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Genotypic and Phenotypic Analysis of *Borrelia burgdorferi* Isolates from The Netherlands

LIESEL M. K. E. NOHLMANS,[1]* ROB DE BOER,[2] ANTON E. J. M. VAN DEN BOGAARD,[1] AND CEES P. A. VAN BOVEN[1]

Department of Medical Microbiology, State University of Limburg, Maastricht,[1] and Department of Pure and Applied Ecology, University of Amsterdam, Amsterdam,[2] The Netherlands

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Sixty-three *Borrelia burgdorferi* isolates recovered from *Ixodes ricinus* ticks collected in 17 locations in The Netherlands and three Dutch human skin isolates were characterized by rRNA gene restriction fragment length polymorphism, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotting (immunoblotting). All three human isolates belonged to *B. burgdorferi* group VS461. Of the tick isolates, 29 (46%) were *B. burgdorferi* sensu stricto, 2 (3%) were group VS461, 19 (30%) were *Borrelia garinii*, and 13 (21%) were different from any previously described genomic species. On the basis of the criteria described, 12 isolates formed a distinct genomic group, designated M19. rRNA gene restriction patterns of the group M19 isolates resembled but were not identical to the *B. garinii* patterns. Hybridization of digested DNA with a flagellin probe confirmed the separation of group M19 from the *B. garinii* isolates. One isolate, M63, was different from all the others. In conclusion, the occurrence of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. burgdorferi* group VS461 in ticks from The Netherlands corresponds with the occurrence of these genomic species among tick isolates from other European countries. However, our findings suggest that *B. burgdorferi* sensu lato probably contains more than three genomic species.

The spirochete *Borrelia burgdorferi* is the causative agent of Lyme borreliosis, a multisystemic disease which is considered now to be the most prevalent tick-borne disease in North America, Europe, and other parts of the world with a temperate climate (42, 49). Clinical manifestations of Lyme borreliosis depend upon the stage of the infection. Erythema migrans (EM), a localized skin lesion, develops first at the site of the tick bite. Subsequent dissemination of spirochetes from the site of inoculation may result in migratory pain in joints and muscles, headache, neuropathy, and cardiac manifestations. Late infection is characterized by chronic arthritis, progressive encephalomyelitis, or acrodermatitis chronica atrophicans (49). Manifestations and disease severity are highly variable, and asymptomatic infection may occur (19, 22, 36, 49). The patterns of disease in Europe and the United States appear to be different. Acrodermatitis chronica atrophicans and neuroborreliosis seem to be more common in Europe, whereas arthritis appears to be more prevalent in the United States (49, 55).

B. burgdorferi was first isolated from *Ixodes scapularis* (formerly known as *Ixodes dammini*) in 1982 by Burgdorfer et al. (14, 57). In 1984, Johnson et al. (25) identified this spirochete as a new species belonging to the genus *Borrelia*. Thereafter, numerous *B. burgdorferi* isolates have been obtained from ticks and humans, especially in the United States and Europe (2, 26, 27, 33–35, 39, 40, 43, 48, 50, 51, 53). The results of several studies in which workers used protein electrophoresis patterns and reactivity with monoclonal antibodies (MAbs) (3, 8, 9, 12, 13, 15, 28, 29, 47, 58), restriction endonuclease analysis (4, 31, 38), sequencing of rRNA (1, 32), PCR (41, 56), plasmid profiles (5, 46), DNA-DNA reassociation (4, 38), and ultrastructure analysis (23) have shown that *B. burgdorferi* is quite heterogeneous. Recently, in a study by Baranton et al. (4) in which isolates from Europe, the United States, and Asia were examined, *B. burgdorferi* sensu lato was split into the genomic species *B. burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and *B. burgdorferi* group VS461 on the basis of DNA relatedness. This classification corresponds to divisions based on other techniques and approaches, as mentioned above. *B. burgdorferi* sensu stricto was found to be the only genomic species in the United States, whereas among European isolates, all three genomic species occurred. Furthermore, strong evidence that the three genomic species of *B. burgdorferi* exert different organotropic and pathogenic potentials has been found (53, 58). In The Netherlands, the predominance of group VS461 and *B. garinii* in skin and cerebrospinal fluid specimens, respectively, has been demonstrated (53).

In this study, we focused on the distribution of the three *B. burgdorferi* species among ticks collected in broadly distributed locations within The Netherlands. Isolates were characterized by (i) restriction fragment length polymorphism of two conserved regions of the chromosomal DNA, namely, that encoding for 16S+23S rRNA and for the flagellin protein, and (ii) protein electrophoresis followed by Coomassie blue staining, Western blotting, and reactivity with MAbs.

