In Vitro Susceptibility Testing of *Borrelia burgdorferi* Sensu Lato Isolates Cultured from Patients with Erythema Migrans before and after Antimicrobial Chemotherapy

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Clinical treatment failures have been reported to occur in early Lyme borreliosis (LB) for many suitable antimicrobial agents. Investigations of possible resistance mechanisms of the *Borrelia burgdorferi* complex must analyze clinical isolates obtained from LB patients, despite their receiving antibiotic treatment. Here, borrelial isolates obtained from five patients with erythema migrans (EM) before the start of antibiotic therapy and again after the conclusion of treatment were investigated. The 10 isolates were characterized by restriction fragment length polymorphism analysis and plasmid profile analysis and subjected to susceptibility testing against a variety of antimicrobial agents including those used for initial chemotherapy. Four out of five patients were infected by the same genospecies (*Borrelia afzelii*, *n = 3*; *Borrelia garinii*, *n = 1*) at the site of the EM lesion before and after antimicrobial therapy. In one patient the genospecies of the initial isolate (*B. afzelii*) differed from that of the follow-up isolate (*B. garinii*). No significant changes in the in vitro susceptibilities became obvious for corresponding clinical isolates before the start and after the conclusion of antimicrobial therapy. This holds true for the antimicrobial agents used for specific chemotherapy of the patients, as well as for any of the additional agents tested in vitro. Our study substantiates borrelial persistence in some EM patients at the site of the infectious lesion despite antibiotic treatment over a reasonable time period. Borrelial persistence, however, was not caused by increasing MICs or minimal borreliacidal concentrations in these isolates. Therefore, resistance mechanisms other than acquired resistance to antimicrobial agents should be considered in patients with LB resistant to treatment.

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Human Lyme borreliosis (LB) represents a multisystem disorder caused by the *Borrelia burgdorferi* complex (36). In much of Europe, LB does not constitute a notifiable disease, but incidence estimations range between 3.9 and 137/100,000 inhabitants/year (23, 35). In Slovenia, the incidence was 168/100,000 in 2002 (National Notifiable Communicable Diseases Surveillance System, Slovenia, unpublished data). Erythema migrans (EM), which develops at the site of the tick bite, occurs in 77 to 90% of LB patients (4, 8). Antimicrobial treatment of early LB manifestations such as EM is commonly successful in >90% of cases (8, 34). However, similar to failures of chemotherapy for *Treponema pallidum* in syphilis (24), clinical treatment failures have been reported to occur in early LB cases for almost every suitable antimicrobial agent (10, 12, 28, 38, 42). Furthermore, the currently available diagnostic techniques do not reliably discriminate among possible reinfection, true endogenous relapse, and coinfection with other tick-borne pathogens (12). These drawbacks together with the phenomenon of resistance to therapy in individual patients undoubtedly contribute to the inconsistencies surrounding the optimal treatment regimens for LB and are often misinterpreted and misused to support prolonged antibiotic treatment regimens. However, relatively few cases of culture-proven treatment failure have been published (19, 22, 28, 29, 37, 38, 39), and the underlying mechanisms of antimicrobial resistance in *B. burgdorferi* sensu lato remain unresolved. The overall culture detection rate of the pathogen in clinical specimens obtained from cutaneous lesions does not usually exceed 40 to 70% of cases under routine laboratory conditions (1, 19, 32, 38, 44). The culture-positive rate falls to <1 and 20% in cases with Lyme arthritis and neuroborreliosis, respectively. Unfortunately, culture is rarely successful after antimicrobial therapy is initiated (18, 21). Despite this challenge, investigations that explore possible resistance mechanisms in *B. burgdorferi* sensu lato must focus on isolates obtained from patients receiving antibiotic therapy. Here, we examined the in vitro susceptibility and molecular biology of *B. burgdorferi* sensu lato isolates cultured from skin biopsy samples of EM patients before and after antimicrobial chemotherapy to explore whether the persistence of the LB spirochete may be caused by increasing acquired antibiotic resistance.
MATERIALS AND METHODS

