

# Genotypic and phenotypic characterisation of *Borrelia burgdorferi sensu lato* strains isolated from human blood

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Lyme borreliosis often presents initially with erythema migrans. *Borreliae* may disseminate from the primary skin lesion, and different organs and systems could be affected. *Borrelia* strains were isolated from blood of 70 patients with Lyme borreliosis, including 10 patients from whom *borreliae* were also isolated from skin. The aim of the present study was to characterise the isolates with regard to their phenotypic and genotypic characteristics. *Borreliae* were cultivated in MKP medium. Species identification and plasmid profiles were determined by pulsed-field gel electrophoresis (PFGE) and protein profiles by SDS-PAGE. Digestion of *Borrelia burgdorferi sensu lato* DNA showed 63 (90%) *B. afzelii* Mla1 and 7 (10%) *B. garinii* Mlg2. No *B. burgdorferi sensu stricto* were isolated. *Borreliae* were isolated from both skin and blood of 10 patients, nine pairs of isolates were identical: seven *B. afzelii* and two *B. garinii*. *B. afzelii* was isolated from the skin and *B. garinii* from blood of the tenth patient. All but one isolate possessed at least one large plasmid and varying numbers of smaller plasmids. Eight (11.4%) of 70 isolates possessed an unusual plasmid profile (2 of 63 *B. afzelii* and 6 of 7 *B. garinii*). *Borreliae* differed in their protein profiles. OspA and OspB proteins were expressed by all *B. afzelii* isolates; 85.7% of *B. garinii* isolates expressed OspA and 71.4% expressed OspB. OspC was expressed by 65% of *B. afzelii* isolates and all *B. garinii* isolates. The ratios of *B. afzelii* and *B. garinii* isolated from blood and skin were similar. These results do not support the hypothesis that *B. garinii* has a higher propensity for haematogenous dissemination than *B. afzelii*. Antigen diversity as well as species and plasmid heterogeneity could play a role in the pathogenesis of the infection, suggesting distinctive strain organotropism.

## Introduction

Lyme borreliosis shows a broad spectrum of clinical manifestations [1]. The aetiological agent, *Borrelia burgdorferi sensu lato*, has been subdivided into several distinct genospecies, and at least three genospecies are associated with human Lyme borreliosis: *B. afzelii*, *B. garinii* and *B. burgdorferi sensu stricto* [2]. In Europe, all three genospecies are associated with Lyme borreliosis, while in the USA, *B. burgdorferi sensu stricto* is reported as the sole human pathogen [3, 4]. Slovenia is an endemic region for Lyme borreliosis [5]. These three borrelial genospecies and also *B. bissettii* have been isolated from Slovenian patients [6, 7].

The pathogenesis of Lyme borreliosis is poorly understood. The initial clinical manifestation is usually erythema migrans, a skin rash at the site of a tick bite [1, 8]. *Borreliae* may disseminate from the primary skin lesion and different organ systems may be affected, including central and peripheral nervous system, joints, skin, heart and eyes [1, 9–12]. Dissemination is thought to be haematogenous and may be asymptomatic [7, 13]. Isolation of spirochaetes from blood has been reported infrequently, possibly suggesting that spirochaetaemia is sporadic and inconsistent [14–17]. Limited information is available on genotypic and phenotypic characteristics of European strains isolated from blood [7, 14]; published data are mainly restricted to findings from the USA. Recently, Wormser and co-workers reported on the distinctive genetic characteristics of *B. burgdorferi sensu stricto* isolated from blood of patients with erythema migrans [18].

This report describes the genotypic and phenotypic characteristics of 70 blood isolates from patients residing in Slovenia. To our knowledge this study represents the largest number of blood isolates analysed until now, and the first series of European blood isolates.

## Materials and methods

### Bacterial isolates and culture conditions

Of 70 *Borrelia* isolates from blood in the period 1994–2000, 68 originated from patients who presented with solitary or multiple erythema migrans, one was from a patient with erythema migrans and facial palsy, and one was from a patient with meningitis without skin involvement. In 29 of 70 patients, two clinical samples (blood and skin) were taken. The skin specimens of 3 × 3 mm, obtained from erythema migrans skin lesions on the same day as a blood sample, were inoculated immediately into modified Kelly-Pettenkofer (MKP) medium and transported to the laboratory. At the same time citrated blood samples (5 ml) were also transported to the laboratory, where they were centrifuged at 800 rpm for 10 min and whole plasma was inoculated into MKP medium. Samples were incubated at 33°C and examined weekly for the presence of spirochaetes by dark-field microscopy [7, 19].