MATERIALS AND METHODS

***Borrelia* isolates and culture procedures.** In this study, a total of 66 Dutch *B. burgdorferi* isolates were analyzed: 63 *Ixodes ricinus* isolates (M01 to M63) and three human isolates (MH01, MH02, and MH03) recovered from skin biopsies of three Dutch patients with EM. The tick isolates had been obtained during a 1989 surveillance to study the occurrence of *B. burgdorferi* in *I. ricinus* in The Netherlands (35). In this surveillance, nymphal and adult ticks were collected from several parts of the country between June and September inclusive by means of flagging. Figure 1 presents the 17 geographic locations from which the 63 tick isolates used were

* Corresponding author. Present address: Laboratorium voor de Volksgezondheid, Postbus 9025, 6800 EG Arnhem, The Netherlands. Phone: 085 777880. Fax: 085 777279.

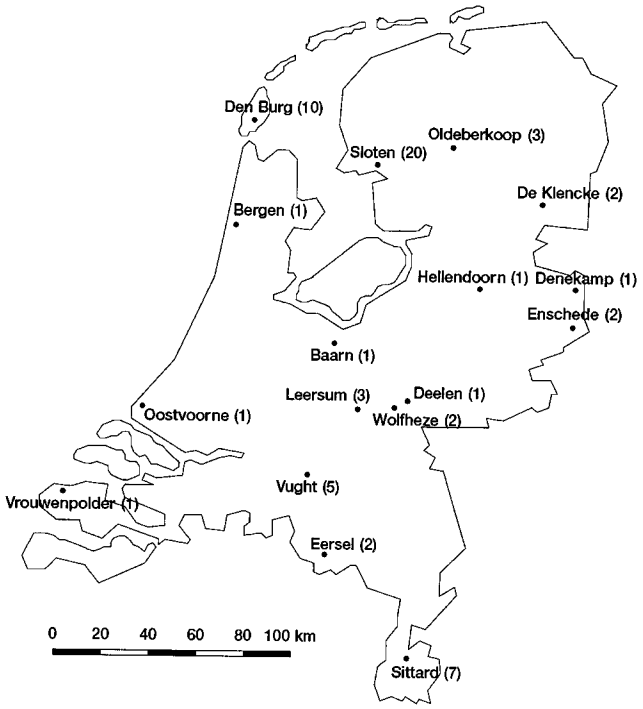

FIG. 1. Geographic locations of *I. ricinus* collection sites in The Netherlands and number of *B. burgdorferi* isolates (in parentheses) recovered from each site.

recovered. The number of *I. ricinus* isolates per geographic location ranged from 1 to 20. All 66 isolates used in this study were identified as *B. burgdorferi* with a modified immunofluorescent antibody assay using the serum from a patient with Lyme borreliosis as described by Wilske et al. (61). *B. burgdorferi* was isolated from ticks and human specimens by using BSKII medium (6) with the addition of neomycin, sulfamethoxazole, and trimethoprim, as described previously (35) and, after one passage in BSKII medium without antibiotics, was stored at −76°C. As references, we used the American tick isolate B31 (ATCC 35210) and the German EM isolate PKo (kindly provided by B. Wilske, Max von Pettenkofer Institute, Munich, Germany), which are known to belong to the genomic species *B. burgdorferi* sensu stricto and group VS461, respectively (4, 53, 58). These two reference isolates were passaged once in BSKII in our laboratory and then stored at −76°C. For the purpose of this study, all isolates were thawed and passaged once in BSKII medium, and then 7- to 10-day-old cultures were used for protein and DNA preparation.

DNA preparation. For the preparation of chromosomal DNA, approximately 5×10^{10} cells were suspended in TES (25 mM Tris-HCl [pH 8.0], 10 mM EDTA, 15% [wt/vol] sucrose), incubated for 15 min in the presence of lysozyme (≥0.5 mg/ml on ice), and shaken after the addition of sodium dodecyl sulfate (SDS; 0.5%). Ammonium acetate (2.2 M) was added, and the resulting precipitate was separated by centrifugation (20,000 × *g* for 10 min). The pellet was resuspended, treated with RNase (0.1 mg/ml, 1 h, 37°C), and subsequently treated, in the presence of SDS (1%), with proteinase K (0.12 mg/ml, 1 h, 37°C). The DNA was extracted with phenol-chloroform (two or three times), precipitated by adding 0.1 volume of 3 M sodium acetate and 2 volumes of 96% ethanol, and resuspended in water.

