A NEW PATHOGENIC *LEPTOSPIRA*, NOT READILY CULTIVATED

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ABSTRACT

ALEXANDER, AARON D. (Walter Reed Army Institute of Research, Washington, D.C.), HERBERT G. STOENNER, GARNETT E. WOOD, AND ROBERT J. BYRNE. A new pathogenic *Leptospira*, not readily cultivated. *J. Bacteriol.* **83**:754-760. 1962. — A pathogenic *Leptospira* was isolated from water of the Grand River, (S.D.) that differed significantly from other known leptospirae in that it could not be cultivated in conventional leptospiral media. Growth was promoted in Fletcher's medium modified to contain 20% rabbit serum. The isolate, after several serial passages, was lethal for hamsters. It could not be adapted to grow in the chick embryo. Guinea pigs and calves inoculated with the isolate developed febrile and antibody responses but showed no other overt signs of disease. The strain was identified on the basis of cross-agglutination and agglutinin-adsorption tests as a new subserotype of *Leptospira naam* and was therefore designated as *L. naam*, subserotype *dakotii*.

During an epidemiological investigation of a focus of human leptospiral infection, a pathogenic *Leptospira* was isolated that could not be cultivated in conventional leptospiral media. In this regard, it differed remarkably from other known leptospirae.

The strain was isolated from a water sample obtained in July 1958 from the Grand River, near Little Eagle in the north central sector of South Dakota. In the summer of 1956, this area had served as the locus of a small outbreak of leptospirosis that occurred among members of a swimming party. The etiology of the outbreak, established in retrospect, was attributed to *Leptospira pomona* on the basis of serological tests on patients' sera (Jellison, Stoenner, and Berg,

1958). A water sample taken from the Grand River in September 1957 contained pathogenic leptospirae, presumably *L. pomona*. A guinea pig inoculated with the sample developed agglutinins for *L. pomona*. These findings indicated that the contamination of this stream with leptospirae might not be a transient occurrence, and additional samples of water from the original focus of human infection were examined. Characteristics of the strain isolated in the 1958 study are described in this report.

MATERIALS AND METHODS

Examination of water. Samples of water were collected from each of three different sites along the Grand River at four different times between March and November 1958. The samples were shipped in chemically refrigerated insulated packages to the Rocky Mountain Laboratory, Hamilton, Mont. Two guinea pigs (200 to 300 g) and two hamsters (50 to 100 g) were employed for each of the 12 samples. Each animal was inoculated intraperitoneally with 3 ml of sample and thereafter examined daily for signs of disease; temperatures of guinea pigs were taken daily. Samples of blood obtained from animals showing signs of disease were cultured for leptospirae. After observation for 39 to 45 days, animals were bled and their sera were tested by the rapid plate method (Stoenner, 1954) for antibodies against *L. pomona*, *L. icterohaemorrhagiae*, *L. canicola*, *L. hebdomadis*, *L. bataviae*, *L. grippityphosa*, *L. ballum*, *L. autumnalis*, *L. sejroe*, and *L. pyrogenes*. Attempts were then made to isolate leptospirae from serologically positive animals.

Culture techniques. Experimental media were distributed in 5.0-ml amounts in 15 × 125 mm screw-cap test tubes. In the initial phase of study, the inoculum consisted of 1 or 2 drops of blood or a 10% suspension of pooled blood,

liver, and kidney tissues from infected hamsters. In a few instances, direct cultures were made with 1-mm³ sections of hamster kidney. In later studies conducted with fluid media, cultures in semisolid media were also employed as inocula. The inoculum comprised 10% of the volume of subculture. All trials were conducted in duplicate or triplicate; in some experiments, quadruplicate or quintuplicate culture tubes were used. Except as noted, cultures were incubated at 28 to 30 C. Three or four dark-field examinations were made at weekly intervals before cultures were discarded as negative.

