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Research paper

Imbalanced presence of *Borrelia burgdorferi* s.l. multilocus sequence types in clinical manifestations of Lyme borreliosis

E. Claudia Coipan [a,*], Setareh Jahfari [a], Manoj Fonville [a], G. Anneke Oei [b], Lodewijk Spanjaard [b], Katsuhisa Takumi [a], Joppe W.R. Hovius [c], Hein Sprong [a]

[a] *Laboratory for Zoonoses and Environmental Microbiology, National Institute for Public Health and Environment (RIVM), Bilthoven, The Netherlands*
[b] *Department of Medical Microbiology, Academic Medical Center, Amsterdam, The Netherlands*
[c] *Center for Experimental and Molecular Medicine, Academic Medical Center, Amsterdam, The Netherlands*

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ABSTRACT

In this study we used typing based on the eight multilocus sequence typing scheme housekeeping genes (MLST) and 5S-23S rDNA intergenic spacer (IGS) to explore the population structure of *Borrelia burgdorferi* sensu lato isolates from patients with Lyme borreliosis (LB) and to test the association between the *B. burgdorferi* s.l. sequence types (ST) and the clinical manifestations they cause in humans. Isolates of *B. burgdorferi* from 183 LB cases across Europe, with distinct clinical manifestations, and 257 *Ixodes ricinus* lysates from The Netherlands, were analyzed for this study alone. For completeness, we incorporated in our analysis also 335 European *B. burgdorferi* s.l. MLST profiles retrieved from literature. *Borrelia afzelii* and *Borrelia bavariensis* were associated with human cases of LB while *Borrelia garinii*, *Borrelia lusitaniae* and *Borrelia valaisiana* were associated with questing *I. ricinus* ticks. *B. afzelii* was associated with *acrodermatitis chronica atrophicans*, while *B. garinii* and *B. bavariensis* were associated with neuroborreliosis. The samples in our study belonged to 251 different STs, of which 94 are newly described, adding to the overall picture of the genetic diversity of *Borrelia* genospecies. The fraction of STs that were isolated from human samples was significantly higher for the genospecies that are known to be maintained in enzootic cycles by mammals (*B. afzelii*, *B. bavariensis*, and *Borrelia spielmanii*) than for genospecies that are maintained by birds (*B. garinii* and *B. valaisiana*) or lizards (*B. lusitaniae*). We found six multilocus sequence types that were significantly associated to clinical manifestations in humans and five IGS haplotypes that were associated with the human LB cases. While IGS could perform just as well as the housekeeping genes in the MLST scheme for predicting the infectivity of *B. burgdorferi* s.l., the advantage of MLST is that it can also capture the differential invasiveness of the various STs.

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1. Introduction

Lyme borreliosis (LB) is the most prevalent vector-borne disease in Europe (ECDC, 2011), with an incidence that has increased in the past few years (Stanek et al., 2012). The causative agents – Lyme spirochetes – are bacteria belonging to the *Borrelia burgdorferi* sensu lato complex. They are transmitted by ticks of genus *Ixodes* (Burgdorfer et al., 1982) and are maintained in enzootic cycles by different vertebrate hosts (Kurtenbach et al., 2006; Mannelli et al., 2012).

The disease presents itself under a wide range of clinical manifestations in humans. These manifestations start, in many cases, with *erythema migrans* (EM) — a rash at the site of the tick bite, and progress on some occasions towards disseminated manifestations such as cardiovascular, neurologic, or arthritic ones. At least five genospecies of *B. burgdorferi* s.l. have been shown to be pathogenic to humans — *Borrelia afzelii*, *Borrelia garinii*, *Borrelia burgdorferi* sensu stricto, *Borrelia spielmanii*, and *Borrelia bavariensis* (Stanek et al., 2012). Current knowledge is that *B. afzelii* is predominantly involved in cutaneous manifestations, being often found in the localized incipient form of the infection and in *acrodermatitis chronica atrophicans* (ACA), *B. garinii* in neurological manifestations, and *B. burgdorferi* in articular ones (Stanek et al., 2012). Thus, these two last genospecies are, apparently, more invasive, being able to disseminate from the inoculation site to other tissues or/and to survive the early immune response. However, there is not a clear-cut differentiation between members of these genospecies in terms of symptoms. Next to that, there have been reports of disseminated disease (e.g. *neuroborreliosis* (NB)) without any characteristic *erythema migrans* symptoms (Reik et al., 1986); that would imply that, while being more invasive, some strains can be overlooked when only testing EM biopsies.

Furthermore, it is not yet known if all the *Borrelia* genotypes within the pathogenic genospecies present in questing ticks can cause disease in humans or if there is only a subset of them that is pathogenic

* Corresponding author.
E-mail address: claudia.coipan@rivm.nl (E.C. Coipan).

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(infectious). From a public health perspective, it is important to be able to differentiate between the infectious and non-infectious *Borrelia* spirochaetes or between the invasive and non-invasive ones. Discriminating between these types could be useful for disease risk assessment and management.

Research on *B. burgdorfer*i s.s. in North America has shown that some major sequence types of the outer surface protein C (*ospC*) and certain sequence types of 16S–23S rRNA intergenic spacer are more frequently found in disseminated cases of LB (Dykhuizen et al., 2008; Strle et al., 2011; Wormser et al., 2008). More recently, Hanincova et al. (2013) have shown significant associations between clusters of sequence types (clonal complexes) of *B. burgdorferi* s.s. and localized or disseminated forms of LB. It seems, thus, that the genetic makeup of the pathogenic spirochetes is determinant for the symptomatology they cause. Consequently, genotyping the bacteria might hold the answer to the question of differential infectivity and invasiveness of *B. burgdorferi* s.l.