Restriction enzyme digests, agarose gel electrophoresis, and Southern blot. DNA was digested with *Hin*dIII or *Eco*RV endonuclease (Pharmacia) as described in the instructions of the manufacturer. Electrophoresis was performed with 0.8% agarose in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA [pH 8.0]) at 90 V for 3 h. As a molecular mass standard, *Hin*dIII-digested λ DNA was included in each gel, showing six clearly visible fragments (23.13 to 2.02 kbp). DNA samples of the two reference isolates (B31 and PKo) were also included in each gel. DNA fragments were transferred to Hybond membranes (Amersham International, Berks, England) by means of a horizontal capillary system as described in the instructions of the manufacturer.

Hybridization of DNA fragments. Hybridization of transferred fragments was performed essentially as described by Grimont et al. (21). 16S+23S rRNA from *Escherichia coli* (Boehringer, Mannheim, Germany) and a 730-bp fragment of the flagellin gene of *B. burgdorferi* B31, which had been prepared as described by Gassmann (20) (kindly provided by S. Rijpkema, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands), end-labelled with [γ-^{32}P]ATP (Amersham) by using T4 polynucleotide kinase (Pharmacia) as described in the manufacturer's instructions, were used as probes. DNA fragments were hybridized at 60°C, and membranes were washed five times with 2× SSC, containing 0.1% SDS, at 55°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). Membranes were autoradiographed with Kodak X-Omat S film and an intensifying screen at −70°C. The gene restriction patterns were classified by visual inspection on the basis of similarity.

Protein electrophoresis. Whole-cell sonicates of the 68 *B. burgdorferi* isolates were prepared essentially as described by Craft et al. (18). The protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (30). In preparing the sample buffer, 2-mercaptoethanol (5% final concentration) was used as the reducing agent. The separation gel (pH 8.8) contained 11% acrylamide. The concentration of acrylamide in the stacking gel (pH 6.8) was 4%. Electrophoresis was performed in a Protean II slab cell (Bio-Rad Laboratories) on 0.5-mm gels with 5 μg of protein per lane. *B. burgdorferi* B31 and low-molecular-mass standards (LMW calibration kit; Pharmacia) were run with each gel. Gels were either stained with Coomassie brilliant blue (R-250; Merck AG, Darmstadt, Germany) or immediately used for blotting. The relative molecular mass (M_r) of major proteins in the ranges of 30 to 36 kDa and 22 kDa were determined by comparing their electrophoretic mobility with that of the standard proteins.

Western blot (immunoblot). Proteins were transferred from the gels to nitrocellulose paper in a Multiphor II Novablot (Pharmacia) by use of a transfer buffer prepared by the method of Towbin et al. (52). Electrophoresis was carried out at 0.8 mA/cm^2 of the trans-unit for 1 h. After transfer, the blots were blocked overnight with 1% bovine serum albumin in phosphate-buffered saline (PBS), subsequently immersed in PBS with the addition of 0.5% Tween 20 (PBS-T), and incubated at room temperature for 30 min. The blots were then incubated with MAb at room temperature for 60 min. MAb was diluted 1:50 (anti-OspA and anti-flagellum) or 1:10 (anti-OspB) in PBS-T. After washing three times with PBS-T, the blots were transferred to a 1:600 dilution of alkaline phosphatase-labelled anti-mouse immunoglobulin G (A1902; Sigma Chemical Co., St. Louis, Mo.) and incubated at room temperature for 60 min. The blots were washed twice with PBS-T, incubated with Tris-$MgCl_2$ (pH 9.5), and transferred to a dimethylformamide

buffer solution containing 165 μg of bromo-chloro-indolyl-phosphate (B8503; Sigma) per ml and 330 μg of nitroblue tetrazolium (N6876; Sigma) per ml. After 5 min of incubation, the enzymatic reaction was stopped by rinsing the blots in Tris-EDTA (pH 7.5).

MAbs. The following MAbs (Symbicom, Umea, Sweden) directed against *B. burgdorferi* B31 were used: H9724 (7) against periplasmic *Borrelia* flagella, H5332 (11) and H3TS (8) against different epitopes of the OspA protein, and H6831 (10) against the OspB protein.

Statistics. χ^2 test and Fisher's exact test were used to test the significance of differences between proportions.