Animal passage. The infective agent was serially passed in hamsters (30 to 60 g). The inoculum consisted of a 10% suspension of infective tissue in a phosphate-buffered (pH 7.4) isotonic salt solution and was administered intraperitoneally in a 0.5-ml dose. In the first ten passages, the inoculum was prepared from infected hamster kidneys harvested 20 to 55 days postinoculation. Subsequent passages were made with suspensions of pooled blood, liver, and kidney tissues obtained from animals 3 to 5 days postinoculation.

Serological procedures. As noted before, in attempts to isolate leptospirae from water samples, the rapid plate test was used to test animal sera. Subsequently, the basic serological procedure employed was the microscopic agglutination test of Schuffner and Mochtar. This procedure, the method of preparing antisera in rabbits, and the techniques of agglutinin-adsorption tests are described in detail elsewhere (Alexander et al., 1954).

RESULTS

Isolation. None of the hamsters or guinea pigs, inoculated with any of the 12 samples of water from the Grand River, showed signs of disease. One of the samples obtained in July 1958 incited the production of agglutinins in the two inoculated hamsters but elicited no antibodies in guinea pigs inoculated at the same time. Animals inoculated with the 11 other samples remained serologically negative. Sera from the two affected hamsters showed titers of 1:10 and 1:40 against *L. icterohaemorrhagiae* when tested with the rapid plate method. An examination of the kidney suspension by dark-field microscopy revealed leptospirae. A 10% kidney suspension prepared from one of the serologically positive hamsters

was injected into two hamsters, and four tubes of Vervoot's (Wolff, 1954) medium were inoculated with small sections of kidney tissue. The isolated strain failed to grow in vitro in initial cultures but was established in inoculated animals. In subsequent passages in hamsters, leptospirae were regularly seen in suspensions of kidneys from passage animals. Repeated attempts to establish growth of the strain in Vervoot's medium and, in some instances, Fletcher's medium were unsuccessful (Gochenour et al., 1953). The strain was maintained through serial passages in hamsters. Infected animals used in the sixth passage were forwarded to the WHO/FAO Leptospirosis Reference Laboratory, Division of Veterinary Medicine, Walter Reed Army Institute of Research, Washington, D.C., for additional studies of the isolate.

Experimental infections. Hamsters used in the first three passages of the Grand River isolate showed no apparent signs of disease, and those killed for passage had no gross pathological changes. Leptospirae could be readily seen on dark-field microscopic examinations of kidney tissues made at different times up to the 55th day postinoculation. However, the infected kidneys appeared essentially normal.

After the ninth passage, the virulence of the strain was enhanced, and death was produced regularly in hamsters (30 to 40 g) at approximately 9 days postinfection. Dead or moribund hamsters did not show the typical manifestations of leptospirosis, e.g. jaundice or "butterfly" lungs. The most notable gross tissue manifestations were an enlarged hemorrhagic liver and blanched kidneys. The possibility of the coincidental presence and transmission of a viral etiological agent for the hamster disease was considered. For this purpose, kidneys from infected hamsters were triturated with a Ten Broeck grinder and suspended in physiological salt solution to provide a 10% tissue suspension. The concentration of leptospirae, estimated by microscopic examination according to the method of Chang (1947), was 16×10^6 organisms per milliliter. Cultures on conventional blood agar and thio-glycolate media did not yield bacteria. Oleandomycin and streptomycin were added to portions of the suspension in final concentrations of 10 and 50 mg per ml, respectively. The treated suspension, as well as an untreated control, was incubated at 30 C for 6 hr and then inoculated into

groups of hamsters. Disease could not be induced with antibiotic-treated suspensions nor were leptospirae recovered from hamsters inoculated with these suspensions. Typical disease was provoked in control animals in which leptospirae were subsequently demonstrated. Therefore, the disease syndrome could be related only to the leptospiral infection.

After the 25th hamster passage, an increase in concentration of leptospirae in tissues from infected hamsters was generally noted. A few scattered pinpoint hemorrhages in lungs appeared sporadically in these animals; otherwise, the gross pathology did not differ from earlier passage animals.