### SDS-PAGE

For protein analysis, spirochaetes were cultured and washed in buffer (PBS/Mg). After the final centrifugation, the pellet was resuspended in buffer containing sodium-dodecyl sulphate 2.5% and 2-mercaptoethanol, boiled for 10 min and electrophoresed on polyacrylamide 12% gel. Proteins were stained with Coomassie Brilliant Blue. The molecular masses of borrelial proteins were calculated by comparison with molecular mass standards (16–110 kDa; BioRad) [7, 20].

### Pulsed-field gel electrophoresis (PFGE)

Total genomic DNA was extracted by a gel insert method as described previously [7, 21]. Briefly, spirochaetes were washed in buffer (Tris-NaCl), resuspended in low melting temperature agarose and pipetted in the plug moulds. Plugs were incubated in lysis solution containing lysozyme 1 mg/ml at 37°C for 24 h, and in digestion buffer containing proteinase K 0.5 mg/ml at 50°C for 72 h, both with gentle shaking. Plugs were stored at 4°C [7, 21].

For plasmid analysis, plugs were electrophoresed with pulse times ramped from 0.9 to 3 s for 35 h. Gels were stained with ethidium bromide and photographed. DNA molecular markers from 0.1 to 200 kb (Sigma) were used as size markers for comparison [7, 21].

For species identification, plugs were digested with

*Mlu*I (30 U) restriction endonuclease at 37°C for 24 h. The digested DNA was electrophoresed with a pulsed time ramped from 1 to 40 s for 24 h. DNA molecular markers from 50 to 1000 kb (Sigma) were used as size markers for comparison [7, 21]. Gels were stained with ethidium bromide and photographed. *Mlu*I large restriction fragment patterns (LRFP) were used to identify species according to Belfaiza *et al.* [2] and Picken *et al.* [6].

### Statistical analysis

Data were compared by Fisher's exact two-tailed test or Yates' corrected  $\chi^2$  test, with the significance level set at  $p < 0.05$ .

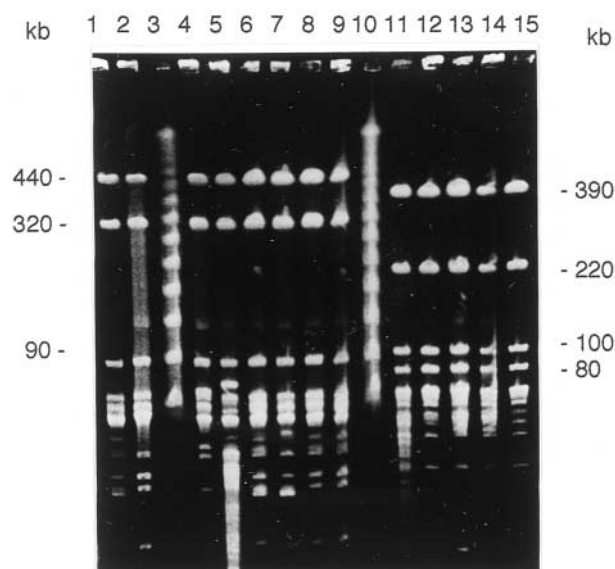
## Results

### Species identification

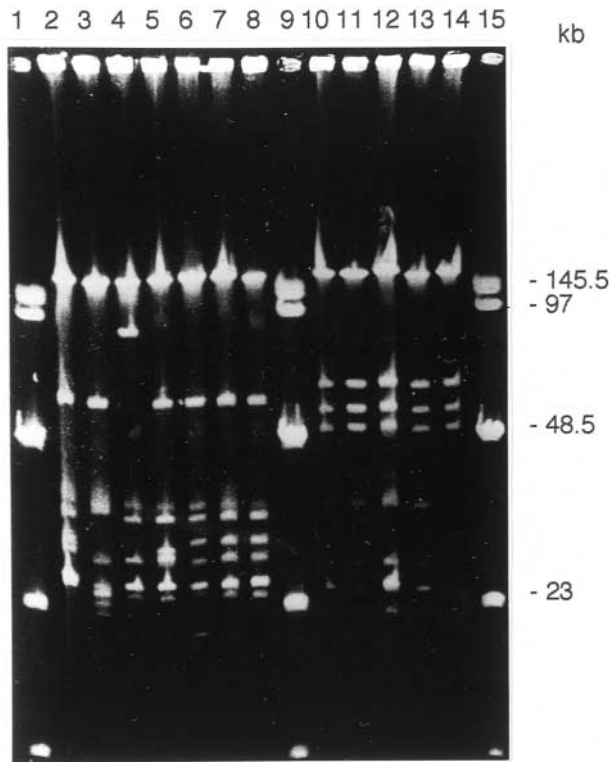
Digestion of 70 *B. burgdorferi sensu lato* isolates from blood showed that 63 strains (90%) were *B. afzelii* and 7 (10%) were *B. garinii*. No *B. burgdorferi sensu stricto* or any other species was isolated.

