to amoxicillin *in vitro*. The MIC and MBC of amoxicillin for *B. miyamotoi* evaluated by microscopy were 16 to 32 mg/liter and 32 to 128 mg/liter, respectively. Since *B. miyamotoi* is susceptible to doxycycline, azithromycin, and ceftriaxone *in vitro*, our data suggest that these antibiotics can be used for the treatment of HTBRF. Oral amoxicillin is currently used as an alternative for the treatment of HTBRF; however, since we found that the *B. miyamotoi* strains tested were resistant to amoxicillin *in vitro*, this issue warrants further study.

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

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The relapsing-fever spirochete *Borrelia miyamotoi* was first described in Japan in 1995 (1). While it is phylogenetically closely related to other relapsing-fever (RF) spirochetes, such as *Borrelia hermsii*, *Borrelia turicatae* (transmitted by soft ticks), and *Borrelia recurrentis* (transmitted by body lice), it is transmitted by hard-bodied *Ixodes* ticks across the temperate zone (2). *Ixodes* ticks concomitantly transmit spirochetes belonging to the *Borrelia burgdorferi sensu lato* group, which are known to cause Lyme borreliosis (3). The incidence of *B. miyamotoi* in *Ixodes* ticks is lower than that of *B. burgdorferi sensu lato*, with infection rates ranging from 0 to 15.4% in the United States and as high as 4% in Europe and Japan (2, 4, 5). RF *Borrelia* spirochetes cause a variety of diseases, which are characterized by episodes of high fever separated by periods of relative well-being. The clinical presentation of disease caused by *B. miyamotoi*, however, appears to differ from that of classical RF, since relapsing-fever episodes have been observed only in 10% of patients infected with *B. miyamotoi*, and their levels of spirochetemia are calculated to be low (6, 7). Since most patients with *B. miyamotoi* infection are usually treated with antibiotics, this might be an underestimation of the naturally occurring relapses. The disease caused by *B. miyamotoi* is therefore consid-

ered a separate clinical entity and has been designated both *Borrelia miyamotoi* disease (BMD) (7) and hard-tick-borne relapsing fever (HTBRF) (8). Clinical cases of HTBRF were first described in Russia in 2011 (6), followed by cases in the United States and Japan (9–11). On average, fever episodes last for 3 days and are accompanied by flu-like symptoms, such as headache, chills, abdominal discomfort, arthralgia, and myalgia. Meningoencephalitis caused by *B. miyamotoi* has been described in three patients receiving B-cell-depleting therapy in the Netherlands, Germany, and the United States (9, 10, 12).

Treatment of HTBRF is currently empirically based on standard regimens used for the treatment of Lyme borreliosis, although no clinical guidelines exist. The antimicrobial susceptibility of *B. miyamotoi* has not yet been elucidated, due to difficulties with the cultivation of *B. miyamotoi* spirochetes *in vitro*, which has been reported only recently (13–15). In contrast, the *in vitro* susceptibilities of the causative agents of Lyme borreliosis and several RF *Borrelia* spirochetes have been extensively studied (16–19). The antimicrobial susceptibility of spirochetes is conventionally shown as MICs—measured either by direct (dark-field) microscopy or by colorimetric assays—or measurement of the minimal bactericidal concentrations (MBCs) after 72 h of exposure to antibiotics (18, 20, 21). As part of the current study, we aimed to determine the *in vitro* antibiotic susceptibility of *B. miyamotoi* in comparison to those of *B. burgdorferi sensu lato* and *B. hermsii*, by determining the MICs and MBCs of the antibiotics most commonly used in the treatment of Lyme borreliosis by both dark-field microscopy and colorimetric assays. This is important because it will help guide future antibiotic treatment of HTBRF.