Clinical information and primary culture of clinical isolates. Between 1995 and 2000 a total of 3,421 patients >18 years of age were diagnosed clinically with typical EM by experienced physicians at the LB Outpatients’ Clinic, Department of Infectious Diseases, University Medical Centre, Ljubljana, Slovenia, according to slightly modified Centers for Disease Control and Prevention criteria as outlined by Arnez et al. (2). Most of these individuals were enrolled in prospective studies on the assessment of clinical and microbiological efficacy of treatment with different antimicrobial agents. The study protocols included initial biopsy of EM at first visit before the institution of antibiotic therapy and a second biopsy at the same anatomic site approximately 2 months (range, 1.3 to 3 months) later as reported elsewhere (39, 40). Biopsy samples were taken under sterile conditions and immediately cultured in modified Barbour-Stoenner-Kelly (BSK) medium at 33°C for 9 weeks as described previously (27). Weekly subcultures were inoculated into fresh modified BSK medium and were examined by dark-field microscopy. An overall recovery rate for B. burgdorferi sensu lato from the initial skin biopsy samples from these patients was 50%. In 19 out of 1,148 biopsy samples, PCR products were still available for analysis in this in vitro study. None of the patients reported a second tick bite, and all declared having taken their medication as prescribed at the first visit. No clinical signs of treatment resistance were obvious in these patients at the time of second biopsy. The 10 isolates were then subjected to further molecular typing and to detailed susceptibility testing. Information on available clinical and laboratory data for the patients is summarized in Table 1.

Subculture procedures. Stools cultures of the B. burgdorferi sensu lato isolates were propagated in modified BSK medium as follows. Aliquots of 1.5 ml of the stock were resuspended in modified BSK medium at 33°C for 9 weeks as described previously (27). Weekly subcultures were inoculated into fresh modified BSK medium and were examined by dark-field microscopy. An overall recovery rate for B. burgdorferi sensu lato from the initial skin biopsy samples from these patients was 50%. In 19 out of 1,148 biopsy samples, PCR products were still available for analysis in this in vitro study. None of the patients reported a second tick bite, and all declared having taken their medication as prescribed at the first visit. No clinical signs of treatment resistance were obvious in these patients at the time of second biopsy. The 10 isolates were then subjected to further molecular typing and to detailed susceptibility testing. Information on available clinical and laboratory data for the patients is summarized in Table 1.

Plasmid profile analysis. Plasmid profile analysis was performed on passage 4 of all isolates as described by Xu and Johnson (43). Purified plasmid DNA was loaded on a 1% agarose gel and run by applying a voltage of 6 V/cm and a pulse time ramped from 0.9 to 2.5 s for 26 h with a CHEF-DRII apparatus (Bio-Rad Laboratories). To determine the plasmid sizes, low-range and MidRange I PFGE markers (Biodescent Labs) were used as molecular weight standards. The gels were stained as outlined above and photographed, and the relative sizes of plasmid bands were calculated by Wincam gel styler software version 1.0 (Cybertech, Berlin, Germany).

Broth microdilution susceptibility testing. Borrelia stock cultures were cultured in modified BSK medium at 33°C until log phase of growth and adjusted to 2.5 × 10^6 borreliae/ml as determined by enumeration with a Kova counting chamber. Biopsy samples were taken under sterile conditions and immediately cultured in modified Barbour-Stoenner-Kelly medium at 33°C in 5% CO2 for an additional 3 weeks (14). After gentle agitation, 5 to 10 ml of the culture was removed from the stock and used for inoculation. Ceftriaxone and doxycycline served as control substances, and streptomycin (ATCC 35210) served as control organism in order that our data could be related to our recent publications on the in vitro susceptibility of borreliae (11–14, 17).

Determination of MICs. For quantification of bacterial growth we applied kinetic measurement of indicator color shift at 562 and 630 nm by use of a commercially available enzyme-linked immunosorbent assay reader (PowerWave 200; Bio-Tec Instruments) in combination with a calculation program (Microwin 3.0; Microtek) at 0, 24, 48, and 72 h of incubation. Growth of samples and controls finally was determined by decrease of absorbance after 72 h (Et72) in comparison to the initial values (Et0). The well was reported negative for growth if Et72 > (Et0 × 10%). Colorimetric MICs were reported as the medians of three experiments performed on different days.

Determination of MBCs. Following 72 h of incubation with the antibiotic, aliquots (20 μl) were taken from all vials without growth and were diluted 1:1,000-fold with BSK medium below the MIC. Subcultures were incubated at 33°C in 5% CO2 for an additional 3 weeks (14). After gentle agitation, 5 to 10 high-power fields were then examined by dark-field microscopy for the presence or absence of spirochetes. The minimal borreliacidal concentration (MBC) was defined as the lowest concentration of the antimicrobial where no spirochetes could be detected (100% killing) after 3 weeks of subculture (14, 17). MBCs were reported as the medians of three experiments performed on different days (Table 1).

Statistical analysis. To detect possible differences in MIC and MBC data of the different genospecies, the Kruskal-Wallis test was applied using Primer of Bistatistics software, version 5.0 (The McGraw-Hill Companies), for statistical calculation.

