JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1984, p. 151–154
0095-1137/84/080151-04$02.00/0

Vol. 20, No. 2

Genetic Relationship of Lyme Disease Spirochetes to *Borrelia*, *Treponema*, and *Leptospira* spp.

FREDERICK W. HYDE* AND RUSSELL C. JOHNSON

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455

Received 14 November 1983/Accepted 21 February 1984

Genetic studies were performed on the following spirochetes: three Lyme disease spirochetes isolated from *Ixodes* ticks and from human spinal fluid; three species of North American borreliae; four species of *Treponema*; and two species of *Leptospira*. The mol% G+C values for Lyme disease spirochetes were 27.3 to 30.5%, similar to values of 28.0 to 30.5% for *Borrelia* species but different from the values for *Leptospira* or *Treponema* species which ranged from 35.3 to 53%. Lyme disease spirochetes represent a new species of *Borrelia*, with DNA homologies of 31 to 59% with the three North American strains of *Borrelia* studied. These studies also showed that Lyme disease spirochetes from three sources constituted a single species, with DNA homologies ranging from 76 to 100%. A high degree of relatedness was also seen between the three North American borreliae, with homology varying from 77 to 95%, indicating that these spirochetes represent a single species. Lyme disease spirochetes and *Borrelia* species exhibited almost no homology with *Leptospira* and *Treponema* species (0 to 2%). Plasmids were detected in the three Lyme disease spirochetes and in the three North American borreliae.

Lyme disease was first recognized in 1975 (2, 21) and occurs in the spring, summer, and fall, beginning with a red skin lesion, erythema chronicum migrans (ECM) (2, 21). Accompanying this are headache, myalgia, malaise, and arthralgias, which last for several weeks. Weeks or months after the early clinical phase of the disease, patients may develop neurological or cardiac abnormalities (21). Patients may go on to develop intermittent attacks of arthritis which may become chronic, resulting in destruction of bone and cartilage and in the large joints (21).

Epidemiological studies suggested the involvement of an unknown agent transmitted by the deer tick, *Ixodes dammini*. Penicillin improved or eliminated the symptoms, suggesting involvement of a penicillin-sensitive bacterium (21). The organism responsible for the disease was discovered during a tick and pathogen survey on Shelter Island in New York. Sixty-one percent of the *I. dammini* ticks examined contained spirochetes that were antigenically reactive with sera from patients in convalescent stages of Lyme disease (6). The same spirochete was isolated from blood, cerebrospinal fluid, and skin lesions of patients with Lyme disease, confirming the role of these spirochetes in this malady (2, 21). Clinical and epidemiological similarities between Lyme disease and ECM in Europe led Barbour et al. to study *Ixodes ricinus*, the tick incriminated as the vector of ECM in Switzerland. Thirty six percent of the *I. ricinus* ticks examined contained spirochetes that were similar, if not identical, to the New York-isolated Lyme disease spirochetes (1).

Lyme disease in the United States covers three geographical areas: the Northeast and the Midwest, where *I. dammini* carries the spirochetes, and the west, with *Ixodes pacificus* ticks serving as the vector. Isolated cases of Lyme disease have been reported in Texas, Arkansas, and Georgia and suggest that a third tick, *Ixodes scapularis*, may be the epidemiological vector involved in the southeast (7). The Lyme disease spirochete has been isolated from white-tailed deer, white-footed mice, and raccoon in the Long Island area (21). The relationship of the spirochete to its vector and animal hosts is unclear.

* Corresponding author.

We report here on the genetic relatedness of the Lyme disease spirochete to *Borrelia*, *Treponema*, and *Leptospira* spp.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Borrelia turicatae*, *Borrelia parkeri*, and Lyme disease isolates from *I. dammini* and *Ixodes ricinus* ticks were gifts of Alan Barbour and Willy Burgdorfer, Rocky Moutain Laboratories, Hamilton, Mont. Lyme disease isolates 297 from infected human cerebrospinal fluid and 272 from skin lesion were gifts of Allan Steere of Yale University, New Haven, Conn. *Borrelia hermsii*, *Treponema phagedenis*, *Treponema denticola*, *Leptospira interrogans* serovar *hardjo*, and *Leptospira biflexa* serovar *patoc I* are stock strains maintained in our laboratory.

