

Newly Recognized *Leptospira* Species ("Leptospira inadai" serovar lyme) Isolated from Human Skin

GEORGE P. SCHMID,^{1*} ALLEN C. STEERE,² ARNOLD N. KORNBLATT,² ARNOLD F. KAUFMANN,¹
C. WAYNE MOSS,¹ RUSSELL C. JOHNSON,³ KARI HOVIND-HOUGEN,⁴ AND DON J. BRENNER¹

Division of Bacterial Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30333¹;
Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06510²; Department of
Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455³; and National Veterinary
Laboratory, DK-1870 Copenhagen V, Denmark⁴

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***Leptospira* strain 10, which represents a new *Leptospira* species, was isolated from a skin biopsy of a patient with Lyme disease. Although pathogenic for laboratory animals, the organism was not considered to have a significant role in the patient's illness.**

In August 1981, in an attempt to recover the then-unknown etiologic agent of Lyme disease, we isolated a *Leptospira* organism from a patient with Lyme disease. Attempts to recover the organism from other patients were unsuccessful, and serologic tests discounted the newly isolated organism as the etiologic agent of Lyme disease. Subsequently, another newly recognized spirochete, *Borrelia burgdorferi*, was documented as the cause of Lyme disease (2, 7, 9). In this report, we describe the *Leptospira* organism and how it differs from previously described *Leptospira* organisms.

The patient, a 69-year-old man, was bitten by a nymphal tick (*Ixodes dammini*) on 1 May 1981. One week later, he developed a severe frontal headache, myalgia, fever (39.4°C), anorexia, and fatigue. The following week, in addition to the early symptoms, he had migratory arthralgia in large and small joints. For the next 4 weeks, he continued to have daily fevers, headache, scalp tenderness, and backache. On 16 June 1981 he developed eight annular skin lesions; the largest, 30 cm in diameter, surrounded the tick bite. These lesions were typical of erythema chronicum migrans and secondary annular skin lesions. A skin biopsy specimen was taken from the outer edge of the large lesion, snap-frozen in an acetone-dry ice bath, and stored at -70°C until cultured. The patient responded promptly to tetracycline therapy.

The skin biopsy was processed for culturing in the following manner. On the day of processing, the sample was thawed in a water bath at room temperature, removed aseptically from the storage vial, and ground by using a sterile mortar and pestle with sterile physiologic buffered saline and a small amount of sterile Alundum. A 0.1-ml portion of the resulting suspension was inoculated into Ellinghausen-McCullough-Johnson-Harris semisolid medium (10) and incubated aerobically at 31°C. On the same day, skin biopsies from four other patients and whole blood samples from six other patients were also cultured.

After several days of incubation, spirochetes were observed by dark-field microscopy in the skin biopsy culture. Typical of leptospire, the spirochetes were about 10 µm long, were tightly coiled with hooked ends, and had rotating

motility. Subsequently, to eliminate the possibility that *Leptospira* strain 10 was a contaminant, we tested an aliquot of the original suspension which had been frozen immediately after processing for the presence of *Leptospira* organisms by direct immunofluorescence; *Leptospira* organisms were seen in every field (400×). None of the other 10 specimens initially processed on the same day with the same reagents were culture positive for *Leptospira* organisms.

The serovar status of the isolate was determined by agglutinin absorption testing (10). To be considered a new serovar within an existing serogroup, an isolate must react to at least 6% of the homologous titer of antiserum to all members of the serogroup. Conversely, antiserum against the isolate must retain 10% or more of its homologous titer after cross-absorption with other members of the serogroup. When tested against hyperimmune rabbit antisera to the type serovars of all recognized *Leptospira interrogans* serogroups, *Leptospira* strain 10 did not react to 6% of the homologous titer of any. Cells of strain 10 were agglutinated only by hyperimmune antiserum against *L. interrogans* serovar *celledoni* and only at a low titer (1:400). The homologous titer for *L. interrogans* serovar *celledoni* in this antiserum was 1:25,600. Cells of *L. interrogans* serovar *celledoni* were not agglutinated by antiserum against strain 10.

To assess the virulence of *Leptospira* strain 10 and its ability to produce a skin lesion, we inoculated one African green monkey (*Cercopithecus aethiops*) and one spider monkey (*Ateles paniscus*) intradermally and subcutaneously with a total inoculum of 10⁸ organisms. Eight weanling hamsters (*Mesocricetus auratus*) were inoculated intradermally and intraperitoneally with a total inoculum of 10⁷ organisms. Three 250-g guinea pigs (*Cavia porcellus*) were inoculated intraperitoneally with 10⁵ organisms. Both monkeys were blood culture positive on day 6 after inoculation, but neither developed a skin lesion. Two hamsters were blood culture positive several hours after inoculation but not upon subsequent culturing. *Leptospira* strain 10 was isolated from cultures of the brains of two hamsters that were killed 2 days after inoculation. The organism was not isolated from any of the other hamsters after inoculation, and no hamster developed a skin lesion. One guinea pig appeared ill and had a positive blood culture 96 h after

* Corresponding author.