RESULTS

rRNA gene restriction patterns. Our isolates could be subdivided into five groups. The first group was represented by a single isolate, M63, a tick isolate that was different from all the others. Cleavage of its DNA with *Hin*dIII resulted in four fragments hybridizing with the 16S+23S rRNA probe. Two of these fragments (2.1 and 0.6 kbp) were common to all other isolates. The sizes of the two unique fragments were in the region of 1 to 1.5 kbp and, roughly, 3 kbp, respectively. Digestion of M63 DNA with *Eco*RV resulted in three fragments, one of which (3.2 kbp) was common to all other isolates. The other two were in the region of 6 to 7 kbp and, roughly, 3 kbp, respectively. The second group contained *B. burgdorferi* B31 and 29 of our tick isolates. These isolates revealed a *Hin*dIII and *Eco*RV restriction pattern identical to that of genomic species *B. burgdorferi* sensu stricto as described by Baranton et al. (4). Two of these isolates, M11 and M13, both collected near Sloten, The Netherlands, had an additional *Hin*dIII fragment of approximately 2.4 kbp, just like some of the isolates described by Baranton et al. (4). The third group comprised two tick isolates, M10 and M55, and all three of our skin isolates. The *Hin*dIII and *Eco*RV restriction patterns of these isolates were identical to that of the PKo reference isolate and thus belong to group VS461 (4, 15, 58). The *Hin*dIII restriction patterns showed six fragments (Fig. 2, lane 1), i.e., one fragment in addition to those described by Baranton et al. (4). This additional fragment was in the range of 0.9 to 1.5 kbp. The fourth group, comprising 19 tick isolates, had the characteristics of *B. garinii*, i.e., a 5.3-kbp *Eco*RV fragment and a 1.3-kbp *Hin*dIII fragment. Again, we consistently found an additional *Hin*dIII fragment which was not described by Baranton et al. (4), this time in the region of 1.6 to 1.7 kbp. The fifth group, comprising 12 *I. ricinus* isolates, produced restriction patterns which were different from those observed in the *B. garinii* group in that all isolates showed an additional weak band of approximately 0.8 kbp and slightly lower molecular masses of the other four fragments obtained after cleavage with *Hin*dIII (Fig. 2, lanes 2 and 3). Furthermore, the largest fragment observed after digestion with *Eco*RV had a higher molecular mass than the corresponding band observed in the *B. garinii* group (Fig. 3A, lanes 4 to 6 and 7 to 9, respectively). This fifth group was designated the M19 group.

Flagellin gene restriction patterns. *Eco*RV digests were also hybridized with the flagellin probe (Fig. 3B). The restriction patterns of the group M19 isolates were quite uniform (one band in the region 3 to 7 kbp) and different from those of the *B. garinii* isolates, which were uniform among themselves (one band in the region of 10 to 20 kbp). The distinction between *B. garinii* and the group M19 isolates was thereby confirmed.

Protein patterns and reactivity with MAbs. Although Coomassie blue-stained SDS-PAGE protein patterns showed extensive heterogeneity, a limited number of constant patterns concerning several major proteins in the range of 30 to 36 kDa were distinguished (Fig. 4). The distribution of these four protein patterns as well as the corresponding reactivities with MAbs among the five genomic groups is presented in Table 1. The 29 isolates showing the genotypic characteristics of *B. burgdorferi* sensu stricto were quite uniform concerning major protein patterns and reactivity with MAbs H5332 and H3TS directed against OspA and with MAb H6831 directed against OspB. The major protein patterns of the five group VS461 isolates were also uniform and different from the *B. burgdorferi* sensu stricto isolates. These isolates produced major bands at 33 and 35 kDa. None of these isolates were reactive with the MAb H5332, H3TS, or H6831. The *B. garinii* isolates and the group M19 isolates could not be distinguished unambiguously on the basis of protein patterns. A common feature of all these isolates was the absence of reactivity with MAb H6831, directed against OspB, and the absence of a protein band in the 34-kDa range, which separated them from all the others. The isolates were variable concerning the molecular mass of OspA (either 33 or 34 kDa) and their reactivity with MAb H5332, but all four conceivable combinations of these characters (33-kDa protein–H5332$^+$, 33-kDa protein–H5332$^-$, 34-kDa protein–H5332$^+$, and 34-kDa protein–H5332$^-$) were found. Different combinations were found quite often at the same geographic location. A 33-kDa OspA was in 12 cases combined with negative H5332 reactivity and in 9 cases with positive H5332 reactivity. Only 1 of the 10 isolates with a 34-kDa OspA had positive H5332 reactivity (Fisher's exact test, $P > 0.10$, two sided). *B. garinii* had the 33-kDa variant of OspA much more frequently than the group M19 isolates (Fisher's exact test, $P < 0.001$, two sided). Also, reactivity with MAb H5332 was seen relatively more often in the *B. garinii* isolates (Fisher's exact test, $P = 0.05$, two sided). Thirty-seven of the 66 isolates had a prominent protein band at about 22 kDa, corresponding with the OspC region (20 to 25 kDa) (59). The intensity of this band was scored (−, +, or ++) without awareness of the genetic