RESULTS

The antibiotic susceptibilities of all *Borrelia* strains tested, expressed as MICs determined by colorimetric assays and as MICs and MBCs determined by dark-field microscopy, are shown in Tables 1 and 2. In short, the colorimetric MIC_1 and MIC_2 were similar to the microscopic MICs or one to three 2-fold dilutions lower. Figure 1 shows, as an example, the correlation between microscopy and the drop in absorbance (compared to that for the positive control) with doxycycline. The correlation between normalized MIC_2 values and microscopy results is shown in Fig. S2 in the supplemental material. The MICs of the various antimicrobials for the *B. burgdorferi sensu lato* strains and for the RF *Borrelia* strains were comparable (within two 2-fold dilutions of each other), with the exception of amikacin, which had a MIC for *B. miyamotoi* LB-2001 (512 to 1,024 mg/liter) that was much higher than the MICs for *B. miyamotoi* HT31 and *B. hermsii* HS1 (32 to 64 mg/liter). As expected, all *Borrelia* strains tested were resistant to amikacin. In addition, both *B. miyamotoi* strains demonstrated greater sensitivity to doxycycline and azithromycin, equal susceptibility to ceftriaxone, and relative resistance to amoxicillin compared to *B. burgdorferi sensu lato* strains (Table 1). In contrast to those for both *B. burgdorferi sensu lato* strains tested, the amoxicillin MICs for *B. miyamotoi* isolates HT31 and LB-2001, and for *B. hermsii* HS1, were above the clinical breakpoint of $\leq$4 mg/liter, indicating that the RF *Borrelia* strains tested should be regarded as resistant to amoxicillin *in vitro*. To exclude the possibility that this difference was due to the addition of fetal calf serum (FCS) to modified Kelly-Pettenkofer (MKP) medium, which is needed for relapsing-fever *Borrelia* strains, we tested the amoxicillin MIC using *Borrelia afzelii* (PKo) in both MKP medium alone and MKP medium supplemented with 10% heat-inactivated FCS (MKP-F medium), compared to *B. hermsii* (HS1) in MKP-F medium. We showed that there was no difference in the susceptibility of PKo to amoxicillin, as determined by microscopy (data not shown) and colorimetry (see Fig. S1 in the supplemental material), whether it was tested in MKP or MKP-F medium. Furthermore, the difference in the amoxicillin MIC between PKo and *B. hermsii* HS1 was also observed when MKP-F medium was used. The MBCs of all antimicrobial agents were one to two dilution steps above the microscopic MICs except for amikacin, for which the MICs and MBCs were identical (Table 2). In some cases, growth was observed

TABLE 1 MICs of different antimicrobial agents for selected *Borrelia burgdorferi sensu lato* and relapsing-fever *Borrelia* strains[a]

Antimicrobial and method	MIC range[b] (mg/liter)				
	B. burgdorferi sensu lato		Relapsing-fever *Borrelia* strain		
	PKo	B31	HT31	LB-2001	HS1
Amoxicillin					
Microscopy	4	1–2	32	16–32	16–32
MIC_1	2	0.5	8	8	8
MIC_2	2	2	16	8	8
Doxycycline					
Microscopy	2	2	0.5	0.125–0.25	0.5
MIC_1	1	0.125	0.25	0.0625	0.25
MIC_2	1	0.5	0.5	0.125	0.25
Ceftriaxone					
Microscopy	0.125	0.06–0.125	0.0625–0.25	0.25–0.5	0.25–0.5
MIC_1	0.0625	0.03125	0.03125	0.03125	0.125
MIC_2	0.0625	0.03125	0.0625	0.125	0.125
Azithromycin					
Microscopy	0.0128	0.0128	0.003–0.006	0.003	0.0128
MIC_1	0.0128	0.003	0.003	0.003	0.0128
MIC_2	0.0128	0.0256	0.003	0.003	0.006
Amikacin					
Microscopy	256	512	64	512	32–64
MIC_1	512	512	32	512	64
MIC_2	256	512	64	1,024	64

[a]PKo, *B. afzelii* reference strain; B31, *B. burgdorferi sensu stricto* reference strain; HT31, *B. miyamotoi* tick isolate (Japan); LB-2001, *B. miyamotoi* tick isolate (United States); HS1, *B. hermsii* reference strain.
[b]Ranges are results of tests that were conducted in quadruplicate.

at the highest available concentration; therefore, the exact MBC could not be determined and was marked as greater than the highest concentration tested.