TABLE 1. Antibody titers against *Leptospira* strain 10 and *Borrelia burgdorferi* in serum samples

Date (mo/day/yr) of serum sampling	Disease activity	Antibody titer against:				
		Leptospira strain 10			<i>B. burgdorferi</i>	
		MAT ^a	IFA ^b		IFA	
			IgM ^c	IgG	IgM	IgG
6/17/81	Acute	16	16	32	256	128
7/10/81	Convalescent	32	32	64	64	256
10/16/81	Remission	ND ^d	8	32	32	32

^a MAT, Microscopic agglutination test.

^b IFA, Indirect immunofluorescence assay.

^c IgM, Immunoglobulin M.

^d ND, Not done.

inoculation. These results demonstrated the ability of *Leptospira* strain 10 to establish an infection in four animal species.

The susceptibility of strain 10 to 225 µg of 8-azaguanine per ml and its ability to grow at 13 and 30°C were determined by previously described methods (4, 6). Strain 10 did not grow in the presence of 8-azaguanine, a characteristic of pathogenic *Leptospira* strains, but did grow at both 13 and 30°C, a characteristic of saprophytic *Leptospira* strains.

The fatty acid content of the whole cell was determined by gas-liquid chromatography. The fatty acid composition of *Leptospira* strain 10 was 40% oleic acid (18:1Δ9) and 22% palmitic acid (16:0), similar to those of the pathogenic serovars *L. interrogans copenhageni*, *L. interrogans pomona*, and *L. interrogans celledoni* and the saprophytic *L. biflexa* serovar *patoc*. The ratio of monosaturated 16-carbon acids (16:1Δ9/16:1Δ11) did not clearly fall in the range of either saprophytic or pathogenic *Leptospira* strains (5).

The guanine-plus-cytosine (G+C) content of the DNA was calculated from the thermal midpoint of DNA denaturation in a spectrophotometer (8). Strain 10 had a DNA G+C content of 43 mol%. *Leptonema illini* has a DNA G+C content of 54 mol%; *Leptospira parva* has a DNA G+C content of 49 mol%; *L. interrogans* serovar *celledoni* has a DNA G+C content of 41 mol%; and *Leptospira biflexa* serovar *patoc* has a DNA G+C content of 36 mol%. Thus, the DNA G+C content of strain 10 lay in the intermediate range of the DNA G+C contents of the members of the family *Leptospiraceae*.

In an unpublished study of the DNA relatedness of members of the family *Leptospiraceae*, strain 10 was compared with 5 serovars of *L. biflexa* and 39 serovars of *L. interrogans*, *L. parva*, and *Leptonema illini* (P. H. Yasuda, A. G. Steigerwalt, K. R. Sulzer, A. F. Kaufmann, F. Rogers, and D. J. Brenner, manuscript in preparation). DNA relatedness was determined by the hydroxyapatite method, with hybridization reactions being carried out at both an optimal reassociation temperature of 55°C and a stringent reassociation temperature of 70°C. Strain 10 was found to have <10% relatedness to any of the other organisms in the study group.

When examined by electron microscopy, *Leptospira* strain 10 organisms were 7 to 11 µm long and had a wavelength of about 0.7 µm. Many cells contained electron-lucent inclusions. The ends were usually hooked. One flagellum was inserted at each end of the cell and wound around the cytoplasmic body for about six wavelengths. The cells were covered by a surface layer which, when separated from slightly damaged cells, formed striated tubules. Cyto-

plasmic tubules were not seen in spirochetes accidentally damaged during preparation for electron microscopy. The basal knob on flagella freed by Teepol treatment had two pairs of disks. Despite having a wavelength comparable to that of *Leptonema* cells, the relatively short length, the lack of cytoplasmic tubules, and the structure of the flagellar basal knobs of the cells examined were most compatible with those of *Leptospira* cells (1, 3).

Antibodies against both *Leptospira* strain 10 and *B. burgdorferi* were detected in serum samples from the patient whose skin biopsy was culture positive (Table 1). The antibody titers against *Leptospira* strain 10, however, were considerably lower than those typically detected in leptospirosis patients, whereas the antibody titers against *B. burgdorferi* were consistent with the serologic diagnostic criteria for Lyme disease. We feel that the clinical presentation of the patient as well as the laboratory data indicate that the patient had Lyme disease. Despite the animal inoculation data indicating that *Leptospira* strain 10 is a pathogen, we do not believe that the available evidence supports an etiologic role for this organism in the patient's illness. We do believe, however, that the evidence supports the conclusion that *Leptospira* strain 10 was intrinsically present in the skin biopsy specimen and was not a contaminant introduced during specimen collection and processing.

Based on morphology, strain 10 is a *Leptospira* strain. Other characteristics of this organism, particularly its low degree of DNA relatedness to other *Leptospira* species, justifies the conclusion that it is a new serovar (*lyme*) of a new species ("*Leptospira inadai*"). A formal proposal of the name of the organism will occur in a comprehensive taxonomic study of leptospires (Yasuda et al., in preparation). The proposed serovar name is derived from the locale of the patient's residence. The proposed species name is in honor of Ryokichi Inada, the Japanese physician who led the first successful study documenting the etiologic agent of Weil's disease.

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