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The expanding Lyme *Borrelia* complex—clinical significance of genomic species?

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Abstract

Ten years after the discovery of spirochaetes as agents of Lyme disease in 1982 in the USA, three genomic species had diverged from the phenotypically heterogeneous strains of *Borrelia burgdorferi* isolated in North America and Europe: *Borrelia afzelii*, *B. burgdorferi* sensu stricto (further *B. burgdorferi*), and *Borrelia garinii*. Whereas *B. burgdorferi* remained the only human pathogen in North America, all three species are aetiological agents of Lyme borreliosis in Europe. Another seven genospecies were described in the 1990s, including species from Asia (*Borrelia japonica*, *Borrelia turdi*, and *B. tanukii*), North America (*Borrelia andersonii*), Europe (*Borrelia lusitaniae* and *Borrelia valaisiana*), and from Europe and Asia (*Borrelia bissettii*). Another eight species were delineated in the years up to 2010: *Borrelia sinica* (Asia), *Borrelia spielmanii* (Europe), *Borrelia yangtze* (Asia), *Borrelia californiensis*, *Borrelia americana*, *Borrelia carolinensis* (North America), *Borrelia bavariensis* (Europe), and *Borrelia kurtenbachii* (North America). Of these 18 genomic species *B. afzelii*, *B. burgdorferi* and *B. garinii* are the confirmed agents of localized, disseminated and chronic manifestations of Lyme borreliosis, whereas *B. spielmanii* has been detected in early skin disease, and *B. bissettii* and *B. valaisiana* have been detected in specimens from single cases of Lyme borreliosis. The clinical role of *B. lusitaniae* remains to be substantiated.

Keywords: *Borrelia afzelii*, *Borrelia burgdorferi*, *Borrelia garinii*, clinical relevance, genomic species

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Introduction

Lyme borreliae may be considered postmodern pathogens, because the illness they cause varies, does not have a predictable incubation period or course, and is likely to have a variable response. Protean manifestations and the absence of techniques to identify the organism in cases of Lyme borreliosis lead to bizarre ideas, and fantasies [1–3].

From Lyme Spirochaete to *Borrelia burgdorferi* Sensu Lato

In 1982, after the discovery of Lyme spirochaetes in hard ticks from Long Island, NY, USA [4], the aetiology of Lyme disease was confirmed by the cultivation of these spirochaetes from skin, blood and cerebrospinal fluid (CSF) of patients [5,6]. The Lyme spirochaete was identified as a new species of the genus *Borrelia* [7]. It very quickly became evident that not only did the clinical presentation of a *Borrelia burgdorferi* infection in Europe differ somewhat from that in North America, but so did the isolates from Lyme borreliosis patients and from ticks [8–10]. It was observed that an increasing number of European isolates of Lyme borreliae from patients and ticks were phenotypically heterogeneous and differed from the American type strain of *B. burgdorferi*. Thus, it was concluded that *B. burgdorferi* may comprise different genomic species, which, however, share common epitopes that are recognized by certain monoclonal antibodies. A serotyping system based on monoclonal antibody reactivity against the outer surface protein OspA was introduced. At the subspecies level, heterogeneity was demonstrated by restriction endonuclease analysis, hybridization with whole *B. burgdorferi* DNA or specific probes, and plasmid analysis. Genetic analysis of the broad variety of phenotypically defined strains was required in order to identify genotypic clusters [11]. The first result of an approach to classify Lyme borreliae on the basis of genomic

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criteria was the delineation of three DNA groups, namely of genospecies *B. burgdorferi* sensu stricto (further *B. burgdorferi*), *Borrelia garinii* sp. nov., and group VS461; all of these strains were associated with clinical Lyme borreliosis [12]. In a similar study, genomic fingerprinting by an arbitrarily primed PCR with *Borrelia* isolates predominantly from *Ixodes* species and mice from North America, Europe and Japan delineated three *Borrelia* groups [13]. These results were in complete agreement with the results of the previously cited study [12]. However, two isolates were distinct from all of the other strains in the collection but were clearly members of the genus *Borrelia* [13]. Later, group VS461 strains were identified with monoclonal antibodies and named *Borrelia afzelii*. On the basis of a small number of *Borrelia* isolates from the skin of patients suffering from acrodermatitis chronica atrophicans, a skin manifestation of European Lyme borreliosis, but also recovered from erythema migrans, it was stated that *B. afzelii* sp. nov. is the only member of this group to result in acrodermatitis chronica atrophicans [14].