All isolates identified as *B. afzelii* had a unique LRFP, Mla1 (440, 320 and 90 kb) and all isolates of the *B. garinii* genospecies had a unique LRFP, Mlg2 (390, 220, 100 and 80 kb). Some of these isolates are shown in Fig. 1.

Borreliae were isolated from blood and skin of 18 of the 29 patients from whom two samples had been taken for investigation. In 10 patients, borreliae isolated from blood and skin were available for further characterisa-



**Fig. 1.** PFGE of *Mlu*I restriction digests of some *B. afzelii* (lanes 1–2, 4–9) and *B. garinii* (11–15) isolates from blood. Lanes 3 and 10, molecular size markers (50–1000 kb; Sigma).



**Fig. 2.** Plasmid profiles of some *B. afzelii* (lanes 2–8) and *B. garinii* (10–14) isolates from blood. Lanes 1, 9 and 15, molecular size markers (9.4–145.5 kb; Sigma).

tion; in the remaining eight patients, only skin isolates were available for further analysis. At species level, skin and blood isolates were identical in nine patients (seven *B. afzelii* and two *B. garinii*), whereas in one patient with erythema migrans, *B. afzelii* was isolated from skin and *B. garinii* from blood. All skin isolates from the remaining eight patients were identified as *B. afzelii*.

### Plasmid analysis

The plasmid content of all 70 blood isolates was analysed. Plasmid profiles varied among the isolates, but all except one possessed a large 50–65-kb plasmid and varying numbers of smaller plasmids. Plasmid profiles of some isolates are shown in Fig. 2.

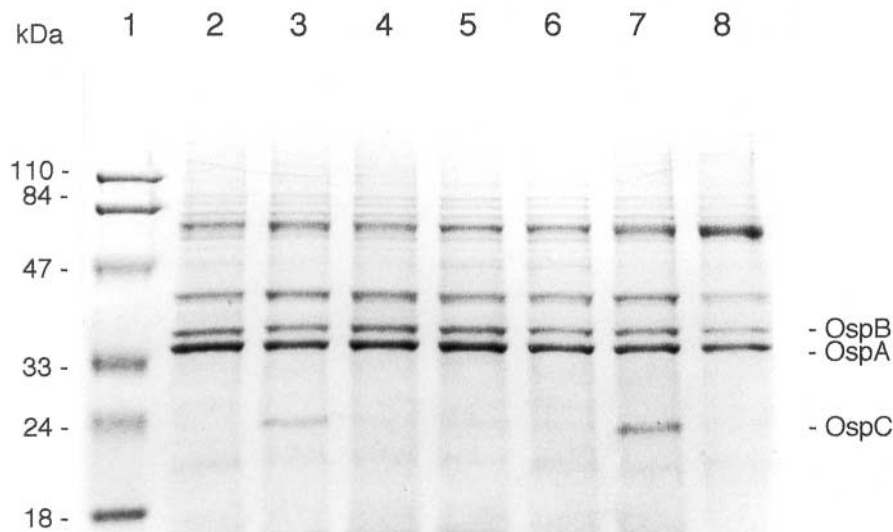
Eight (11.4%) of 70 isolates had unusual plasmid contents, which were observed more often in *B. garinii* isolates than in *B. afzelii* (6 of 7 versus 2 of 63, respectively;  $p = 0.0008$ ). One *B. afzelii* isolate possessed a plasmid dimer of *c.* 100 kb and lacked the large 50–65-kb plasmid (Fig. 2, lane 4). Another *B. afzelii* isolate possessed three copies of the large 50–65-kb plasmid. All six *B. garinii* isolates possessed three copies of the large 50–65-kb plasmid (Fig. 2, lanes 10–14). The possession of three copies of the large 50–65-kb plasmid of the Mlg2 subgroup of the *B. garinii* genospecies was not unusual in this study.