Borrelia hermsii, *B. turicatae*, *B. parkeri*, and Lyme disease isolates from *I. dammini* and *I. ricinus* ticks, and human isolate 297 were grown in BSK medium a modified Kelly medium (22). *Treponema* spp. were grown in an anaerobic medium developed by Smibert (20). *Leptospira* spp. were grown in a bovine serum albumin-Tween 80 medium (3).

Radiolabeling of spirochetes. Five hundred milliliters of appropriate medium was inoculated with 5 ml of a log-phase culture of spirochetes and incubated at the temperature optimal for the spirochete being labeled. Lyme disease isolates, *Borrelia* spp., and *Treponema* spp. were labeled with 100 μCi of [*methyl*-^{3}H]thymidine (ICN Pharamaceuticals, Covina, Calif.) for 8 to 10 days at 34°C. *Leptospira* spp. were labeled with 100 μCi of [8-^{3}H]adenine due to their inability to incorporate pyrimidines. *Leptospira* spp. were incubated for 8 to 10 days at 30°C.

Chromosomal DNAs were alternately labeled with [^{14}C]dATP by in vitro nick translation with a nick translation kit purchased from Bethesda Research Laboratories, Rockville, Md.

Isolation of chromosomal DNA. Spirochetes were harvested by centrifugation at 16,000 × *g* for 30 min in a GSA head with a Sorvall RC2-B centrifuge. Cells were washed by resuspension in 10 ml of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and centrifuged at 12,000 × *g* for 30 min.

Washed cells were suspended in 5 ml of TE buffer, 0.5 μl of 5% sodium dodecyl sulfate was added, and the suspension was mixed and placed in a 65°C waterbath (to inactivate DNases and complete cell lysis) for 10 min. Pronase (200 μl, 5 mg/ml) was added and mixed and incubated at 37°C for 1 h. The mixture was deproteinized with phenol and chloroform followed by treatment with 50 μl of boiled RNase (10 mg/ml) for 1 h at 37°C. The mixture was again extracted with phenol and chloroform, and the DNA was precipitated by adding 1/10 volume of 2 M sodium acetate (pH 8.0) and 2.5 volumes of cold 95% ethanol. Precipitation was completed by placing the mixture at −20°C for 1 h. The DNA was then collected by centrifugation at 12,000 × *g* for 30 min and resuspended in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate).

Determination of moles percent guanine plus cytosine. Moles percent guanine plus cytosine was determined by thermal denaturation studies (12, 14). DNA (50 μg) was suspended in SSC and placed in a thermocuvette of a Gilford 2400-S spectrophotometer equipped with a thermoprogrammer. The temperature of the cuvette was raised 0.5°C/min, with hyperchromicity developing as the DNA denatured. The melting temperature was obtained from the absorbance graph as being the midpoint of the curve, at which 50% of the DNA was denatured. The moles percent guanine plus cytosine was calculated by the equation mol% G+C = $(T_m - 69.3) \times 2.44$ (14), where T_m is the midpoint of thermal denaturation.

DNA-DNA hybridization and calculation of percent homology. DNA preparations were reannealed in situ by the method of Denhardt (8). DNAs were sheared to an average length of 500 bases by DNase I treatment for 5 min. Average fragment size was determined by electrophoresis, with phage lambda DNA digested with restriction enzyme *Hin*dIII as molecular weight standards. DNA samples were then denatured by boiling for 5 min.

