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Molecular characterisation of Lyme disease borreliae using RAPD analysis and 16S rDNA sequencing

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Summary. - Here we report the use of random amplified polymorphic DNAs (RAPDs) and sequence analysis of the genes encoding for the small subunit ribosomal RNA (16S rDNA) for the characterisation of *Borrelia burgdorferi* sensu lato strains recovered from *Ixodes ricinus* and from Lyme disease patients. All strains examined were assigned to the species *Borrelia garinii*. However, both RAPDs and 16S rDNAs revealed a level of genetic variation among the strains which appears higher than expected for a bacterial species. In addition, the data obtained agree with the clonal theory applied to *Borrelia burgdorferi* s.l. for explaining some traits of its epidemiology. According to this theory, particular strains should spread rapidly, leading to the diffusion of bacteria with a particular chromosomal genotype. Our results reveal high genetic variation even among strains isolated in the same period from a restricted geographic area. Moreover, the data here reported indicate that clonal diffusion of antigenic characteristics could also occur.

Key words: Borrelia burgdorferi, Borrelia garinii, Lyme disease, RAPD, 16S rDNA.

Riassunto *(Caratterizzazione molecolare di borreliae del gruppo* Burgdorferi *mediante analisi dei RAPDs e sequenziamento del 16S rDNA).* - La presente nota riporta i risultati ottenuti attraverso il sequenziamento dei geni codificanti per l'RNA della subunità ribosomale minore (16S rDNA) e l'analisi dei *random amplified polymorphic DNAs* (RAPDs) di ceppi di *Borrelia burgdorferi* sensu lato ottenuti da *Ixodes ricinus* e da pazienti affetti da malattia di Lyme. Tutti i ceppi esaminati sono stati assegnati alla specie *Borrelia garinii*. Tuttavia, tanto i RAPDs quanto l'analisi del 16S rDNA hanno rilevato livelli di variabilità superiori a quanto atteso per una specie batterica. Inoltre, i dati ottenuti risultano concordi con i modelli che prevedono una diffusione clonale dei ceppi di *Borrelia burgdorferi* s.l. Secondo questi modelli, i ceppi si diffonderebbero rapidamente, portando alla diffusione di batteri caratterizzati da particolari genotipi cromosomici. Di fatto i nostri dati rilevano alti livelli di variabilità anche fra ceppi isolati nella medesima area geografica e nello stesso periodo. Inoltre, i dati qui riportati suggeriscono che alcune caratteristiche antigeniche potrebbero diffondersi con modalità clonale analogamente a quanto sembra avvenire per le caratteristiche genetiche specificate a livello cromosomico.

Parole chiave: Borrelia burgdorferi, Borrelia garinii, malattia di Lyme, RAPD, 16S rDNA.

Introduction

Three different species of *Borrelia burgdorferi* sensu lato *(B. burgdorferi* sensu stricto, *B. garinii, B. afzelii)* have been associated with Lyme disease in Europe [1-4]. Within each species, a high level of genetic variation is also present among the different strains. From the epidemiological point of view, an accurate characterisation of the strains recovered in a particular area is thus very important, and comparison of strains from different areas would allow to detect their origin and investigate their capacity for geographic diffusion. From the diagnostic point of view, when testing serological methods, it is important to use isolates only after an accurate (genetic, molecular, and biochemical) characterisation.

Two methods are currently used for the rapid identification of *B. burgdorferi* s.l.: a surface immunofloresence assay (SIFA) for serological identification [5], and PCR for molecular identification [6, 7]. During an epidemiological survey, 12 strains of *B. burgdorferi* s.l. were characterised using both SIFA and PCR (data recorded at the Section of Microbiology, DMCSS, Università di Bologna). The two methods gave unmatching results: PCR identified all the examined strains as *B. garinii,* whereas SIFA assigned to this species only 6 strains (SIFA+); the remaining 6 strains (SIFA-) could not be assigned to any of the known species of *B. burgdorferi* s.l. The primary aim of the present study was thus to obtain a molecular characterisation of the 12 strains. This was achieved by sequence analysis of the genes encoding for the small subunit ribosomal RNA

(16S rDNA) [8]. A secondary goal was to estimate the genetic variation among these strains. This was achieved by random amplified polymorphic DNA (RAPD) analysis [9]. In particular, the study attempted to estimate the relationships between the strains examined, relating these to the antigenic response to SIFA.