Guinea pigs weighing approximately 300 g were inoculated with infective tissue suspensions from the 2nd, 17th, and 119th passage hamsters, respectively. On each occasion, two animals were used. Guinea pigs inoculated with material from the 2nd hamster passage showed a slight febrile reaction 5 to 7 days postinoculation; otherwise, the animals appeared to be normal. Tissue suspensions from the 17th and 119th passage hamsters were lethal for hamsters, but a 2.0-ml dose, administered intraperitoneally into guinea pigs, failed to provoke overt disease. However, leptospirae were demonstrated in the blood of the inoculated animals cultured on the fourth day postinoculation. At necropsy 32 to 34 days after inoculation, all tissues appeared normal and leptospirae could not be revealed in kidneys by microscopic and cultural examinations. Two guinea pigs inoculated with tissues from the 17th hamster passage developed titers of 1:10 against *L. icterohaemorrhagiae*, as shown by the microscopic agglutination technique.

Chick embryos (9 to 10 days old), inoculated with 0.5-ml amounts of hamster-lethal tissue suspension (via the allantoic cavity), survived. Attempts to adapt the strain to grow in the chick embryo were unsuccessful.

Because of the fastidious growth characteristics of the isolate, an immediate question was posed about the possible etiological relationship of this *Leptospira* to the occurrence of *hebdomadis* group antibodies in cattle. These antibodies have been observed frequently and extensively in bovine herds in the United States; however, repeated cultural attempts to demonstrate leptospirae in serologically positive animals were, with rare exceptions, unsuccessful (Alexander and

Evans, *in press*). Therefore, tests were conducted to determine if the isolate could provoke *hebdomadis*-group agglutinins in cattle. Two calves were each inoculated subcutaneously with 5.0-ml suspensions of tissue from infected hamsters of the 16th passage. A transient fever, 103.4 and 104.5 F, but no other sign of disease was seen on the ninth day postinoculation. During a 28-day observation period, repeated attempts to isolate leptospirae by inoculating hamsters with blood and urine were unsuccessful. Serum samples obtained during the second to fourth week postinoculation had agglutinin titers of 1:25 against *L. icterohaemorrhagiae*, *L. canicola*, and *L. ballum*. Sera from both calves were negative up to the tenth day. No serological reactions were elicited against serotypes in the *hebdomadis* group.

Cultural studies. The primary objective of cultural studies was the development of a medium that would maintain the Grand River isolate *in vitro* and provide a source of antigen requisite for serological identification of the strain. No attempt was made to determine the essential nutrients for the isolate. In this study, conventional media and conditions of incubation were varied or modified on an empirical basis. Stuart's liquid (Stuart, 1946) and Fletcher's semisolid media were principally used. Modifications and variables employed either singly or in combination included the following: source of serum, rabbit, cow, horse, and sheep; inactivation of serum, 30 to 45 min at 56 and 100 C; serum concentration, 10 to 25%; pH, 7.15 to 7.9; incubation temperature, 30 and 37 C; agar concentration (in Fletcher's medium), 0 to 0.15%; additives, yeast lysate (120 to 900 mg/100 ml), thiamine hydrochloride (40 mg), liver extract (70e to 140 mg/100 ml), charcoal (150 mg/100 ml), gelatin (150 mg/100 ml), Lipomul (a commercial emulsion consisting of cottonseed oil, dextrose, lecithin, and oxyethylene oxypropylene polymer, Upjohn Company, Kalamazoo, Mich., 1 to 10%), and TEM-4t (a water-dispersible combination of palmitic, oleic, and stearic acids, Hachmeister Co., Pittsburgh, Pa., 0.5 mg). Cox's (Cox and Larson, 1957), Chang's (1947), and Earle's (1943) fluid media and modifications thereof were used in more limited trials.