Denatured bacterial DNA (10 μg) was placed on nitrocellulose filters (45 μm; HAWPO25; Millipore Corp., Bedford, Mass.) that had been dipped in 20× SSC and allowed to dry. The disks were then dried in vacuo at 80°C overnight. Hybridization was performed by placing a DNA disk into a vial containing 1 ml of Denhardt prehybridization buffer (2× SSC with 0.02% [wt/vol] each of bovine serum albumin, polyvinylpyrrolidone [molecular weight, 25,000], and Ficoll 400 [8]) to prevent nonspecific adherence of DNA to filters and incubated at 65°C overnight. Heat-denatured probe DNA (500 ng; 10,000 cpm/μg) was added and the incubation was continued at 60°C for 24 h. After incubation, the vials were placed on ice, and the disks were washed for 10 min with 2× SSC three times. Washed disks were dried with a heat lamp, and the radiolabeled content was determined with a Beckman scintillation counter. Percent homology (relative binding ratio) was calculated by the following equation: % homology = 100 × {[cpm (DNA under test) − cpm (background hybridization)]/[cpm (100% control) − cpm (background hybridization)]}.

Examination of spirochetes for extrachromosomal DNA. Plasmid DNA was isolated by a modification of the method of Radloff (19). Ethanol-precipitated spirochete DNA was suspended in 3 ml of TE buffer and mixed with 3.55 g of cesium chloride (Sigma Chemical Co., St. Louis, Mo.). One milliliter of 5-mg/ml ethidium bromide was added and overlaid with mineral oil. The DNA preparations were centrifuged at 38,000 rpm for 60 h. Satellite bands were visualized with UV light and were removed from the gradient by needle puncture. Ethidium bromide was extracted in the dark with one volume of NaCl-saturated isopropanol followed by two extractions with isopropanol in room light. Samples were diluted twofold with water and ethanol precipitated. DNA was harvested by centrifugation at 16,000 × *g* for 20 min.

TABLE 1. Characterization of spirochetal DNAs

Organism	T_m (°C)	Mol% G+C[a]
Ixodes dammini spirochete	81.8	30.5
Ixodes ricinus spirochete	80.9	28.3
Human cerebrospinal fluid isolate	80.5	27.3
Borrelia hermsii	81.8	30.5
Borrelia turicatae	81.5	29.8
Borrelia parkeri	80.8	28.0
Treponema denticola	84.0	36.0
Treponema vincentii	88.5	46.8
Treponema scoliodontum	84.0	36.0
Treponema phagedenis	84.0	36.0
Treponema pallidum (Nichols)	83.5–91.0	35–53[b]
Leptospira interrogans	84–86	35–40[c]
Leptospira biflexa	85.3	39[c]

[a] Calculated from thermal denaturation studies by using the formula given in the text (13). Results are the average of three experiments.
[b] From reference 15.
[c] From reference 10.

RESULTS

Moles percent guanine plus cytosine for *B. hermsii*, *B. turicatae*, *B. parkeri*, *I. dammini* spirochetes, *I. ricinus* spirochetes, Lyme disease human cerebrospinal fluid isolate, *Treponema vincentii*, *T. scoliodonitum*, *T. phagedenis*, and *T. denticola* are shown in Table 1. *I. dammini* and *I. ricinus* tick isolates and the human spinal fluid isolate had mol% G+C of 30.5 to 27.3%. These values are similar to the mol% G+C for *B. hermsii*, *B. turicatae*, and *B. parkeri*, of 28.0 to 30.5%. The mol% G+C of the Lyme disease spirochetes and the borreliae differ from those of *Leptospira* and *Treponema* spp., which ranged from 35 to 53% (10, 15, 16).

The degree of relatedness among spirochetes as suggested by mol% G+C was confirmed by DNA homology studies. When radioactive DNA from the *I. dammini* spirochete was used as a probe, DNA homologies of 100 to 76% were observed among the Lyme disease spirochetes, and homologies of 59 to 37% were seen with the *Borrelia* spp. (Table 2). The above results indicated that Lyme disease spirochetes are of the same species and also constitute a new *Borrelia* species. These results were confirmed by using radiolabeled DNA from *B. hermsii* and *B. turicatae* as probes. Homologies of 95 to 77% existed among the *Borrelia* (Table 3). With *B. turicatae* DNA as probe, *B. parkeri* and *B. hermsii* showed 95 and 81% homology, respectively. The *B. hermsii* DNA was 58 to 31% related to the DNA of the Lyme disease spirochetes (Table 3). A temperature profile study of homology between *B. parkeri* and human cerebrospinal fluid isolates exhibited increasing homologies of 38 to 41% as annealing temperature was decreased from 65 to 50°C, respectively. As suggested by the moles percent guanine plus cytosine values (17), no significant homology (0 to 2%) was detected between the *I. dammini* spirochete or *B. hermsii* with the *Treponema* or *Leptospira* spp. surveyed.