DISCUSSION

***In vitro* antibiotic susceptibility.** In this study, we describe, to our knowledge for the first time, the *in vitro* susceptibilities of *B. miyamotoi* to the antibiotics most commonly used in the treatment of HTBRF and Lyme borreliosis. Microdilution-based methods have been extensively studied by others and have been shown to be robust methods for standardized testing of the antimicrobial susceptibility of *Borrelia* species (18, 20, 21). Using similar methods, we demonstrated that *B. miyamotoi* strains HT31 and LB-2001 are susceptible to azithromycin, doxycycline, and ceftriaxone but—compared to the *B. burgdorferi sensu lato* strains tested—resistant to amoxicillin *in vitro*.

TABLE 2 MBCs of different antimicrobial agents for selected *Borrelia burgdorferi sensu lato* and relapsing-fever *Borrelia* strains[a]

Antimicrobial	MBC range[b] (mg/liter)				
	B. burgdorferi sensu lato		Relapsing-fever *Borrelia* strains		
	PKo	B31	HT31	LB-2001	HS1
Amoxicillin	16–32	64	128	32	>64
Doxycycline	8–16	>16	4	1–2	>4
Ceftriaxone	>0.5	0.25–>1	>1	1–2	2
Azithromycin	0.025	>0.05	>0.0125	0.0125	≥0.05
Amikacin	256–512	512	64	512	32–64

[a]MBCs, minimum bactericidal concentrations. PKo, *B. afzelii* reference strain; B31, *B. burgdorferi sensu stricto* reference strain; HT31, *B. miyamotoi* tick isolate (Japan); LB-2001, *B. miyamotoi* tick isolate (United States); HS1, *B. hermsii* reference strain.
[b]Ranges are results of tests that were conducted in quadruplicate.