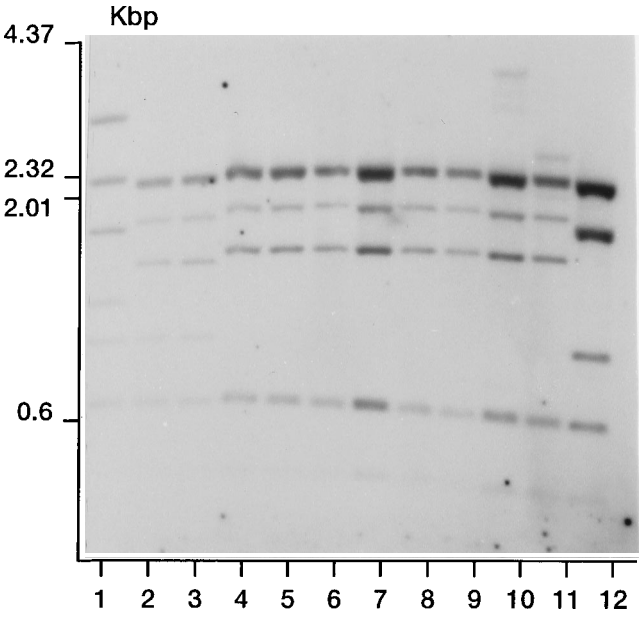

FIG. 2. Gene restriction patterns of *B. burgdorferi* isolates recovered from ticks collected in The Netherlands after cleavage with *Hin*dIII. DNA digests were hybridized with 16S+23S rRNA of *E. coli*. Lanes: 1 and 12, controls PKo and B31, respectively; 2 and 3, isolates typed as group M19; 4 to 11, isolates typed as *B. garinii*.

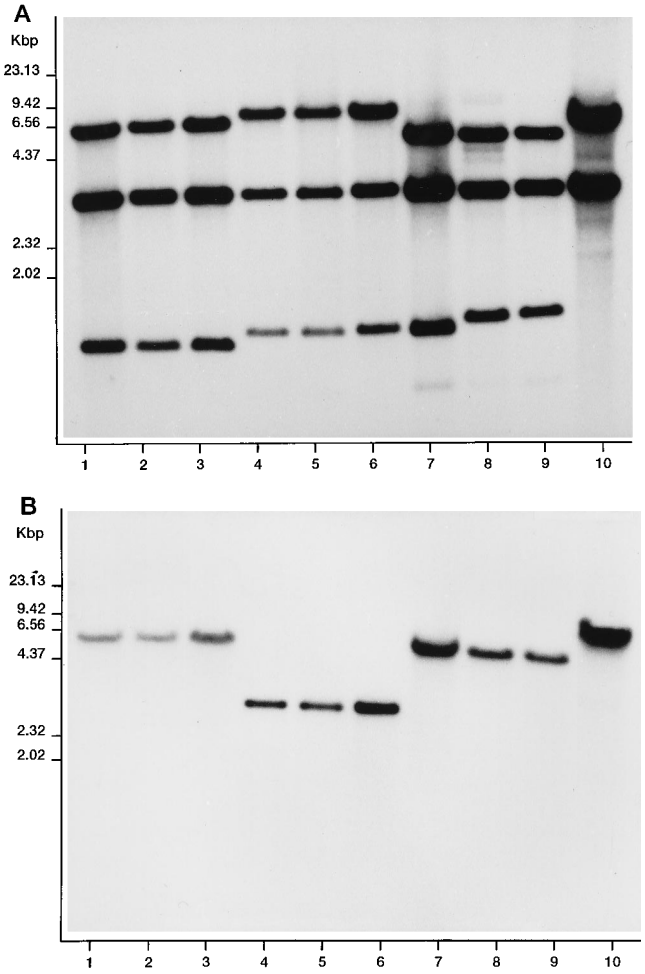

FIG. 3. Gene restriction patterns of *B. burgdorferi* isolates recovered from ticks collected in The Netherlands after cleavage with *Eco*RV. (A) Hybridization of DNA digests with 16S+23S rRNA of *E. coli*; (B) hybridization of the same digests as those of panel A with the flagellum probe. Lanes: 1 and 10, controls PKo and B31, respectively; 2 and 3, isolates typed as *B. burgdorferi* group VS461; 4 to 6, isolates typed as group M19; 7 to 9, isolates typed as *B. garinii.*