RESULTS

Identification of genospecies. The results of genomic DNA RFLP analysis and the PCR-RFLP analysis of the rfaA-trnB spacer region of the clinical isolates are depicted in Fig. 1. Three out of five patients (Table 1, patients 1, 3, and 4) were infected by B. afzelii at the site of the EM lesion before and after antimicrobial therapy as shown by the presence of species-specific 460-, 320-, and 90-kb bands in PFGE-RFLP analysis (Fig. 1, top). In patient 2 B. garinii was cultured from the first skin biopsy sample and from the follow-up specimen as demonstrated by the characteristic 220- and 80-kb bands. Interestingly, B. afzelii was cultured from the primary skin biopsy sample from patient 5 followed by growth of B. garinii from the follow-up specimen sampled from the initial site of the EM

(continued)
TABLE 1. Clinical information and laboratory data for five patients with EM and culture-confirmed persistent *B. burgdorferi* sensu lato infection after conclusion of antimicrobial chemotherapy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical information</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (F, M)</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>43</td>
<td>38</td>
<td>53</td>
<td>68</td>
<td>36</td>
</tr>
<tr>
<td>Symptom(s) at first visit</td>
<td>MEM</td>
<td>EM</td>
<td>EM</td>
<td>EM, BL, PFP</td>
<td>EM, BL</td>
</tr>
<tr>
<td>Tick bite remembered</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Time (days) between tick bite and onset of EM</td>
<td>10</td>
<td>26</td>
<td>NK</td>
<td>NK</td>
<td>19</td>
</tr>
<tr>
<td>No. of EM lesions</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Size of EM (cm)</td>
<td>7 by 12; 6 by 8</td>
<td>10 by 16</td>
<td>7 by 11</td>
<td>9 by 12</td>
<td>7 by 12</td>
</tr>
<tr>
<td>Systemic complaint(s)</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Time (days) between onset of symptoms and treatment</td>
<td>1</td>
<td>11</td>
<td>5</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td><strong>Laboratory data</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IgM-IFT (titer)</td>
<td>256</td>
<td>128</td>
<td>Neg.</td>
<td>Neg.</td>
<td>Neg.</td>
</tr>
<tr>
<td>Time (wks) between first biopsy and positive culture</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Genospecies cultured after first biopsy</td>
<td><em>B. afzelii</em></td>
<td><em>B. garinii</em></td>
<td><em>B. afzelii</em></td>
<td><em>B. afzelii</em></td>
<td><em>B. afzelii</em></td>
</tr>
<tr>
<td>Time (days) between start of treatment and second biopsy</td>
<td>40</td>
<td>39</td>
<td>55</td>
<td>56</td>
<td>69</td>
</tr>
<tr>
<td>Time (wks) between second biopsy and positive culture</td>
<td>4</td>
<td>5</td>
<td>2.5</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Genospecies cultured after second biopsy</td>
<td><em>B. afzelii</em></td>
<td><em>B. garinii</em></td>
<td><em>B. afzelii</em></td>
<td><em>B. afzelii</em></td>
<td><em>B. garinii</em></td>
</tr>
</tbody>
</table>

a Abbreviations: F, female; M, male; MEM, multiple EM; BL, borrelial lymphocytoma; PFP, peripheral facial palsy; NK, not known; i.v., intravenously; p.o., orally; Neg., negative; IFT, indirect fluorescent antibody test; IgM, immunoglobulin M; IgG, immunoglobulin G. 

b Fever, headache, and/or myalgia.