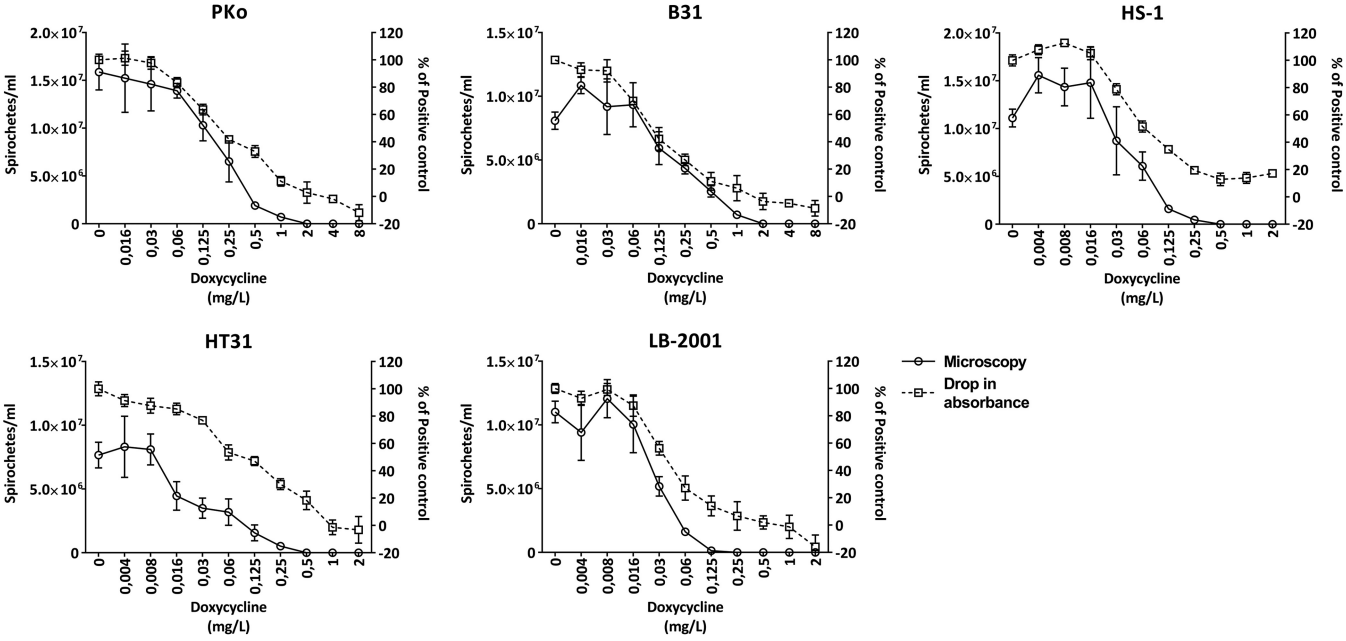

FIG 1 Correlation between microscopy and the drop in absorbance with doxycycline. Shown is the correlation between microscopy results and the drop in absorbance (A_{562}/A_{630})—as a percentage of that of the positive control (MIC_2)—after 72 h of incubation with a medium containing a 2-fold dilution series of doxycycline. The microscopy results, expressed as the number of spirochetes per milliliter, are given along the left *y* axis. The drop in absorbance, as a percentage of the decrease in absorbance of the positive control, is given along the right *y* axis and was calculated as $[(E_{t0} - E_{t72})/(E_{POS,t0} - E_{POS,t72})] \times 100$. Symbols and error bars represent means ± standard deviations. PKo, *B. afzelii* reference strain; B31, *B. burgdorferi sensu stricto* reference strain; HS1, *B. hermsii* reference strain; HT31, *B. miyamotoi* tick isolate from Japan; LB-2001, *B. miyamotoi* tick isolate from the United States.

Although multiple pharmacokinetic and dynamic features are important for the efficacy of an antibiotic to eradicate *B. miyamotoi*, it should be noted that the MICs of ceftriaxone, azithromycin, and doxycycline for *B. miyamotoi* isolates are well below the serum trough levels (22) that are reached by the dosages of these antibiotics recommended in international guidelines (23). In contrast, amoxicillin MICs for *B. miyamotoi* isolates (8 to 32 mg/liter) were comparable to, or higher than, the peak serum levels (8 to 10 mg/liter) reached by the dosage of amoxicillin recommended in international guidelines (22, 23). Since no clinical guidelines exist for the treatment of HTBRF, and clinical experience is limited, our *in vitro* findings are of great interest and warrant further study, including *in vivo* studies.

Of note, the susceptibilities of *B. afzelii* and *B. burgdorferi sensu stricto* isolates in this study to various antibiotics, including amoxicillin, were comparable to those determined in previous studies (Table 3) (17, 18, 24, 25). Also, the susceptibilities of *B. hermsii* and *B. miyamotoi* to doxycycline, ceftriaxone, and azithromycin that we report here are in line with previous studies, which showed similar susceptibilities of other RF *Borrelia* species to tetracyclines, cephalosporins, and macrolides (16, 19, 26–29). It should be mentioned that only a few studies have described the antimicrobial sensitivities of RF *Borrelia* spirochetes, such as the louse-borne relapsing-fever spirochete *B. recurrentis* and the soft-tick-borne relapsing-fever spirochetes *B. hermsii* and *B. turicatae* (16, 19, 26). Previous studies reported susceptibility of *B. hermsii*, *B. recurrentis*, and *B. turicatae* to antibiotics of the beta-lactam group (19, 26, 27); however, neither of these studies tested amoxicillin. Interestingly, one study did show that *B. hermsii* was less susceptible than *B. burgdorferi sensu stricto* to ampicillin (29), supporting our findings. It is known that the effect of beta-lactam antibiotics is temperature dependent, and previous studies revealed a 16-fold-lower efficacy of penicillin during incubation at 36°C than at 38°C and showed that loss of activity during incubation is attributable to chemical instability (30). This might suggest that the *in vitro* resistance of RF *Borrelia* spirochetes to amoxicillin that we describe here could be attributed partially to experimental conditions (17). However, the difference in susceptibility to amoxicillin between the RF

TABLE 3 MICs of different antimicrobial agents for *Borrelia burgdorferi sensu lato* in the literature and the current study