The gold standard for genotyping of *B. burgdorferi* nowadays is multilocus sequence typing (MLST), based on eight housekeeping genes on the chromosome, which undergo slow evolution and show nearly neutral variation (Margos et al., 2008; Urwin and Maiden, 2003). Previous studies (Coipan et al., 2013a) have shown that the 5S–23S rDNA intergenic spacer (IGS) can also discriminate among the genospecies of *B. burgdorferi* s.l. and that it can also detect genetic differentiation among the bacteria of various geographic provenience.

In this study we used typing based on MLST and IGS to explore the population structure of *B. burgdorferi* s.l. isolates from patients with LB and to address the issue of association between the *B. burgdorferi* s.l. sequence types (ST) and the clinical manifestations they cause in humans. Firstly, we examined whether all the bacterial STs found in questing ticks can cause disease in humans or it is only a small subset that is responsible for the reported cases of LB. Assuming that the pathogenicity of the bacteria is a characteristic determined primarily by their genetic composition, we used MLST and IGS as genetic proxies for infectivity/invasiveness of a strain. Secondly, we wanted to test whether there are *B. burgdorferi* s.l. STs that are more frequently encountered in human cases than we would expect based on their frequency in questing ticks. If that were to be true, it would imply that some of the bacterial STs are more infectious than others. The last hypothesis we tested is that of differential dissemination/persistence ability of *B. burgdorferi* s.l., for which we calculated the probabilities of various STs of causing disseminated/persistent clinical manifestations in humans.

2. Material & methods

2.1. Borrelia isolates

Isolates of *B. burgdorferi* from 183 Lyme borreliosis cases across Europe, with distinct clinical manifestations, and 257 *Ixodes ricinus* lysates from The Netherlands, were analyzed for the present study. The European isolates were selected primarily to give as wide as possible diversity of *Borrelia* in human samples, based on their country of origin.

2.2. DNA extraction and screening of questing ticks

DNA extraction from the individual questing ticks was done by alkaline lysis in ammonium hydroxide, as described previously (Schouls et al., 1999), while the DNA extraction from the bacterial cultures was performed using DNeasy® Blood & Tissue Kit (QIAGEN N.V., Venlo, The Netherlands).

Screening of the questing ticks for *B. burgdorferi* s.l. was done by qPCR with outer surface protein A (*ospA*) and flagellin B (*flaB*) targeted primers, as described (Heylen et al., 2013).

The majority of the ticks included in this study was represented by nymphs, as they constitute most of the acarological risk (Coipan et al., 2013b) for acquiring LB.

2.3. Multilocus sequence typing (MLST)

All 440 isolates of *B. burgdorferi* s.l. were sequenced and typed using the MLST procedure described by Margos et al. (2008), except that the elongation times were 60 s. Briefly, eight loci on the bacterial chromosome (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *rplB*, *recG*, and *uvrA*) were amplified and subsequently sequenced in forward and reverse directions.

Trimming and manual cleaning of sequences was performed in Bionumerics 7.1. (Applied Math, Belgium).

In frame alignment of sequences was made with TranslatorX (Abascal et al., 2010), using the program MUSCLE (Edgar, 2004) with bacterial and plant plastid genetic code.

2.4. 5S–23S typing

For 220 of these samples we also performed PCR targeting the variable 5S–23S rDNA intergenic spacer region (IGS). The PCR was performed according to the protocol described in Coipan et al. (2013a). Alignment of the sequences was made using MAFFT (Katoh and Toh, 2008) and the sequences were trimmed to nucleotides between position 438,838 and 439,196 (359 nucleotides) of whole genome sequence of *B. afzelii* strain PKo (GenBank entry CP002933). An IGS sequence type was defined here as a group of *Borrelias* within a genospecies in which all members share an identical sequence. We assigned a unique number to each of the IGS sequence types.

2.5. Multilocus sequence analysis

Each unique allele type of the housekeeping genes received a number. Alleles that were identical to the ones already existing in MLST.net received the same number, while the new ones received numbers over 500. Similarly, the STs resulting from concatenating the alleles at all eight loci, were assigned unique numbers that either matched the known ones in MLST.net or had values higher than 1000 if they were previously unknown.

To gain more information regarding the distribution of the STs in questing ticks we incorporated in our analysis also 335 European *B. burgdorferi* s.l. MLST profiles that we retrieved from publications (Hoen et al., 2009; Margos et al., 2008, 2009; Vollmer et al., 2011), the full dataset analyzed consisting, thus, of 775 isolates.

2.6. Phylogenetic analysis and clustering

The cluster analysis was performed on the IGS sequences and on each of the eight MLST loci, as well as on the concatenated sequences of the latter ones.

Best-scoring maximum likelihood trees were generated using the PhyML online platform (Guindon et al., 2010), with a general time reversible (GTR) model of DNA evolution and Subtree-pruning–regrafting (SPR) and Nearest Neighbor Interchanges (NNI) for tree improvement, with 100 bootstraps.

We identified clusters of *Borrelia* isolates in the phylogenetic tree. For this, we retained, using Biopython 1.65, only the clusters with a strong bootstrap support (i.e. greater than 90%) in the best-scoring phylogenetic tree.

2.7. Rarefaction analysis

To test our hypothesis of the differential pathogenicity (whether all the *Borrelia* spirochetes found in ticks are infectious to humans or the ones that are found in clinical manifestations are only a subset) we counted distinct STs of *B. burgdorferi* s.l. identified in clinical samples. Similarly, we counted distinct STs identified in tick samples. We ran a rarefaction analysis, implemented in EstimateS 9 (Colwell, 2013) for individual-based abundance data, with 100 runs, randomization of individuals without replacement, and extrapolation to 2000 individuals. We

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