Expansion of the Lyme *Borrelia* Complex during the 1990s

Genomic fingerprinting of *B. burgdorferi* sensu lato strains by pulsed-field gel electrophoresis (PFGE) showed that all isolates used in this study were recognized by one band (135 kbp), each of the *B. garinii* isolates by two bands (220 and 80 kbp), and each of the *B. afzelii* isolates by three bands (460, 320 and 90 kbp). Whilst there were differences in the PFGE patterns among *B. burgdorferi* and *B. garinii* isolates, the patterns of *B. afzelii* isolates were all similar [15]. The number of genomic species was further expanded by the characterization of borreliae isolated from *Ixodes ovatus* ticks in Japan. A new species, apparently not a human pathogen and restricted to Japan [16], was hence named *Borrelia japonica* [17]. Another study focused on the ribosomal genes of *B. burgdorferi* [18], using restriction polymorphism analysis of PCR products obtained with primers at the 3′-end of the first *rrf* gene and at the 5′-end of the second *rrl* gene. An amplicon, 226–266 bp in length, was generated from the *B. burgdorferi* strains tested. Restriction polymorphism analysis of the resulting amplicons with the nuclease *Mse*I permitted identification of the established species *B. burgdorferi*, *B. garinii*, *B. afzelii*, and *B. japonica* (formerly group F63B), and the identification of four new genomic groups. Two of these genomic groups were European strains, and the other two were North American strains. The method developed in that study could be applied for rapid screening of strain collections and for epidemiological and medical purposes [19]. With a similar approach, a new species, named *Borrelia andersonii*, was identified [20]. Genomic typing of borrelial strains isolated from *Ixodes tanuki* and *Ixodes turdus* ticks in Japan revealed two new genospecies, named *Borrelia tanukii* and *Borrelia turdi* [21].

Some researchers recognized the greater variety of *B. burgdorferi*, the sole North American aetiological agent of Lyme borreliosis, which is also present in Europe. The multiplicity of genospecies in Europe might indicate that Lyme borreliae emerged in Europe. However, according to *ospC* typing, there was a closer relationship between the European strains than between those in North America, supporting the reverse conclusion, that *B. burgdorferi* was introduced to Europe from America [22,23]. Despite this, a different view on the origin of *B. burgdorferi* has recently been published [24].

Nevertheless, more genospecies were described. *Borrelia* strains isolated from *Ixodes ricinus* ticks in Switzerland, The Netherlands, and the UK of genomic groups VS116 and M19 were carefully characterized, and their taxonomic status was assessed; as a result of this, new genospecies was proposed, *Borrelia valaisiana* sp. nov., type strain VS116 [25].

Isolates of another genomic species, PotiB2, isolated from *I. ricinus* ticks in Portugal, were studied in detail, and this resulted in the proposal of a new species, *Borrelia lusitaniae*, type strain PotiB2 [26].

Not only was diversity among European *Borrelia* strains being being re-examined, but atypical strains of North American origin, previously designated genomic group DN127, were closely analysed, and it was found that they cluster separately from *B. burgdorferi*. The conclusion was that genomic group DN127 should be referred to as a new species, *Borrelia bissettii* sp. nov., and that other related but distinct strains, which require further characterization, should be referred to as *Borrelia* spp. [27].

Up to this point, ten species within the *B. burgdorferi* sensu lato complex have been recognized, but only three—*B. afzelii*, *B. burgdorferi*, and *B. garinii*—were widely accepted human pathogens. These pathogenic *Borrelia* species were characterized by their vectors, geographical distribution, and organotropism [28].

Expansion of the Lyme *Borrelia* Complex in the New Millenium

The newly described genospecies *B. valaisiana*, a *Borrelia* species isolated from *I. ricinus* ticks in some countries of Europe [25], was also identified in specimens from wild rodents captured on Kinmen Island and from central Taiwan [29]. Borreliae were also isolated from rodents and ixodid ticks collected in southern China. Molecular characterization of

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these isolates from white-bellied rats (*Niviventer confucianus*) and from *Ixodes ovatus* ticks revealed sufficient diversity to justify a new species, *Borrelia sinica* sp. nov., type strain CMN3T [30]. Meanwhile, analysis of *B. lusitaniae* isolates from North Africa, Tunisia and Morocco revealed only moderate diversity, suggesting that *B. lusitaniae* isolates from North Africa constitute a clone of Portuguese origin [31]. Garden dormice were found to be the reservoir hosts of a novel *Borrelia* species. Its unique biological relationship, together with previous genetic characterization, justified designating this dormouse-associated genospecies as a distinct entity, *Borrelia spielmani* sp. nov., which is considered to be a human pathogen, as it was isolated from patients in central Europe [32]. When those strains were further characterized, the novelty was confirmed, and the spelling of the name was corrected to *Borrelia spielmanii*, type strain PC-Eq17N5T [33]. It was further shown that *B. spielmanii* resists human complement-mediated killing by direct binding of the immune regulators factor H and factor H-like protein 1 [34].