Study	Strain(s) (no.)	Method	MIC range (mg/liter)				
			Amoxicillin	Doxycycline	Ceftriaxone	Azithromycin	Amikacin
Hunfeld et al. (20)	Various	Review	0.03–2	0.06–2	<0.01–0.125	0.003–0.03	32–>128
Veinović et al. (18)	*B. burgdorferi sensu stricto* (9)	Microscopy	0.125–2	0.125–2	0.03–0.25	0.027–0.22	32–512
Ruzić-Sabljić et al. (24)	*B. afzelii* (10)	Microscopy	1–4	1–4	0.063–4,0	0.0138–0.0275	64–128
Baradaran-Dilmaghani and Stanek (16)	B31	Microscopy	0.2	0.4	0.013	0.006	ND[a]
Hunfeld et al. (21)	PKo and B31	Microscopy	0.125–0.5[b]	0.25	0.03	<0.01	ND
Boerner et al. (29)	B31 and N34	Microscopy	0.06–1[b]	0.125–2	0.03–0.06	<0.016–0.03	8–32[c]
This study	PKo and B31	Microscopy	1–4	2	0.06–0.125	0.0128	256–512
		Colorimetric assay	0.5–2	0.125–1	0.03–0.06	0.003–0.0256	256–512
Hunfeld et al. (21)	PKo and B31	Colorimetric assay	0.015–2[b]	0.06–1	<0.01–0.06	<0.01–0.06	ND
Boerner et al. (29)	B31 and N34	Colorimetric assay	0.06–2[b]	0.125–1	<0.016–0.125	<0.016	4–32[c]
Morgenstern et al. (25)	B31	Colorimetric assay	0.5	0.25	0.03	0.03[d]	ND

[a]ND, not determined.
[b]Penicillin G was used instead of amoxicillin.
[c]Gentamicin was used instead of amikacin.
[d]Erythromycin was used instead of azithromycin.

Borrelia strains tested and to the *B. burgdorferi sensu lato* strains cannot be explained by the experimental conditions (Table 1; also Fig. S1 in the supplemental material). Nonetheless, our findings do not allow us to suggest that relapsing-fever *Borrelia* spirochetes are resistant to treatment with beta-lactam antibiotics, such as amoxicillin, *in vivo*. First, an immunocompromised patient with *B. miyamotoi* meningoencephalitis was successfully treated using penicillin G (10). Second, since we tested only two *B. miyamotoi* strains, albeit from two different continents, and one *B. hermsii* strain, caution should be used in predicting a general pattern of susceptibility of *B. miyamotoi* or relapsing-fever *Borrelia* spirochetes.

Colorimetric antibiotic susceptibility testing. As part of this study, we tested the *in vitro* susceptibilities of several *Borrelia* strains to various antibiotics by dark-field microscopy, but we also used an existing colorimetric microdilution method, by Hunfeld et al. (21) (yielding MIC_1), in order to strive for a more standardized procedure, as well as a newly described variant to this colorimetric method (yielding MIC_2). The two colorimetric methods used in this study demonstrated largely similar MIC values. Of interest, a significant correlation ($P < 0.01$) was found between the colorimetric MIC_2 measurement and the number of motile spirochetes as determined by microscopy after 72 h of incubation (Fig. S2 in the supplemental material). However, colorimetric assays are known to be affected by laboratory conditions, such as the type of liquid medium used (e.g., Barbour-Stoenner-Kelly [BSK] medium, modified Kelly-Pettenkofer [MKP] medium, or MKP medium supplemented with 10% heat-inactivated fetal calf serum (MKP-F medium), incubation temperatures, and differences in anaerobic or aerobic cultivation. Indeed, we found that in colorimetric assays, the color of the medium of the microtiter wells, including the negative controls, changes in the first 48 h, possibly due to changes in the solubility of CO_2, a volatile acid, at increasing temperatures (31). The resulting change in absorbance affects the outcome of the colorimetric measurement. In the current study, we circumvented this nonspecific color shift in the first 48 h by preincubating the cultivation medium for 72 h at 33°C, as described in Materials and Methods, and by correcting the decrease in absorbance after 72 h for the change in absorbance of the negative control. Importantly, by comparing color shifts to those of the positive control, the newly described MIC_2 is less dependent on experimental conditions, limiting intra- and interexperimental differences, Moreover, especially for *Borrelia* strains with lower replication rates (B31, HT31, and LB-2001), the MIC_2 correlated better to the microscopic MIC. We therefore propose that this method should be used for determining *in vitro* antibiotic susceptibility using a colorimetric change.