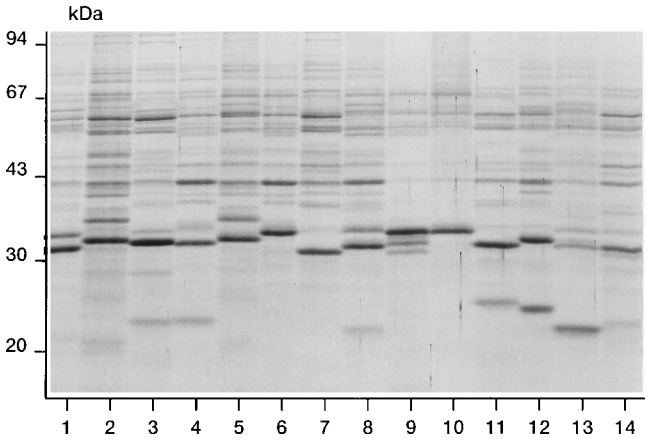

FIG. 4. Coomassie blue-stained SDS-PAGE of whole-cell lysates of *B. burgdorferi* isolated from ticks in The Netherlands. Lanes: 1, control B31; 2, control PKo; 3, 4, and 12, isolates genotyped as *B. garinii*; 5, isolate genotyped as *B. burgdorferi* group VS461; 6, 9, and 10, isolates genotyped as group M19; 7, isolate not genotyped and therefore not included in this study; 8, 13, and 14, isolates genotyped as *B. burgdorferi* sensu stricto; 11, isolate genotyped as group M63.

characters of the isolates. The proportions of 22-kDa negative isolates were significantly different among *B. burgdorferi* sensu stricto (6 of 29 isolates), *B. garinii* (9 of 19 isolates), and the group M19 isolates (11 of 12 isolates) (χ^2 test, degrees of freedom = 2, $P < 0.001$). It is also remarkable that the three group VS461 isolates of human origin were 22 kDa positive (++), whereas the two group VS461 tick isolates were 22 kDa negative.

Geographic distribution of genomic groups. Ticks collected from one location, Sloten, located in the northern part of the Netherlands, yielded 20 isolates. Sixteen of these were *B. burgdorferi* sensu stricto isolates, three were *B. garinii* isolates, and one was a group M19 isolate. The high percentage of *B. burgdorferi* sensu stricto isolates in this sample departs significantly from its representation among the rest of our isolates (χ^2 test, degree of freedom = 1, $P = < 0.001$). The numbers of isolates from the other locations were much smaller and therefore constitute a more balanced sample of tick isolates. A location on the island of Texel, Den Burg, The Netherlands, yielded 10 isolates: 4 were *B. garinii* and 6 were group M19. All seven isolates from Sittard, located in the southern part of the Netherlands, were *B. garinii*. From the remaining locations, we never obtained more than five isolates, and more often than not, the isolates from one location represented two or even three different types.

DISCUSSION

For comparison of our findings with the available information on *I. ricinus* isolates from European countries, the studies of Baranton et al. (4), Wallich et al. (54), and Wilske et al. (58) were consulted. On the basis of DNA-DNA reassociation studies and other genetic characters, Baranton et al. (4) distinguished three genomic species, i.e., *B. burgdorferi* sensu stricto, *B. garinii*, and *B. burgdorferi* group VS461, among 16 *I. ricinus* isolates. Wallich et al. (54) characterized 19 tick isolates which were not included in the Baranton study, and another 17 *I. ricinus* isolates were characterized by Wilske et al. (58), bringing the total to 52. Of these 52 isolates from widely dispersed locations in Europe, 17 (33%) were in the same division as *B. burgdorferi* B31 and thus corresponded to the genomic species *B. burgdorferi* sensu stricto. Of our 63 tick isolates, 29 (46%) were *B. burgdorferi* sensu stricto (χ^2 test, degree of freedom = 1, $P = 0.15$). However, as argued above, a better representation of tick isolates is obtained when the 20 isolates from Sloten are disregarded. Then, 13 (30%) of 43 isolates are *B. burgdorferi* sensu stricto. This percentage also agrees reasonably well with the proportion generally found among European tick isolates (χ^2 test, d.f. = 1, $P = 0.10$). Thus, the genomic species *B. burgdorferi* sensu stricto is commonly found in European *I. ricinus* ticks. Of the three recognized genomic species, this is the only one found to date in North America, where it is associated with rheumatic manifestations that may be relatively rare in European patients with Lyme borreliosis (49, 55). Variability of pathogenic properties apparently also exists within genomic species.