Materials and methods

Twelve strains of *B. burgdorferi* s.l. were included in this study (Table 1). Amplification and sequencing of 16S rDNA was effected on 9 strains (VS102, VS185, VS286, VSBp, K48, 3251/3, 3251/5, 2872/4, PBr). RAPD analysis was effected on all strains. Three strains were from Lyme disease patients (PBr, P/Bi, VSBp), the remaining from *Ixodes ricinus* ticks. It should be noted that BITS was the first *B. burgdorferi* strains isolated from ticks in Italy [10]. Strains 3251/3, 3251/5 and 2872/4 were collected in one month (June 1993) from a restricted area (the forest around the Lamar Lakes, TN). DNA was extracted from the cultured *B. burgdorferi* according to standard procedures [11]. Amplification and sequencing of 16S rDNA was effected using universal eubacterial primers according to the procedures reported in Bandi *et al.* [12] and in Damiani *et al.* [13]. RAPD analysis was effected according to Bandi *et al.* [14] using primers 494New, 495New, NP1, NP3New and D17 [12-15]. The 16S rDNA sequences obtained were aligned with those of representatives of the spirochete group of the eubacteria, and analysed according to distance matrix and parsimony methods [16]. RAPD bands were codified as two state characters (presence/absence) and analysed according to distance matrix methods [16].

Results

The 16S rDNA amplification products obtained from 9 strains of *B. burgdorferi* s.l. were sequenced for about a half of the gene sequence (700 base pairs, bp). These sequences have been deposited in the EMBL Data Library. Alignment with the available *B. burgdorferi* s.l. 16S rDNAs revealed, in general, high similarity of the obtained sequences with those of *B. garinii*. Fig. 1 shows a distance-matrix tree including the 9 sequences obtained together with those of two reference strains of *B. garinii* (G1 and G2) and two of *B. burgdorferi* s.s. The 9 new sequences, and those of G1 and G2, form a monophyletic grouping, supported by bootstrap analysis (93%). Most sequences appear closely related to those of the reference strains, with the exception of the sequence derived from 3251/3 which stems very deeply if compared to the other sequences. Three SIFA+ strains (K-48, 2872/4, VSBp) are grouped in the same cluster, while a fourth one (VS286) groups with SIFA- strains. Parsimony analysis revealed the same groupings, with the SIFA+ K-48, 2872/4, VSBp in the same cluster, VS286 among the SIFA-, and 3251/3 well differentiated from the remaining strains.

Table 1. - Twelve strains of *Borrelia burgdorferi* sensu lato examined by 16S rDNA and/or RAPD analyses, and their response to SIFA test

Strain	Host	Geographic origin	Analyses	SIFA
VS102	*Ixodes ricinus*	Switzerland	16S + RAPD	-
VS116	*Ixodes ricinus*	Switzerland	RAPD	-
VS185	*Ixodes ricinus*	Switzerland	16S + RAPD	-
VS286	*Ixodes ricinus*	Switzerland	16S + RAPD	+
VSBp	man - cerebrospinal fluid	Switzerland	16S + RAPD	+
K-48	*Ixodes ricinus*	Italy	16S + RAPD	+
2872/4	*Ixodes ricinus*	Italy	16S + RAPD	+
3251/3	*Ixodes ricinus*	Italy	16S + RAPD	-
3251/5	*Ixodes ricinus*	Italy	16S + RAPD	-
BITS	*Ixodes ricinus*	Italy	16S + RAPD	+
P/Bi	man - cerebrospinal fluid	Germany	RAPD	+
PBr	man - cerebrospinal fluid	Germany	RAPD	-