The organism failed to grow in Stuart's basal medium enriched with varying concentrations of rabbit, cow, or horse serum used fresh or inactivated at different temperatures. Additional variations in pH and temperature of incubation did

not affect growth. The addition of thiamine hydrochloride or liver extracts to conventional Stuart's medium was similarly ineffective. Other experimental media employed without success were: Chang's medium containing variable concentrations of liver extract and 10 or 20% of either sheep or rabbit serum inactivated at 56 or 100 C; Cox's liquid medium similarly prepared with sheep and rabbit serum; and Earle's medium containing 10% horse serum. The strain did not form colonies on solid medium (1% agar) prepared with Cox's or Stuart's medium containing different concentrations of rabbit serum and incubated at 30 C under normal aerobic conditions or under 1% CO₂. Transient growth was obtained in Stuart's medium (pH 7.15) containing 20% sheep serum inactivated at 56 C. However, growth declined after the fourth subculture. Additional attempts to cultivate the strain in the modified Stuart's medium were not successful. However, Fletcher's semisolid medium, containing 20% sheep serum, supported growth. Growth could be maintained by successive transfers. After incubation at 30 C for 1 to 2 weeks, inoculated cultures showed a disc of growth 1 to 2 cm below the medium surface, characteristic of leptospiral cultures in semisolid medium. Further incubation resulted in a rapid decline in cell populations, with few surviving cells at 3 to 4 weeks. More vigorous growth was obtained when rabbit serum in a final concentration of 20% was used in lieu of sheep serum. In this medium, cultures remained viable during an observation period of 2 to 3 months. Sera used in these successful growth trials were inactivated at 56 C. Heat inactivation of serum at 100 C destroyed the growth factor. At present, the Grand River strain has been maintained in Fletcher's semisolid medium (20% rabbit serum) through 18 transfers; subcultures have generally been made at intervals of 4 to 6 weeks. The use of lesser concentrations of rabbit serum gave decreased growth. Repeated attempts to maintain the strain in ordinary Fletcher's medium (10% rabbit serum) have been unsuccessful.

Although the modified Fletcher's semisolid medium provided a satisfactory milieu for the isolation of the Grand River strain from experimentally infected animals and for the maintenance of the strain *in vitro*, it was not suitable for the production of antigens for use in microscopic agglutination and agglutinin-adsorption

TABLE 1. Cross-agglutination reactions between strain Grand River (GR) and other serotypes

Serotype*	Homologous serum titer†	Titer† with GR	
		Antigen	Antiserum
Grand River.....	102,400		
andamana.....	102,400	—†	—
semaranga.....	25,600	—	—
djatzi.....	25,600	—	—
hyos.....	25,600	—	—
pomona.....	25,600	1,600	—
djasiman.....	25,600	—	—
sentot.....	25,600	400	NT
autumnalis.....	25,600	1,600	1,600
australis.....	25,600	1,600	100
ballum.....	25,600	—	—
canicola.....	102,400	1,600	100
icterohaemor-			
rhagiae (M20)....	102,400	102,400	25,600
zanoni.....	25,600	25,600	NT
alexi.....	25,600	—	—
grippotyphosa.....	25,600	—	—
medanensis.....	6,400	—	NT
javanica.....	6,400	1,600	400
pyrogenes.....	25,600	100	—
jonsis.....	25,600	—	NT
icterohaemor-			
rhagiae (RGA)...	102,400	102,400	6,400
mankarso.....	102,400	102,400	25,600
naam.....	102,400	102,400	102,400
malaya.....	25,600	6,400	NT
sarmin.....	25,600	25,600	6,400

* Type strains of serotypes employed.

† Titer expressed as reciprocals; — = negative at 1:100 dilution; NT = not tested.

tests. A fluid medium, free of particulate matter, was required as a source of antigens for these purposes. As previously noted, comparable concentrations of rabbit or sheep serum in other fluid mediums did not support growth. However, the strain could be grown in Fletcher's medium devoid of agar and containing a 20% concentration of rabbit serum. The maximal cell concentration which appeared after 2 weeks of incubation was approximately 10 to 30 × 10⁶ organisms per ml, which is tenfold less than that obtained generally in fluid cultures of other strains. Less growth was obtained when sheep serum was used in cultures. The employment of serum concentrations in excess of 20 to 25% did not enhance growth. Addition of thiamine hydro-

TABLE 2. Results of cross-agglutinin adsorption tests on strain Grand River with members of the icterohaemorrhagiae serogroup