B. burgdorferi group VS461 appears to be less common. Only 4 (8%) of the 52 tick isolates of the combined studies of Baranton et al. (4), Wallich et al. (54), and Wilske et al. (58) belong to this group. This agrees well with our findings. Only two (3%) of our tick isolates were group VS461. It is, however, in striking contrast with the results of van Dam et al. (53). These authors also isolated *B. burgdorferi* from ticks collected

TABLE 1. Molecular masses of major outer membrane proteins and MAb reactivities of *B. burgdorferi* isolates from The Netherlands in relation to their genomic groups

Genomic group[a]	Origin	Geographic location	No. of isolates	Mol mass (kDa)[b]		Reactivity with MAb:			
				OspA	OspB	H9724 (flagellum)	H5332 (OspA)	H3TS (OspA)	H6831 (OspB)
Group M63	*I. ricinus*	Vught	1	31	34	+	+	+	−
B. burgdorferi sensu stricto	*I. ricinus*	Baarn	1	31	34	+	+	+	+
	I. ricinus	Bergen	1	31	34	+	+	+	+
	I. ricinus	Deelen	1	31	34	+	+	+	+
	I. ricinus	De Klencke	1	31	34	+	+	+	+
	I. ricinus	Eersel	1	31	34	+	+	+	+
	I. ricinus	Hellendoorn	1	31	34	+	+	+	+
	I. ricinus	Leersum	3	31	34	+	+	+	+
	I. ricinus	Sittard	1	31	34	+	+	+	+
	I. ricinus	Sloten	16	31	34	+	+	+	+
	I. ricinus	Vught	3	31	34	+	+	+	+
B. burgdorferi group VS461	*I. ricinus*	Oldeberkoop	1	33	35	+	−	−	−
	I. ricinus	Wolfheze	1	33	35	+	−	−	−
	Human skin	Sittard	3	33	35	+	−	−	−
B. garinii	*I. ricinus*	Den Burg	4	33		+	+ or −[c]	−	−
	I. ricinus	De Klencke	1	33		+	+	−	−
	I. ricinus	Eersel	1	33		+	+	−	−
	I. ricinus	Oldeberkoop	1	33		+	−	−	−
	I. ricinus	Oostvoorne	1	33		+	+	−	−
	I. ricinus	Sittard	6	33		+	+ or −[d]	−	−
	I. ricinus	Sloten	3	34		+	+ or −[c]	−	−
	I. ricinus	Vrouwenpolder	1	33		+	−	−	−
	I. ricinus	Wolfheze	1	33		+	−	−	−
Group M19	*I. ricinus*	Denekamp	1	34		+	+	−	−
	I. ricinus	Den Burg	6	33		+	−	−	−
	I. ricinus	Enschede	2	34		+	−	−	−
	I. ricinus	Oldeberkoop	1	33		+	−	−	−
	I. ricinus	Sloten	1	33		+	−	−	−
	I. ricinus	Vught	1	34		+	−	−	−

[a] Genomic group as defined by rRNA gene restriction patterns after digestion with *Hin*dIII and *Eco*RV. DNA digests were hybridized with radiolabelled 16S+23S rRNA of *E. coli.*
[b] Molecular mass as determined by SDS-PAGE.
[c] One isolate was positive.
[d] Four isolates were positive.