The plasmid profiles of all pairs of isolates of the same species isolated from skin and blood were identical with the exception of the isolates from the patient from whom *B. afzelii* was isolated from skin and *B. garinii* from blood.

### Protein analysis

Borreliae differ with regard to the proteins they express, the amount of the expressed protein and (to a lesser extent) the mol. wt of the expressed protein. Protein profiles of some representative isolates are shown in Fig. 3.

OspA protein was found in 69 (98.6%) of 70 isolates analysed: in all *B. afzelii* and 6 (85.7%) of 7 *B. garinii*



**Fig. 3.** Whole-cell lysate protein profiles of some *B. afzelii* (lanes 2–8) isolates from blood. Lane 1, molecular size markers (18–110 kDa; Sigma).

isolates ( $p = 0.1000$ ). Expression of the OspB and OspC differed between the species, but these differences were not statistically significant for OspC. OspB was expressed by all 46 *B. afzelii* isolates and by 5 (71.4%) of 7 *B. garinii* isolates ( $p = 0.0086$ ), while OspC was found in 41 (65%) of 63 *B. afzelii* isolates and in all *B. garinii* isolates ( $p = 0.0893$ ). The results are summarised in Table 1.

Protein profiles of isolates from blood and skin of individual patients were identical. In the patient from whom *B. garinii* was isolated from blood and *B. afzelii* from skin, the protein profiles were very similar, differing only in the quantity of expressed proteins.

## Discussion

The present study demonstrates some phenotypic and genotypic characteristics of 70 *B. burgdorferi sensu lato* isolates from blood of patients with Lyme borreliosis. To our knowledge, no similar European study on *Borrelia* blood isolates has been published. The large majority of the patients presented with erythema migrans. Thus, the analysed isolates reflect the aetiology of erythema migrans, and they represent a subset of the *Borrelia* strains that disseminate from skin and cause spirochaetaemia. The finding that 61 (87%) of 70 isolates from blood of patients with erythema migrans belong to *B. afzelii*, and 7 (10%) of 70 to *B. garinii*, is nearly identical to the ratio of these two species for skin isolates (86.5% *B. afzelii* and 13.5% *B. garinii*) found in a previous study of 72 patients with erythema migrans [7]. This similarity does not support the hypothesis that *B. garinii* has a higher propensity for haematogenous dissemination than *B. afzelii*, or that more frequent involvement of central nervous system is a consequence of more frequent spirochaetaemia with *B. garinii* than with *B. afzelii*.

The results of the present study corroborate previous findings that borreliae disseminate haematogenously early in the course of infection [13, 17]. However, several questions remain to be answered, including how frequently borreliae disseminate in European patients, whether spirochaetaemia is continuous or intermittent, whether blood represents a 'hostile environment' damaging to borreliae, and how often borreliae disseminate to and affect other organs.

According to our laboratory experience, borreliae isolated from blood are less easily cultured and frequently grow more slowly than skin isolates. This suggests that blood does not favour borreliae as a suitable environment. Other factors may also affect recovery of borreliae from blood: e.g., the addition of anticoagulant to blood sample tubes. Recently, Wormser *et al.* reported a higher isolation rate from serum than from blood, but with both approaches the volume of the sample was critical [18, 22]. However, in spite of the overall lower yield of blood compared with skin for the isolation of borreliae, in some patients with erythema migrans blood may give a positive culture result while skin does not. This fact was also confirmed by the findings of the present study.

By identifying isolates to the species level, the present study found *B. afzelii* to be the predominant species isolated from blood samples. Investigation of the LRFPS of isolates tested revealed unique LRFPS for *B. afzelii* and also for *B. garinii* blood isolates. Whereas Mla1 has been the sole *B. afzelii* subgroup found until now [6, 7], Mlg2 has been just one of the several LRFPS subgroups of *B. garinii*. According to previous findings, Mlg2 has been the most frequent (but not the only) LRFPS type of *B. garinii* isolated from patients with Lyme borreliosis residing in Slovenia, representing 8 (53.3%) of 15 of *B. garinii* skin isolates and 19 (76%) of 25 of cerebrospinal fluid (CSF) isolates [7, unpublished data]. The results of the present study, demonstrating that all *B. garinii* isolates from blood belonged exclusively to Mlg2 subgroup, are in contrast to previous findings for *B. garinii* skin isolates ( $p = 0.0512$ ) and CSF isolates ( $p = 0.2964$ ). The importance of these findings is limited by the low number of blood isolates examined and the fact that differences were not statistically significant.