Antiserum		Reciprocal of titer* with	
Against	Adsorbed with	Homologous strain after adsorption	Adsorbing strain before adsorption
Grand River (102,400)	icterohaemorrhagiae (M-20)	25,600	25,600
Grand River (102,400)	icterohaemorrhagiae (RGA)	25,600	6,400
Grand River (102,400)	naam	25,600	102,400
Grand River (102,400)	mankarso	102,400	25,600
Grand River (102,400)	sarmin	25,600	6,400
Grand River (102,400)	birkin	25,600	25,600
Grand River (102,400)	ndambari	102,400	6,400
icterohaemorrhagiae (M-20) (102,400)	Grand River	25,600	102,400
icterohaemorrhagiae (RGA)	Grand River	102,400	102,400
naam (102,400)	Grand River	1,600	102,400
mankarso (25,600)	Grand River	102,400	102,400
sarmin (102,400)	Grand River	102,400	102,400
birkin (102,400)	Grand River	102,400	102,400
ndambari (102,400)	Grand River	102,400	102,400

* Homologous titer of unadsorbed serum shown in parentheses. After adsorption with homologous antigen, serum agglutinins were undetectable at 1:100 dilution. Cross-agglutinins against heterologous (adsorbing strain) antigens were undetectable at 1:100 dilution after adsorption with respective heterologous antigens.

chloride, charcoal, or gelatin to Fletcher's fluid medium did not affect the growth of the Grand River strain. The addition of chloroform-soluble extracts of agar resulted in poorer growth; Lipomul,

when added to media, inhibited growth. However, growth appeared to be slightly increased when TEM-4t was added to the media. The leptospiral growth-supporting characteristics of TEM-4t had been noted previously by Vogel and Hutner (*personal communication*). The modified Fletcher's medium, with and without the addition of TEM-4t, was used for serological studies.

Serological studies. The serological response of experimentally infected guinea pigs, cows, and hamsters provided ancillary evidence that the Grand River strain was a member of the icterohaemorrhagiae serogroup. This was further substantiated by results of cross-agglutination tests (microscopic agglutination technique) conducted with the Grand River strain and the homologous antiserum prepared in the rabbit (Table 1). The cross-agglutination reactions of strain Grand River were similar to those shown by members of the icterohaemorrhagiae serogroup. Reciprocal agglutinin-adsorption tests were conducted to determine the antigenic relationships of strain Grand River with various members of the icterohaemorrhagiae serogroup. The results indicate that the antibodies associated with specificity of strain Grand River were unaffected after adsorption with any of the serotypes in the icterohaemorrhagiae group (Table 2). Conversely, adsorption of the various serotype antisera with strain Grand River did not result in a significant reduction in the respective homologous titers except for *L. naam* antiserum. In this case, the homologous titer was reduced 64-fold. A repeat test on the *L. naam* antiserum with the use of two-fold, rather than fourfold, dilutions substantiated the initial findings.

DISCUSSION

Basic methods for the in vitro cultivation of leptospirae were formulated within a few years after the demonstration of the etiology of Weil's disease (Uhlenhuth and Fromme, 1930). Essentially, the media comprise a buffered-salt solution (pH 7.0 to 7.8) fortified with 7 to 15% mammalian serum, the essential growth ingredient. These media have been employed successfully for the isolation and maintenance of numerous antigenic types of pathogenic as well as nonpathogenic leptospirae.

Growth characteristics of leptospirae have been used as a principal taxonomic criterion for the differentiation of the nonpathogenic "water"