Electrophoretic analysis of DNAs of Lyme disease spirochetes (Fig. 1) and the three North American borreliae (Fig. 2) revealed the presence of plasmid DNA. The *I. dammini* spirochete contained two plasmids, whereas the *Borrelia* spp., the *I. ricinus* spirochete, and the human cerebrospinal fluid isolate contained one plasmid.

TABLE 2. DNA homologies between Lyme disease spirochetes and other spirochetes[a]

Organism	Relative binding ratio (60°C)[b]
Ixodes dammini spirochete	100
Ixodes ricinus spirochete	100
Human cerebrospinal fluid isolate	76
Borrelia hermsii	59
Borrelia turicatae	46
Borrelia parkeri	37
Treponema phagedenis	2
Treponema denticola	2
Leptospira interrogans serovar *hardjo*	1
Leptospira biflexa serovar *patoc*	1

[a] Probe, *I. dammini* spirochete DNA.
[b] Relative binding ratio =

$$\frac{\text{cpm of heterologous DNA bound to filters} - \text{background cpm}}{\text{cpm of homologous DNA bound to filters} - \text{background cpm}} \times 100.$$

Results are the average of two experiments.

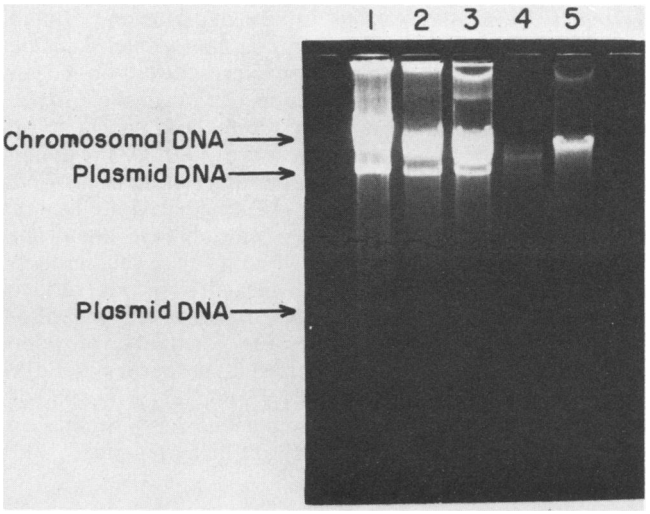

FIG. 1. Plasmid DNA in Lyme disease spirochetes. Lane 1, *I. dammini* spirochete DNA; lane 2, *I. ricinus* spirochete DNA; lane 3, human cerebrospinal fluid isolate DNA; lane 4, human skin lesion (ECM) isolate DNA; lane 5, *Escherichia coli* K-12 DNA negative control.

DISCUSSION

The agent of Lyme disease is a tick-transmitted spirochete that is morphologically similar to both *Borrelia* and *Treponema* spp., with cell dimensions of 4 to 30 μm in length and 0.18 to 0.25 μm in diameter (21). Lyme disease spirochetes appear to contain 14 periplasmic flagella, similar to the 2 to 16 present in treponemes but different from *Borrelia* spp., which contain 30 to 40 periplasmic flagella (11). The antigenic variation, seen in *Borrelia* spp. (5), was not observed in the Lyme spirochetes. However, Lyme disease spirochetes appear to be more *Borrelia*-like since they are tick transmitted and can be cultivated in vitro in modified Kelly medium (BSK) (21).