By multilocus sequence analysis (MLSA), five *B. valaisiana*-related strains isolated from rodents and ticks in southwestern China were eventually classified as a new genospecies of *B. burgdorferi* sensu lato rather than *B. valaisiana*; these strains were named *Borrelia yangtze* [35]. MLSA clarified our understanding of the taxonomy of *B. burgdorferi* sensu lato, by replacing the cumbersome DNA–DNA hybridization method. With this method, a new North American genospecies was delineated, and named *Borrelia californiensis* [36].

In 2009, another three novel species, two from North America and one from Europe, were delineated by MLSA: *Borrelia americana*, *Borrelia carolinensis*, and *Borrelia bavariensis*, respectively. The first genospecies resulted from analyses of isolates from nymphal *Ixodes minor* ticks collected in South Carolina, and showed close relatedness to California strains known as genomospecies 1, associated with *Ixodes pacificus* [37]. The second resulted from the phylogenetic analysis of isolates from rodents from North Carolina, and was therefore named *B. carolinensis* [38]. A formal description of *B. carolinensis* sp. nov., type strain SCW-22T, isolated from rodents and ticks from the southeastern USA, followed [39]. The description of the third novel genospecies resulted from analysis of the rodent-associated ecotypes of *B. garinii*, OspA serotype 4 strains [40], which were sufficiently genetically distinct from bird-associated *B. garinii* strains. Thus, it was concluded that this distinction deserves species status, and the name *B. bavariensis* sp. nov. was suggested [41].

MLSA of the phylogenetic relationships of North American *B. bissettii* disclosed a cluster of strains that were more distant from *B. bissettii* than *B. carolinensis*, with the proposition that these constitute a new *Borrelia* genospecies. The name *Borrelia kurtenbachii* sp. nov. was suggested, in honour of the late Klaus Kurtenbach [42]. Fig. 1 shows the increasing numbers of genospecies defined within the 28 years after the discovery of the 'Lyme spirochaete'.

Methods used for the Identification of Novel *Borrelia* genospecies

The method of whole DNA–DNA hybridization (WDDH) still represents the reference standard for species delineation [33,43]. According to Wayne *et al.* [43], DNA relatedness above 70% and a ΔT_m below 5°C are the basic characteristics within a phylogenetic species. Large restriction fragment length polymorphism analysis with *Mlu*I digestion of *Borrelia* genomic DNA followed by subsequent resolution of the fragments by PFGE provides an alternative to the WDDH approach, and allows for species and strain determination [15,44]. Cultivability, however, is a prerequisite for both of the above techniques, and in addition to this, the application of WDDH for slow-growing bacteria is limited [33]. However, isolation introduces a selection bias, as not all strains grow equally well in culture [45]. To circumvent these problems, improved high-resolution typing methods have been applied to the borreliae. PCR-based restriction fragment length polymorphism (RFLP) has been shown to be an exceptionally valuable method for species and even subspecies discrimination [46]. RFLP of the 5S (*rrfA*)–23S (*rrlB*) intergenic spacer region, as described by Postic *et al.*, has been extensively used as a fast and simple method for molecular typing of *Borrelia* species [19,47,48]. RFLP of the 16S (*rrs*)–23S (*rrlA*) intergenic spacer region, although this region is less diverse, presently allows the characterization of three different ribosomal spacer types (RSTs) [49,50]. In addition to the use of RSTs to explore phylogenetic relationships, correlations of certain subtypes with differences in virulence and ability to disseminate have been described [51,53]. Furthermore, RSTs are in strong, although spatially restricted [54], linkage disequilibrium with *ospC*, another marker that is frequently used in both classic serotyping and genotyping of *Borrelia* [52,55]. As sequence-based methods have largely replaced other methods for microbial population analysis [56], Richter *et al.* [33] applied MLSA to the borreliae. Using seven different loci (*rrs*, *fla*, *groEL*, *hbb*, *recA*, *ospA*, and the *rrf–rrl* spacer), Richter *et al.* [33] successfully confirmed the delineation of *B. spielmanii*, and the results showed excellent correlation with WDDH; also, this approach was less demanding and thus more robust than DNA–DNA reassociation methods [33]. As stated above, MLSA has subsequently been used for the delineation of novel *Borrelia* species such as *B. spielmanii* and *B. carolinensis* [33,38]. MLSA has subsequently