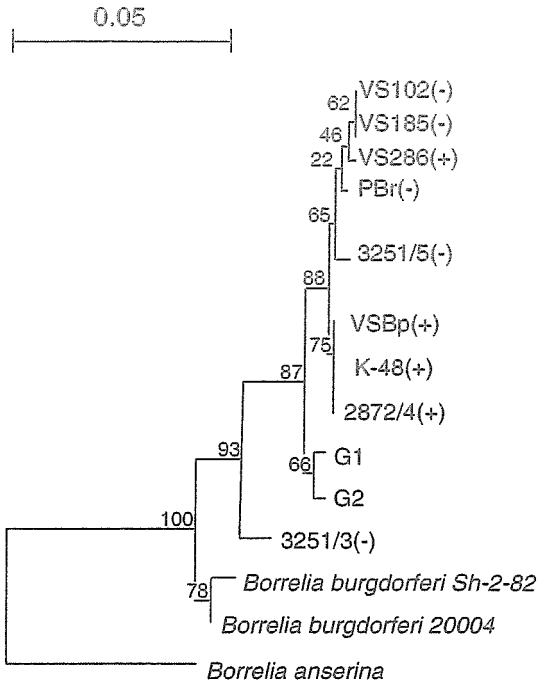

Fig. 1. - Distance matrix tree (Kimura correction; neighbour joining method) illustrating the positioning, as revealed by 16S rDNAs, of the examined borrelia strains in relation to reference strains of *Borrelia garinii* (G1, G2) and of *Borrelia burgdorferi* s.s.(Sh-2-82; 20004). *Borrelia anserina* is included as an outgroup. The numbers at the nodes are the bootstrap confidence values obtained after 100 replicates. The scale bar indicates the distance in substitutions per nucleotide.

In addition to the above 9 strains, three further strains were examined by RAPD analysis. RAPD analysis produced electrophoretic patterns of 20-40 bands, with each strain showing a different pattern. By joining the data produced using 5 primers, a data matrix of 12 X 180 bands was obtained. An example of a distance-matrix tree derived from this data set is shown in Fig. 2. The tree agrees with that based on 16S rDNA. In particular, SIFA+ strains are grouped in a single cluster, with the exception of VS286. Moreover, strain 3251/3 appears well differentiated from most strains, with the exception of VS116 (not included in the analyses of 16S rDNAs), whose branch appears as the deepest one.

Discussion

Based on the 16S rDNA data, 8 out of the 9 strains appear very similar and closely related to the reference strains of *B. garinii* G1 and G2 (Fig. 1). We can thus confirm the identification of these strains as *B. garinii*. The identification of the remaining strain (3251/3) remains uncertain. Compared to the reference strains, 3251/5 shows twice the number of base substitutions. Phylogenetic analysis groups all the examined strains, including 3251/3, with the reference *B. garinii* G1 and G2 with high bootstrap support. However, within this grouping, 3251/3 stem as a very deep branch. RAPD analysis gave results congruent with those revealed by 16S rDNA sequencing, with evidence for a high divergence of strain 3251/3 from the "standard" *B. garinii*. In addition, RAPDs revealed a high degree of divergence of 3251/3 and VS116 from the other strains. It should be noted that some (VS185, VS286, P/Bi, PBr, K-48, BITS, VS116) of the strains included in our study have been examined by other authors [4] through the analysis of the ribosomal intergenic spacer *rrf(5S)-rrl(23S)*. According to these authors, VS116 does not belong to *B. garinii*, and could represent a new species. Thus, while 16S rDNAs agree with RAPDs in indicating 3251/3 as a strain of uncertain position, RAPDs agree with *rrf(5S)-rrl(23S)* data in indicating VS116 as a strain well differentiated from *B. garinii*.