leptospirae from the pathogenic types. The former strains could be cultivated in an egg-yolk or fecal medium, whereas the latter types required mammalian serum. Recently, differences among "water" leptospirae in their cultural requirement for vitamin B₁₂ and serum have been noted (Babudieri and Zardi, 1960/61). To date, the pathogenic leptospirae have not been differentiated from each other on the basis of growth characteristics. They do, however, vary considerably in their ability to grow in conventional leptospiral media. Some types (*L. pomona*, *L. canicola*, and *L. icterohaemorrhagiae*) grow profusely in cultures, whereas others (*L. hyos*, *L. sejroe*, and *L. hardjo* LSU 79) are frequently difficult to cultivate under comparable cultural conditions. These are common observations in all laboratories maintaining multiple stock cultures of leptospiral serotypes. However, these observations have not been generally noted in the monographs and text books on leptospirosis and are rarely or only casually mentioned in the vast literature on leptospirosis. The growth of the Grand River strain differed significantly from the other pathogenic leptospirae, including the more fastidious types, in that it could not be cultivated in a medium containing less than 20% serum. In this regard, it is noteworthy that some leptospiral strains are reported to grow more readily in the presence of higher concentrations (20 to 33%) of serum (Fuchs, 1959; and Noguchi, 1918).

It was evident in this study that the concentration of serum was not the only critical factor for the growth of the Grand River strain in modified Fletcher's media. Other basal media containing comparable amounts of serum did not support growth. The peptone or meat extract present in Fletcher's but not in other media probably supplied an essential nutrient. Other investigators (Babudieri and Zardi, 1960/61; Czekalowski, McLeod, and Rodican, 1954) have noted that growth of leptospirae could be enhanced by the presence of suitable concentrations of some peptone preparations. This aspect was not investigated and requires further study.

The fastidious growth characteristic of the Grand River strain poses questions on the suitability of currently employed leptospiral media for the isolation and identification of pathogenic leptospirae. The presence of other comparable leptospiral types merits consideration in investigation of human and animal leptospirosis. It is interesting to note that in the United States¹ one

of the rare leptospiral isolates from cattle, serologically positive to hebdomadis-group serotypes, was recovered in medium only after several serial passages in cows (Roth and Galton, 1960). Moreover, this isolate, *L. hardjo* strain LSU 79, was difficult to maintain in conventional culture media. Strains which cannot readily be cultivated in vitro have also recently been isolated from cattle by Clark and Kresse (*personal communication*) in epidemiological investigations being conducted in Pennsylvania. Heretofore, some investigators (Gochenour, Gleiser, and Ward, 1958) have believed that the use of laboratory animals for the isolation of leptospirae afforded no advantages over direct culture of tissues and body fluids that could be obtained aseptically. This study indicates that presently used media do not obviate the use of laboratory animals for the isolation of leptospirae. The suitability of the modified Fletcher's medium (20% rabbit serum) as a general-purpose medium requires additional study.

The isolated strain also differed from the recognized serotypes in its antigenic characteristics. Although this isolate possesses distinct antigenic properties not heretofore described, it does have close serological affinities with *L. naam*, a type that grows remarkably well in medium. According to currently proposed taxonomic criteria for the genus *Leptospira* (World Health Organization, 1959), "two strains are considered to belong to different serotypes if, after cross-adsorption with adequate amounts of heterologous antigen, 10% or more of the homologous titer regularly remains in each of the two antisera in repeated tests." A subserotype is defined as "a strain within a serotype where, in repeated tests, less than 10% of the homologous titer remains in one antiserum but 10% or more in the other antiserum after cross-adsorption with heterologous antigen." On this basis, strain Grand River can be classified as a subserotype of *L. naam*. Accordingly, it is proposed that strain Grand River be designated *L. naam*, subserotype *dakotii*.

The potential veterinary and public health significance of *L. naam dakotii* is not known. It is interesting to note that this organism elicited febrile and antibody responses in inoculated calves. One must assume that it is potentially pathogenic for man in view of the marked susceptibility of man to leptospiral infections.

Although *L. naam dakotii* was isolated at a focus of human infection, its possible etiological role in this outbreak could not be affirmed. Serological tests on patients' serum provided presumptive evidence of infection with a strain closely related to *L. pomona*, but did not necessarily rule out infection with *L. naam dakotii*. More likely, the presence of an *L. pomona* serotype and the Grand River isolate at the same site was coincidental. The area is frequented by a variety of domestic and wild animals that may comprise the primary hosts of different leptospiral serotypes. The natural host of *L. naam dakotii* has yet to be determined.

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