Microscopic testing of antibiotic susceptibility. Not unexpectedly, both MIC_1 and MIC_2 were mostly a few dilution steps below the MICs determined by dark-field microscopy. This can be explained by the fact that the colorimetric change reflects a significant decrease in borrelial growth or replication rather than indicating the absolute absence of viable spirochetes. Therefore, colorimetric MIC measurement can be considered a non-labor-intensive, robust, and easy-to-standardize method for testing spirochetal antibiotic susceptibility; however, dark-field microscopy remains the gold standard for the determination of motile spirochetes and MICs. Minimal bactericidal concentrations (MBCs) were determined by inoculating a small amount of medium containing spirochetes that were exposed to antibiotics for 72 h into fresh medium. Our culture methods allow for successful cultivation of as little as 1 spirochete (data not shown), suggesting that this is a sensitive method even for small amounts of viable spirochetes. As shown in Table 2, MBCs in general were 1 to 3 dilution steps higher than the MIC values we found; however, in some cases, they exceeded the highest concentration tested, so that we could not determine the exact MBC for each *Borrelia* strain and antibiotic tested.

Antibiotic treatment of HTBRF. Treatment for HTBRF is currently empirically based on standard antibiotic therapy for Lyme borreliosis and treatment of other relapsing-fever *Borrelia* infections, assuming that the organism has the same antibiotic susceptibilities as these *Borrelia* spp. (8). However, no clinical guideline for the treatment of HTBRF exists yet. Based on our findings and practical considerations, doxycycline (100 mg orally twice daily) seems to be the preferred initial therapy for patients suspected of having a *B. miyamotoi* infection. This would also be an effective treatment against Lyme disease and human granulocytic anaplasmosis, which might be in the differential diagnosis. Doxycycline is contraindicated for children under the age of 9 years and for pregnant and nursing women, and therefore, amoxicillin has been recommended as an alternative oral therapy to tetracyclines. For HTBRF, intravenous therapy with ceftriaxone has been used for immunocompromised patients with central nervous system disease (e.g., meningoencephalitis) (9, 12) or immunocompetent patients (6), and penicillin G has been used successfully as an alternative (10). The outcome of antibiotic therapy for HTBRF has been described only in a few case reports and case series (6, 9, 10, 32), which reported only minor treatment failures. Of interest, recently, one U.S. case report described the complete recovery of a patient with HTBRF even without antibiotic treatment (33). Treatment of 51 U.S. patients with fever after a tick bite, who were retrospectively shown by PCR to have had HTBRF, consisted of doxycycline, ceftriaxone, and, for 7 patients, oral amoxicillin (7). Of these patients, three reported residual fatigue or other symptoms several months after the completion of antibiotic treatment, but whether these patients had received oral amoxicillin or another antibiotic regime was not mentioned. Penicillins, including aminopenicillins such as amoxicillin, have displayed no more than moderate-to-good *in vitro* activity against *B. burgdorferi sensu lato*; nonetheless, amoxicillin is clinically effective in the treatment of Lyme borreliosis. We show that *B. miyamotoi* strains HT31 and LB-2001 are less susceptible to amoxicillin than *B. burgdorferi sensu lato* isolates *in vitro*. Whether these findings are due, for instance, to alterations in known penicillin-binding proteins (PBPs) in RF *Borrelia* spirochetes or to the production of additional PBPs with a lower affinity to penicillins remains to be investigated, as does the *in vivo* translation of our findings.