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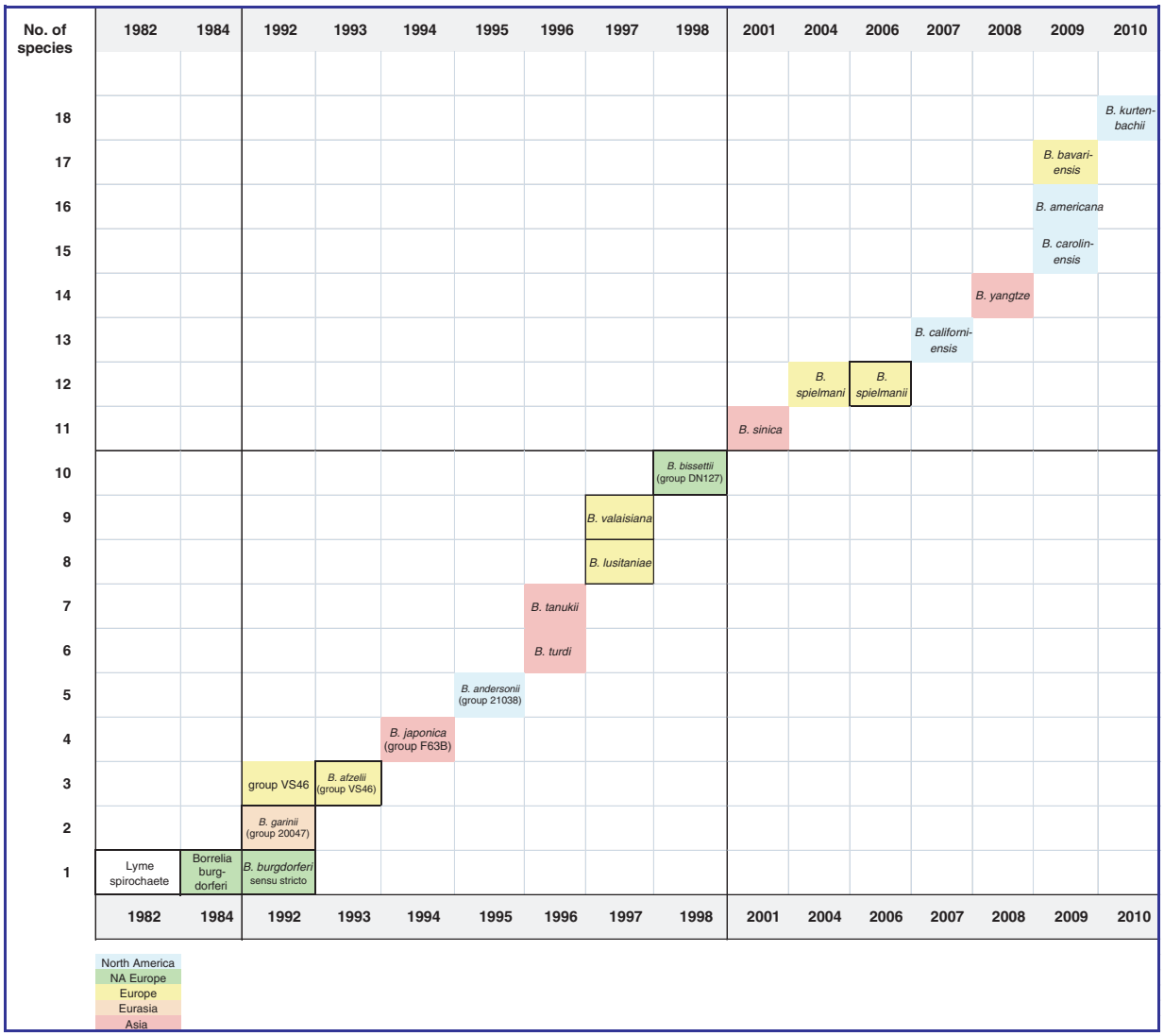