c No significant titer change was noted at the second biopsy.
| Patient and isolate | Drug (test range [H9262 g/ml]) | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC | MIC | MBC |
|---------------------|--------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **Patient 1**       |                                |     |     |     |     |     |     |     |     |     |     |     |
| 1A                  |                                | 0.0156 | 0.5 | 0.0019 | 0.125 | 0.0002 | 0.0312 | 0.25 | 4 | 0.0625 | 16 | 0.0156 | 0.25 |
| 1B                  |                                | 0.0078 | 0.5 | 0.0009 | 0.0625 | 0.0002 | 0.0312 | 0.25 | 8 | 0.0625 | 16 | 0.0312 | 0.5 |
| **Patient 2**       |                                |     |     |     |     |     |     |     |     |     |     |     |
| 2A                  |                                | 0.0039 | 0.5 | 0.0004 | 0.0312 | 0.0002 | 0.0039 | 0.0312 | 0.5 | 0.0625 | 8 | 0.0156 | 0.5 |
| 2B                  |                                | 0.0019 | 0.5 | 0.0004 | 0.0156 | 0.0002 | 0.0019 | 0.0625 | 1 | 0.0312 | 8 | 0.0156 | 0.25 |
| **Patient 3**       |                                |     |     |     |     |     |     |     |     |     |     |     |
| 3A                  |                                | 0.0078 | 0.5 | 0.0009 | 0.0156 | 0.0002 | 0.0312 | 0.0625 | 8 | 0.0312 | 16 | 0.0156 | 0.5 |
| 3B                  |                                | 0.0078 | 0.5 | 0.0019 | 0.0156 | 0.0002 | 0.0625 | 0.125 | 16 | 0.0312 | 1 | 0.0156 | 0.25 |
| **Patient 4**       |                                |     |     |     |     |     |     |     |     |     |     |     |
| 4A                  |                                | 0.0019 | 0.125 | 0.0004 | 0.0312 | 0.0002 | 0.0312 | 0.0625 | 8 | 0.0312 | 10 | 0.0156 | 0.25 |
| 4B                  |                                | 0.0019 | 0.0625 | 0.0004 | 0.0312 | 0.0002 | 0.0156 | 0.0625 | 8 | 0.0312 | 16 | 0.0156 | 0.25 |
| **Patient 5**       |                                |     |     |     |     |     |     |     |     |     |     |     |
| 5A                  |                                | 0.0156 | 0.5 | 0.0039 | 0.125 | 0.0004 | 0.0312 | 0.0312 | 2 | 0.0312 | 8 | 0.0156 | 0.5 |
| 5B                  |                                | 0.0078 | 0.5 | 0.0019 | 0.125 | 0.0009 | 0.0312 | 0.0625 | 4 | 0.0625 | 16 | 0.0312 | 0.25 |

**Reference strain B31 (ATCC 35210)**

- MICs and MBCs are in range of ranges specified for these antimicrobial agents in our recent publications by use of our well-defined assay method for the susceptibility testing of *B. burgdorferi sensu lato*. The MIC and MBC for each isolate are reported as the median of three experiments. Boldface indicates the initial antibiotic treatment regimen.

- The initial MIC and MBC of each isolate obtained during the course of therapy are reported in the median of three experiments. Boldface indicates the initial antibiotic treatment regimen.

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lesion after conclusion of antimicrobial chemotherapy (Fig. 1, top). These results were confirmed by the genospecies determination of the clinical isolates with the use of highly specific PCR-RFLP analysis of the rrfA-rrlB spacer region after digestion with MseI (Fig. 1, bottom). Isolates of patients 1, 3, and 4 and the initial isolate of patient 5 were identified as B. afzelii by generation of fragment sizes of 107, 68, and 50 bp. B. garinii-specific fragment sizes of 107, 95, and 50 bp were recovered from the first and second isolates of patient 2 and the second isolate of patient 5.

Plasmid profile analysis. The plasmid profiles of the 10 clinical isolates as determined by PFGE are depicted in Fig. 2. The number of plasmids present in each isolate varied from 6 to 11, and the plasmid size ranged from approximately 5 to 62 kb. The majority of plasmids were in the 30- to 39-kb size range (n = 26), followed by the 20- to 29-kb range (n = 20), and only one plasmid was in the 50- to 59-kb range. The average number of plasmids per strain was higher for the B. afzelii isolates (7.7; range, 6 to 11) than for the B. garinii isolates, all of which contained six plasmids. The plasmid pattern of the B. afzelii and B. garinii isolates obtained before and after treatment from patient 5 revealed major differences (Fig. 2, lanes 9 and 10) and therefore paralleled the results of the DNA RFLP analysis and PCR-RFLP of the rrfA-rrlB spacer region in these isolates (see above). The B. garinii isolates obtained from patient 2 before and after therapy showed exactly the same plasmid pattern (Fig. 2, lanes 3 and 4). In the remaining patients (1, 3, and 5) the plasmid pattern appeared closely related for each pair of strains. However, the number and size of plasmids varied in the isolates obtained before and after antimicrobial chemotherapy (Fig. 2, lanes 1 and 2, 5 and 6, and 7 and 8).