in The Netherlands. All of the 26 tick isolates that were obtained from only two collection sites were group VS461 (Fisher's exact test, $P < 0.0001$, two sided). van Dam et al. (53) did not specify the two geographic locations where they had collected the ticks. Of the 17 locations where our isolates were collected, only two yielded group VS461. A discrepancy like this would be found purely by chance only once in 25 trials (Fisher's exact test, $P = 0.039$, two sided), not considering the fact that the locations where our isolates were found also yielded other genomic species. In their report, van Dam et al. (53) suggest that different growth and survival rates for the various genomic species in their culture medium may have resulted in an overrepresentation of certain isolates. Even if that explanation is correct, it is still notable that so many group VS461 isolates were obtained. A survey of *B. burgdorferi* in ticks using PCR technology may be less vulnerable to sampling biases than our method, which depends on successful culturing. The scarcity of group VS461 isolates among the tick isolates also contrasts with the preponderance of this type generally found among EM isolates (53, 58). Our data are in good agreement with this general finding. All three of our skin isolates were *B. burgdorferi* group VS461. Apparently, this genomic species is more prone to cause EM and acrodermatitis chronica atrophicans than any of the others (15, 53, 58).

Among the 31 remaining isolates, two types, represented by 19 and 12 isolates, respectively, were distinguished. The distinction was originally based on the restriction fragment length polymorphism patterns produced by the 16S+23S rRNA sequences. Later, it was fully confirmed by the unrelated, conserved region of the genomic DNA, encoding for the flagellin protein, positioned more than 250 kbp away from the rRNA gene cluster (16). One group of isolates is probably conspecific with *B. garinii*. The other one, designated group M19, needs further analysis to determine whether this group constitutes a new genomic species.

Several studies have demonstrated that classification of *B. burgdorferi* isolates using the electrophoretic mobility and MAb reactivity of OspA and OspB correlates well with genetic analysis such as restriction pattern of chromosomal DNA, rRNA restriction pattern, and DNA-DNA hybridization (1, 4, 37, 58). This indicates that the plasmids encoding for OspA and OspB are not commonly exchanged between borreliae that belong to different genomic species. In the present study, only three of the five groups distinguished by restriction fragment

length polymorphism could also be distinguished on the basis of electrophoretic mobility and MAb reactivity of OspA and OspB. However, the number of MAbs used in this study was small. Peter et al. (37), who classified 20 Swiss *I. ricinus* isolates on the basis of polymorphism of OspA and OspB, identified four groups which corresponded to divisions based on genetic analysis. Two of these groups produced a single OspA protein either at 33 or 33.5 kDa. Separation of these two groups was possible on the basis of reactivity with MAb D6, directed against a 12-kDa protein of *B. burgdorferi*. Concerning our *B. garinii* and group M19 isolates, which produced a single OspA at 33 or 34 kDa, further analysis using a broader set of MAbs is needed to determine whether these groups can also be identified on the basis of phenotypic characteristics.

There are indications that protein profiles and antigenic characterization of surface proteins may be less reliable for the classification of *B. burgdorferi* (44, 60) since antigenic changes may occur during in vitro cultivation. These changes may be due to loss of plasmids, gene rearrangements, or gene expression and regulatory processes (17, 24, 45). This was the main reason why we used only low-passage isolates. Wilske et al. (60) noticed that some isolates of *B. burgdorferi* expressed the OspC at the expense of OspA. Twenty-two of our 66 isolates clearly produced a protein band in the OspC region (20 to 25 kDa), but there were always sufficient amounts of OspA detectable to allow its determination. Furthermore, comparison of 2- and 40-passage isolates (data not shown) revealed no differences in the electrophoretic mobilities and MAb reactivities of OspA and OspB, which convinced us that the phenotypic expression of these proteins is maintained during cultivation.

The proportion of isolates producing a 22-kDa band differed significantly among *B. burgdorferi* sensu stricto (79%), *B. garinii* (58%), and the group M19 isolates (5%). This finding may suggest that OspC, in addition to OspA and OspB, may be useful in the classification of *B. burgdorferi* isolates on the basis of Osp profiles. However, the expression of OspC has been shown to be an unstable feature (59). Concerning the percentages described above, it also should be mentioned that we did not verify the identity of the 22-kDa band as OspC by antibody recognition. This is particularly important in light of the findings that in the 20- to 25-kDa range, proteins that are not surface associated may be seen and mistaken for OspC (59). OspC is, besides the flagellin protein, a major target of the early immune response in patients with Lyme borreliosis. Verification of OspC by means of an MAb is important when serodiagnostic antigens should be prepared.

The finding in the present study that *B. burgdorferi* may contain more than three genomic species is important in light of the recent findings that different subspecies are associated with distinct clinical pictures of Lyme borreliosis and that, in particular, *B. garinii* is associated with extracutaneous manifestations (53, 58). Further analysis to more clearly define the status of the M19 group is needed.

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