In conclusion, we demonstrate poor *in vitro* activity of amoxicillin against *B. miyamotoi* strains from two continents and one *B. hermsii* isolate, with MICs and MBCs ranging from 8 to 32 mg/liter and 32 to 128 mg/liter, respectively. This finding contrasts sharply with the results for the *B. burgdorferi sensu stricto* and *B. afzelii* strains tested, for which MICs were 0.5 to 2 mg/liter and 2 to 4 mg/liter, respectively. Therefore, amoxicillin might have to be used with caution in the treatment of HTBRF. Further research should focus on (i) the occurrence of poor *in vitro* activity of amoxicillin against clinical *B. miyamotoi* isolates, (ii) the mechanism underlying the possible resistance of *B. miyamotoi* to amoxicillin, (iii) the *in vivo* efficacy of

TABLE 4 Tested antimicrobial agents and dilution ranges

Antimicrobial	Company[a]	Order no.	Dilution range (mg/liter)		Breakpoint (mg/liter)[b]	Stock concn (mg/ml), diluent[c]
			B. burgdorferi sensu lato	Relapsing-fever *Borrelia* strains		
Amoxicillin	Centrafarm	14029596	0.25–128	0.25–128	≤4	8.89, PBS
Doxycycline	Sigma-Aldrich	D3447	0.03–8	0.004–2	≤4	44.4, DMSO
Ceftriaxone	Sigma-Aldrich	PHR1382	0.008–4	0.008–4	≤8	4.44, PBS
Azithromycin	Sigma-Aldrich	PHR1088	0.0001–0.05	0.0001–0.05	≤2	5.56, DMSO
Amikacin	Sigma-Aldrich	A1774	2–1,024	2–1,024	≤16	11.26, MKP

[a]Locations: Centrafarm, Etten-Leur, the Netherlands; Sigma-Aldrich, St. Louis, MO, USA.
[b]According to the Clinical and Laboratory Standards Institute.
[c]PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; MKP, modified Kelly-Pettenkofer medium. The DMSO concentrations used in our studies did not influence *Borrelia* viability (data not shown).

beta-lactams in animal studies, and (iv) the clinical efficacy of the treatment of HTBRF with beta-lactam antibiotics.

MATERIALS AND METHODS

Bacterial strains and culture medium. The *Borrelia* strains investigated in this study were PKo (a *B. afzelii* skin isolate from Germany), B31 (*B. burgdorferi sensu stricto*; ATCC 35210; tick isolate, United States), LB-2001 (*B. miyamotoi*, murine isolate; Yale University, United States), HT31 (*B. miyamotoi*, tick isolate; Japan) and HS1 (*B. hermsii*, tick isolate; United States). Glycerol stocks of low-passage-number spirochete isolates (<5 passages) were thawed and were cultured in a regular incubator (Memmert, Schwabach, Germany) at 33°C for 4 days to reach the exponential, mid-log phase of growth. Spirochetes were enumerated using a Petroff-Hausser counting chamber and dark-field microscopy. *B. burgdorferi sensu stricto* B31 and *B. afzelii* PKo were cultured using modified Kelly-Pettenkofer (MKP) medium, and *B. miyamotoi* HT31, *B. miyamotoi* LB-2001, and *B. hermsii* HS1 were cultured using modified Kelly-Pettenkofer medium with the addition of 10% heat-inactivated fetal calf serum (MKP-F medium), as described previously (14).

Microdilution assays and antimicrobial drugs. The antibiotics tested belonged to five different classes: penicillins, tetracyclines, macrolides, cephalosporins, and aminoglycosides. These antibiotics are used in standard regimens for the treatment of Lyme borreliosis. Amikacin was used as a negative control, since *Borrelia* spirochetes are known to be resistant to this antibiotic agent (18, 24, 34). Detailed information is shown in Table 4. MICs were determined by a broth microdilution method using 96-well flat-bottom polystyrene microtiter trays (M0687; Greiner) as described by others (18, 21). Four-day spirochete cultures were centrifuged at 10,000 × *g* for 10 min, resuspended in MKP (*B. burgdorferi sensu lato*) or MKP-F (RF *Borrelia* strains) medium containing extra phenol red (25 mg/liter) as a growth indicator, enumerated, and adjusted to 5×10^7 cells/ml. MKP and MKP-F media with extra phenol red were preincubated at 33°C for 72 h in 50-ml polystyrene tubes with an approximately 30% volume column of air above the medium and were sealed with screw caps to achieve a steady equilibrium of the phenol red color indicator in the media. After preincubation, 2-fold serial dilutions of antibiotics were made in 180 μl MKP and MKP-F media with extra phenol red. Twenty microliters of the spirochete suspension was added to wells, achieving a final concentration of 5×10^6 spirochetes/ml in each well. All antimicrobial agents, as well as a negative control (no spirochetes) and a positive control (no antibiotics), were tested in quadruplicate. The microtiter trays were sealed with adhesive plastic and were incubated in a regular incubator (Memmert, Schwabach, Germany) at 33°C for 72 h. Each day, the microtiter wells were gently mixed to disperse the spirochetes evenly throughout the medium.