MIC and MBC determination. The individual in vitro susceptibilities of the 10 clinical isolates to seven commonly used antimicrobial agents including the drugs used for initial treatment of the patients with EM are summarized in Table 2. Clearly, there was some variability in the individual MICs and
MBCs of the various antimicrobial agents belonging to the classes macrolides, β-lactams, and tetracyclines for the different pairs of isolates. Erythromycin and amoxicillin revealed the largest amount of interstrain variability, with MICs and MBCs varying over an 8- to 100-fold range for the different isolates, respectively. Overall, B. garinii isolates tended to be more susceptible than B. afzelii isolates, for which in part β-lactam agents showed higher MICs and MBCs. For amoxicillin, MICs described for in vitro susceptibility testing of bial agents in our recent publications by use of our assay (Table 2) were within the ranges specified for these antimicro-

B burgdorferi, the MIC and MBC ranges of the drugs tested indicating high reproducibility. For the reference strain B31 one (5). In our study, the plasmid pattern also differed in three out of four pairs of isolates belonging to the same genospecies cultured from the corresponding patient’s EM site before and after chemotherapy. This observation requires further investigation but is more likely to result from adaptation of borrelial clones during persistent infection rather than from reinfection of the same body site due to a second and unobserved tick bite within 5 to 10 weeks. Vector-borne pathogens have evolved to adapt and persist in their various hosts (5). Similarly, such adaptation during antibiotic chemotherapy may rapidly result in selection of clonal subtypes of the same borrelial genospecies. Our findings in antibiotically treated EM patients indeed suggest that the population of spirochetes detected after chemotherapy may genetically differ from the initial bacterial population initiating the infection. This observation is in accordance with recent findings that survival of infectious borrelial isolates in antibiotically treated mice is correlated with genetic recombination and diminished levels or complete loss of lp25 and lp28-1, plasmids that are known to carry genes which are important for the infectiousness of borreliae (5). Such attenuated residual borreliae were no longer infectious when transmitted to new mammalian hosts (5). In our strains, we did not test for a potential loss of infectiousness, but our patients did not present with clinical signs of treatment failure or relapse. Similarly, the persistence of group A streptococci and Chlamydia spp. after chemotherapy of infection is not necessarily equivalent to clinical treatment failure (15, 41). In patient 2, however, the plasmid pattern of the two subsequent B. garinii isolates did not change at all despite antibiotic treatment (Fig. 2). Therefore, survival of small numbers of bacteria may result in persisting clinical complaints in some patients. Further investigations are clearly warranted to elucidate effects of potential changes in the infectiousness of persisting borreliae on the clinical course of human LB treated with antimicrobial agents.

In Europe, coinfections with more than one borrelial genospecies have been well documented in molecular epidemiological studies of patients with EM (30, 31). In one of our EM patients (patient 5, Table 1) B. afzelii was cultured from the primary skin biopsy sample, followed by growth of B. garinii from the follow-up specimen after conclusion of antimicrobial chemotherapy (Fig. 1, top). Although these findings could result from a possible double or concomitant infection with B. garinii and B. afzelii following the initial infectious tick bite, a second tick bite that went unnoted cannot be excluded based on our clinical and molecular biological findings. In Europe, human coinfections may be more frequent than previously believed, as 45% of Ixodes ricinus ticks have been shown to be infected with more than one borrelial genospecies (20).

To date, neither MIC definitions, test conditions, nor the inocula for the in vitro susceptibility testing of B. burgdorferi sensu lato are standardized (7, 12). Previous studies (6, 7) and our own experience (11–14, 17), however, clearly indicate that a microdilution method with BSK medium and incubation for 72 h holds promise for standardization of antimicrobial susceptibility testing of borreliae. To relate our study data to our recent publications on the in vitro susceptibility of borreliae...
agents for the species level (11, 17, 29, 33). Correspondingly, there was a vitro susceptibilities to some antimicrobial agents on the geno-

vation of study provides compelling evidence that, although rare, sur-

prolonged effective antibiotic therapy (33). In summary, our critical concentrations for these substances to become ineffec-

no clinical relevance, as they commonly do not exceed the antimicrobial agents. These minor differences, however, are of can also differ in their individual susceptibilities to various antimicrobial agents. These minor differences, however, are of no clinical relevance, as they commonly do not exceed the critical concentrations for these substances to become ineffec-

tive and therefore cannot explain survival of spirochetes during prolonged effective antibiotic therapy (33). In summary, our study provides compelling evidence that, although rare, sur-

vival of B. burgdorferi sensu lato can occur in antibiologically treated individuals with EM after antimicrobial chemotherapy. Spirochete persistence in these patients was not caused by increasing MICs or MBCs for B. burgdorferi sensu lato. Instead, our findings corroborate those of Hansen et al. (9) and Pfister et al. (25) in relapsed patients with early LB, demonstrating that isolates cultured after the conclusion of roxithromycin and ceftriaxone therapy remain sufficiently susceptible to these agents in vitro. These findings, however, do not rule out phen-

otypic resistance mechanisms similar to those assumed to cause relapse in syphilis and leptospirosis (24, 37).

REFERENCES


venereol. 72:297–300.