Our work has shown that, on the basis of moles percent guanine plus cytosine and DNA homology studies, Lyme disease spirochetes constitute a new species of *Borrelia*. In addition, Lyme disease spirochetes isolated from different geographical regions constitute a single species of *Borrelia*, as defined by Brenner (4). These results are in agreement with those obtained by Schmid et al. (19a). Close similarities exist between Lyme disease spirochetes isolated in this country and spirochetes isolated from *I. ricinus* ticks in Switzerland (1). An unexpected finding was that the three North American *Borrelia*, *B. hermsii*, *B. turicatae*, and *B. parkeri*, actually constitute a single species of spirochetes. Additional genetic studies must be performed to elucidate the relationship of North American borreliae to similar organisms found in other geographical areas. However, these studies are difficult to perform due to the inability to grow these spirochetes in vitro.

The early manifestation of Lyme disease has been charac-

TABLE 3. DNA homology between *Borrelia hermsii* and other spirochetal DNAs

Organism	Relative binding ratio (60°C)[a]
Borrelia hermsii	100
Borrelia turicatae	86
Borrelia parkeri	77
Ixodes dammini spirochete	58
Ixodes ricinus spirochete	44
Human cerebrospinal fluid isolate	31
Treponema denticola	0
Treponema vincentii	0
Treponema scoliodontum	0
Leptospira interrogans serovar *hardjo*	0
Leptospira interrogans serovar *canicola*	0
Leptospira biflexa serovar *illini*	0
Leptospira biflexa serovar *patoc*	0

[a] See Table 2, footnote *b* for the formula used to calculate relative binding ratio. Results are the average of two experiments.

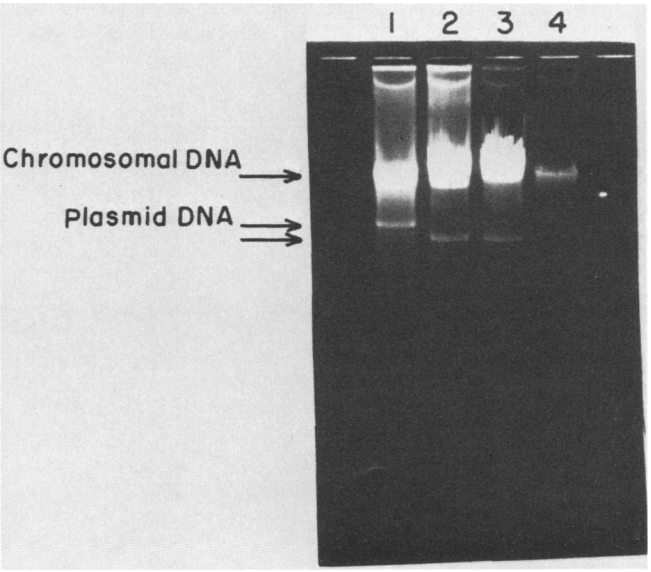

FIG. 2. Plasmid DNA in North American borreliae. Lane 1, *B. hermsii* DNA; lane 2, *B. turicatae* DNA; lane 3, *B. parkeri* DNA; lane 4, *Escherichia coli* K-12 DNA negative control.

terized by ECM skin lesions in patients from the United States and Europe. Arthritis is a common sequelae in the United States; in Europe, arthritis is believed to be an uncommon sequelae of the infection. Although the *I. dammini* spirochete and the *I. ricinus* spirochete appear to be genetically homologous, the clinical manifestations resulting from infections by these organisms differ somewhat. This may be a result of extrachromosomal-mediated properties. We found plasmids in the three Lyme disease organisms studied. Plasmids have been shown to mediate such properties as antibiotic resistance and may also encode surface antigens. The presence of plasmid in Lyme disease spirochetes and *Borrelia* spp. sugests the potential to develop important plasmid-mediated biological properties. Of the other pathogenic spirochetes, only *Treponema pallidum* has previously been shown to contain plasmids (18). Studies on characterization of these plasmids are being pursued.