FIG. 1. The increasing number of genospecies of the *Borrelia burgdorferi* sensu lato complex. Genospecies that are proven agents of Lyme borreliosis are framed in bold; those suspected of having clinical relevance are framed by a fine line. The colours indicate the geographical areas where the various genospecies were detected. NA Europe, North America and Europe.

been used for the delineation of novel *Borrelia* species and, in the recent past, several systems were designed, differing in the choice of genes. For example, a set of five loci (*rrf–rrl* spacer, *rrs*, *fla*, *ospA*, and *p66*) was used to clarify the taxonomic status of 16 uncharacteristic *Borrelia* isolates [38]. However, for the most recent delineation of *B. kurtenbachii*, Margos *et al.* [42] used another set of genetic loci (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*), all located on the linear chromosome.

Clinical Relevance of Genomic Species

Although the *B. burgdorferi* sensu lato complex now comprises up to 18 *Borrelia* species, only three of them are clearly pathogenic for humans, namely *B. afzelii*, *B. burgdorferi*, and *B. garinii* (including *B. garinii* OspA type 4, recently named *B. bavariensis*). They can cause localized skin manifestations and disseminated infection by invading various tissues, including the nervous system, the joints, and the skin. *B. spielmanii* was isolated repeatedly from lesional skin of erythema migrans patients [1]. There are also reports on the isolation or detection of *B. bissettii* and *B. valaisiana* from specimens of patients suffering from Lyme borreliosis [57,58]. It is, however, unclear what role *B. lusitaniae* plays, as the described clinical manifestations do not match with the known features of Lyme borreliosis [59]. However, the predominant pathogens in central Europe are *B. afzelii* and *B. garinii* (including *B. garinii* OspA type 4, recently named *B. bavariensis*). Among nearly 500 skin

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isolates from patients with erythema migrans in Slovenia, 89% were identified as *B. afzelii*, 11% as *B. garinii*, and only 0.4% as *B. burgdorferi* [1]. According to the detection rates from CSF of patients suffering from Lyme neuroborreliosis in Europe, *B. garinii* is most often the causative agent, followed by *B. afzelii* and *B. burgdorferi*. *B. bissettii* was isolated only once, and the presence of *B. valaisiana* was confirmed in three cases [1].

A comparison of the epidemiological and clinical characteristics of patients with erythema migrans caused by either *B. afzelii* or *B. garinii* yielded 200 consecutive adult patients with skin isolates of *B. afzelii* and 53 with isolates of *B. garinii*. It was found that *B. garinii* patients were older, had skin lesions more often located on the trunk but less often on the extremities, had shorter incubation and faster evolution of erythema migrans, more often had local and certain systemic symptoms, more often had abnormal liver function test results, and more frequently showed seropositivity [60].

In a long-term Slovenian study of adults with culture-confirmed Lyme neuroborreliosis, isolates were similarly identified as either *B. garinii* or *B. afzelii*. It was found that patients with *B. garinii* isolated from their CSF had a distinct clinical presentation from that of patients with *B. afzelii*. *B. garinii* causes what, in Europe, is appreciated as typical early Lyme neuroborreliosis (Garin–Bujadoux–Bannwarth syndrome), whereas the clinical features associated with *B. afzelii* are much less specific and more difficult to diagnose [61]. Results from North America, where *B. burgdorferi* is the only pathogenic agent of Lyme borreliosis, showed that, among the subtypes differentiated by RFLP analysis of the 16S–23S rDNA intergenic spacer region, there was a predominance of genotypes 1 and 2 (70%). Similarly, these were much more likely to cause disseminated infection than genotype 3 isolates [62]. Correlation of *B. burgdorferi* subtypes with clinical presentation has been substantiated by others, and might hold the key to explaining the protean manifestations of Lyme borreliosis [53]. Although many new species have been described, their pathogenic role remains questionable. On a cautionary note, in those exposed to frequent tick bites, exposure to these newer members of the genus might result in seroreactivity that could obscure or complicate clinical diagnosis.

The results of a more recent study showed that a distinct subset of just four of 16 *ospC* genotypes were responsible for more than 80% of cases of early disseminated Lyme borreliosis [63].

Conclusions

To date, our understanding of European Lyme borreliosis has depended on studies undertaken in limited specialist centres, where clinical isolates can be obtained [60,61,64–67]. To gain more comprehensive insights into the relationships of genospecies and genotypes of Lyme borreliae with clinical presentations, it is essential to initiate concerted multicentre action within Europe.

Transparency Declaration

The authors have no conflicts of interest to declare.

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