In 10 patients presenting with erythema migrans borreliae were isolated not only from blood but also from skin. In seven of these patients both isolates were typed as *B. afzelii*, in two patients as *B. garinii*, while in one patient *B. afzelii* was isolated from skin and *B. garinii* from blood. Different genospecies, isolated from different samples in the same patient, point to the possibility that the host could have been infected with different species simultaneously. In this patient, skin and blood samples were not examined by PCR, so there is no evidence of *B. garinii* presence in the skin as well as *B. afzelii* in the blood. Host infection with

**Table 1.** Expression of OspA, OspB and OspC in 70 *B. burgdorferi sensu lato* isolates from blood

Species n	Number (%) of isolates that expressed		
	OspA	OspB	OspC
<i>B. afzelii</i> 63	63 (100)	63 (100)	41 (65)
<i>B. garinii</i> 7	6 (85.7)	5 (71.4)	7 (100)
All 70	69 (98.6)	68 (97)	48 (68.6)

two or three *Borrelia* species has been reported previously, and was usually confirmed by PCR [23–25]. The agreement of different methods of typing *B. burgdorferi sensu lato* has not yet been assessed critically.

Heterogeneity of plasmid profiles seems to be a natural characteristic of *B. burgdorferi sensu lato* strains [26]. The present study found 8 (11.4%) of 70 isolates with unusual plasmid contents (either plasmid dimer or multiple copies of the large 50–65-kb plasmid). Of the eight isolates two were *B. afzelii* and six were *B. garinii*. Unusual plasmid content has also been found in low percentages of skin and CSF isolates from Slovenian patients (in 13.5% and 7.5% respectively) [7, 27]. It was surprising that six (85.7%) of seven *B. garinii* isolates from blood had three copies of the large plasmid. Such high incidence of unusual plasmid content among the human strains has not been reported previously. In the present study the frequency of an unusual plasmid profile was significantly higher in *B. garinii* isolates than in *B. afzelii* blood isolates ( $p = 0.0008$ ), but the number of *B. garinii* isolates examined was low. The role of (unusual) plasmids in the pathogenesis of borreliosis remains an open question.

When all the isolates were compared, the presence of plasmid dimer or multiple copies of the large 50–65-kb plasmid appeared to be 'unusual', but if these plasmid profiles are compared with those of subgroup Mlg2 of genospecies *B. garinii* it seems not to be unusual. It will be interesting to analyse more *B. garinii* Mlg2 isolates from other human specimens, ticks and animals.

Phenotypic characteristics of blood isolates as found in the present study are demonstrated by protein profiles (Fig. 3 and Table 1). OspA was expressed by all *B. afzelii* and 6 (85.7%) of 7 *B. garinii* isolates, OspB was expressed by all *B. afzelii* and 5 (71.4%) of 7 *B. garinii* isolates, while OspC was expressed by 41 (65%) of 63 *B. afzelii* and by all *B. garinii* isolates. Differences between the two borreliosis species were statistically significant for the expression of OspB ( $p = 0.0113$ ) but not for OspA and OspC. Expression of Osp proteins according to distinctive *Borrelia* species has been reported rarely [7, 28]. More data have been published on the associations of different Osps in strains from various natural environments [29] and many studies have been performed to analyse the role of the Osp proteins [30, 31]. Differences in their expression may indicate distinctive organotropism of the strains [7, 28], but their exact role in the pathogenesis of Lyme borreliosis has yet to be elucidated. There is no doubt that Osp proteins are important antigens with the ability to stimulate the host immune system for antibody production [20]. Antigenic heterogeneity and heterogeneity regarding the amount of the expressed antigen found among *B.*

*burgdorferi sensu lato* strains distinctively influence the host immune system, have an impact on the sensitivity and specificity of serological tests, and may profoundly influence the efficacy of future Lyme borreliosis vaccines [20, 32].

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