Colorimetric MIC measurements. Absorbance was measured at 562 nm and 630 nm at 0, 24, 48, and 72 h using a commercially available enzyme-linked immunosorbent assay (ELISA) reader (PowerWave 200; Bio-Tek Instruments, USA), and absorbance values were calculated by dividing the absorbance at 562 nm by the absorbance at 630 nm. Colorimetric change was calculated by comparing the absorbance after 72 h (E_{t72}) and initial absorbance values (E_{t0}) for each concentration of the antimicrobial agent corrected for the absorbance of the negative control (by subtracting the change in absorbance found in the wells of the negative control from the change in absorbance in each well). Two calculations of the colorimetric MIC were used and compared. MIC_1, as described by others, is defined as the lowest concentration of antibiotics where ($E_{t0} - E_{t72}$) is <10% of E_{t0} (21). It has been established that the absence of borrelial growth in microtiter wells results in a decrease in absorbance of <5 to 10% compared to the initial absorbance values of the microtiter well after 72 h (17, 20, 21, 35). However, this measurement is also dependent on the initial absorbance, which is independent of borrelial growth and can differ between experiments. Also, low replication rates of some *Borrelia* strains lead to a smaller decrease in absorbance compared to the initial absorbance values. Therefore, we also applied an alternative calculation, MIC_2, in which the MIC was calculated by comparing the drop in absorbance ($E_{t0} - E_{t72}$) to the drop in absorbance of the positive control (no antibiotics) ($E_{POS,t0} - E_{POS,t72}$), and the MIC was set arbitrarily at 25%. Thus, MIC_2 was defined as the lowest concentration of antibiotics where ($E_{t0} - E_{t72}$) is <25% of ($E_{POS,t0} - E_{POS,t72}$). This method is solely dependent on borrelial growth and is independent of the initial absorbance and replication rate.

MIC measurement by dark-field microscopy. Spirochetes were counted in all wells after 72 h by dark-field microscopy, as described previously (14). Briefly, 5 μl from each well was put on a microscopy slide and covered by a coverslip. Slides were blinded, and motile spirochetes were counted in six separate microscopy fields. The MIC was defined as the lowest concentration of the antimicrobial agent at which no motile spirochetes were observed by dark-field microscopy. One spirochete per microscopy field equals a concentration of 2.5×10^5/ml. Since we counted 6 separate fields, the lower detection limit using this method was 4×10^4 spirochetes/ml.

Determination of MBCs. MBCs were determined by taking medium from wells one concentration below the MIC (as determined by dark-field microscopy) up to two wells above the MIC (in which minimal spirochetal growth could still be discerned) and diluting these samples 1:75 to a total volume of 1.35 ml of fresh MKP or MKP-F medium in 2-ml screw-cap tubes, as described by others (20). These samples were evaluated for growth after 3 weeks. The MBC was defined as the lowest concentration of the antimicrobial agent at which no motile spirochetes could be detected by dark-field microscopy. The lower detection limit using this counting method was approximately 4×10^4 spirochetes/ml, as described above.

Statistical analysis. A Pearson correlation was used to calculate the correlation between normalized (0 to 1) MIC_2 results and normalized (0 to 1) microscopy results. All analyses were performed using PASW Statistics software, version 19.0 (SPSS Inc., Chicago, IL, USA), and a P value of <0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00535-17.

SUPPLEMENTAL FILE 1, PDF file, 2.3 MB.

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