LITERATURE CITED

1. **Barbour, A. G., W. Burgdorfer, S. F. Hayes, O. Peter, and A. Aeschlimann.** 1983. Isolation of a cultivatable spirochete from *Ixoides ricinus* ticks of Switzerland. Curr. Microbiol. **8**:123–126.
2. **Benach, J. L., E. M. Bosler, J. P. Hanrahan, J. L. Coleman, G. S. Habicht, T. F. Bast, D. J. Cameron, J. L. Ziegler, A. G. Barbour, W. Burgdorfer, R. Edelman, and R. A. Kaslow.** 1983. Spirochetes isolated from the blood of two patients with Lyme disease. N. Engl. J. Med. **308**:740–742.
3. **Bey, R. F., and R. C. Johnson.** 1978. Protein free and low-protein medium for the cultivation of *Leptospira*. Infect. Immun. **19**:562–569.
4. **Brenner, D. J.** 1981. Introduction to the *Enterobacteriaceae*, p. 1105–1127. *In* M. P. Starr, H. Stolp, H. G. Truper, A. Balows, and H. G. Schlegel (ed.), The prokaryotes. Springer-Verlag, New York.
5. **Burgdorfer, W.** 1976. The epidemiology of relapsing fevers, p. 191–200. *In* R. C. Johnson (ed.), Biology of parasitic spirochetes. Academic Press, Inc., New York.
6. **Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis.** 1982. Lyme disease: a tick-borne spirochetosis? Science **216**:1317–1319.
7. **Burgdorfer, W., and J. E. Kierans.** 1983. Ticks and Lyme disease in the United States. Ann. Intern. Med. **99**:121.
8. **Denhardt, D. T.** 1966. A membrane filter-technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
9. **Felsenfeld, O.** 1971. Borrelia: strains, vectors and human borreliosis. Warren H. Green, Inc., St. Louis, Mo.
10. **Haapala, D. K., M. Rogul, L. B. Evans, and A. D. Alexander.** 1969. DNA base composition and homology studies of *Leptospira*.. J. Bacteriol. **98**:421–428.
11. **Hovind-Hougen, K.** 1976. Treponema and Borrelia morphology, p. 7–18. *In* R. C. Johnson (ed.), Biology of parasitic spirochetes. Academic Press, Inc., New York.
12. **Mandel, M., and J. Marmur.** Use of UV absorbance-temperature profile for determining guanine plus cytosine content of DNA. Methods Enzymol. **12**:195–206.
13. **Marmur, J., and P. Doty.** 1961. Isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
14. **Marmur, J., and P. Doty.** 1962. Determination of base composition of DNA from its thermal denaturation temperature. J. Mol. Biol. **5**:109–118.
15. **Miao, R., and A. H. Fieldsteel.** 1978. Genetics of *Treponema*: relationship between *Treponema pallidum* and five cultivatable treponemes. J. Bacteriol. **133**:101–107.
16. **Miao, R., and A. H. Fieldsteel.** 1980. Genetic relationship of *Treponema pallidum* and *Treponema pertenue*, two non-cultivable human pathogens. J. Bacteriol. **141**:427–429.
17. **Moore, R. L.** 1974. Nucleic acid reassociation as a guide to genetic relatedness among bacteria. Curr. Top. Microbiol. Immunol. **64**:105–128.
18. **Norgard, M. V., and J. N. Miller.** 1981. Plasmid DNA in *Treponema pallidum*: potential for antibiotic resistance by syphilis bacteria. Science **213**:553–555.
19. **Radloff, R., W. Baver, and J. Vinograd.** 1968. A dye-buoyant density method for the detection and isolation of close circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Natl. Acad. Sci. U.S.A. **59**:838.

19a. **G. P. Schmid, A. G. Steigerwalt, S. E. Johnson, A. G. Barbour, A. C. Steere, I. M. Robinson, and D. J. Brenner.** 1984. DNA characterization of the spirochete that causes Lyme disease. J. Clin. Microbiol. **20**:155–158.

20. **Smibert, R. M.** 1976. Cultivation, composition and physiology of avirulent treponemes, p. 49–56. *In* R. C. Johnson (ed.), Biology of parasitic spirochetes. Academic Press, Inc., New York.
21. **Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista.** 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. **308**:733–740.
22. **Stoenner, H. G., T. Dodd, and C. Larsen.** 1982. Antigenic variation in *Borrelia hermsii*. J. Exp. Med. **156**:1297–1311.