Strain 3251/3 was isolated from *I. ricinus* specimens collected from the forest around the Lamar Lakes in June 1993. During the same month other two strains (3251/5, 2872/4) were isolated from *I. ricinus* from the same forest. These latter strains were unambiguously assigned to *B. garinii*, and show high sequence similarity with the reference strains G1 and G2. Thus, in the same host species, at the same time and in the same area, well differentiated strains of *B. burgdorferi* were present. It should be noted that the percentage nucleotide difference in 16S rDNAs between strain 3251/3 and the other two Lamar strains (3251/5 2872/4) is about 1%. According to the rates of molecular evolution so far calibrated for 16S rDNA [12,17], this nucleotide difference could be explained by an evolutionary separation of 25-50 million years, and is usually observed between bacteria assigned

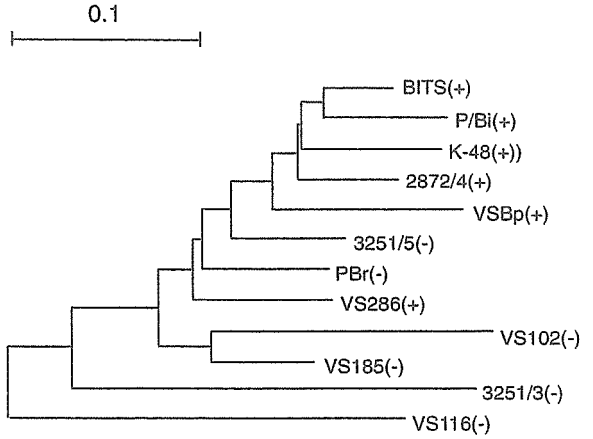

Fig. 2. -Similarity tree (simple matching coefficient (SM); neighbour joining method) illustrating the relationships among the examined borrelia strains as revealed by RAPDs. No outgroup was included (midpoint rooting). The scale bar indicates the dissimilarity according to 1-SM.

to different species. On the whole, the above data agree with the hypothesis of a clonal epidemiology for *B. burgdorferi* s.l., which suggests the spread of strains with particular chromosomal genotypes [18]. Of course, the differences observed among the Lamar strains could have arisen after local evolution from a common ancestor. However, RAPD analysis does not reveal any grouping of strains derived from a particular area. Finally, we should consider that while strains 3251/3 and 3251/5 are both SIFA-, strain 2872/4 is SIFA+. Thus, the two closely related strains (as evidenced by 16S rDNA) 3251/5 and 2872/4 appear to have different antigenic profiles. This agrees with the data reported in by several authors on the rapid evolution of antigenic variation in *B. burgdorferi* s.l.

SIFA serological test for the identification of *B. burgdorferi* subspecies is based on the use of polyclonal antibodies. The antigens recognised by SIFA are not completely characterised; in any case, the most prominent surface antigens are the target of this method [5]. In man, the response against the main outer surface proteins of *B. burgdorferi* (OspA and OspB) is not constant, while the ability of human sera to recognise surface antigens of *B. burgdorferi* in SIFA tests is constant six months post-infection [5]. We can thus assume that the antigens recognised by SIFA are at least in part different from OspA and OspB. RAPD and 16S rDNA analyses support this hypothesis. Both rDNA and RAPD trees show most SIFA+ strains grouped. This agree with the possibility that the genes controlling them are subjected to vertical (and clonal) transmission together with rDNA genes and with chromosomal DNA (from which we can assume that most RAPDs are generated). On the other hand, OspA and OspB genes are located on plasmid DNA, and are subjected to extensive intra- and inter-genic recombination leading to non-clonal evolutionary dynamics [18-20]. Thus, the data here reported suggest that antigen coding genes, different from Osp genes, could be present in the genome of *B. garinii* and could be located on the chromosomal DNA, or on plasmids not subjected to extensive recombination and orizontal transmission phenomena (like the plasmid carrying Osp genes). Future attempts towards the development of vaccines and serological tests for Lyme disease should thus consider the possibility of finding antigens which are more stable than Osp-A and -B.

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