Identification of Two Leptospiral Serotypes New to the United States

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RECENTLY, two leptospiral serotypes not found previously in the United States were isolated from the kidneys of wild animals collected in southwest Georgia (*1*). The identity of these two serotypes, one a member of the *mitis-hyos* serogroup, the other belonging to the *australis* A serogroup, is recorded in this paper.

Relatively few leptospiral serotypes have been isolated in the United States, probably because of the limited number of medical and veterinary laboratories providing appropriate diagnostic service. *Leptospira icterohemorrhagiae*, the first serotype isolated in this country, was obtained from wild rats in 1917 (*2*) and from man in 1922 (*3*). Since that time, four other serotypes, *Leptospira canicola* (*4, 5*), *Leptospira pomona* (*6–8*), *Leptospira autumnalis* Fort Bragg (*9*), and *Leptospira ballum* (*10*), have been identified in the United States. Serologic evidence has indicated that infection with strains related to *Leptospira bataviae* (*11*), *Leptospira sejroe* (*12, 13*), *Leptospira grippotyphosa* (*14*), and *Leptospira pyrogenes* (*15*) may exist, but these serotypes have not been isolated.

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Four of the cultures of leptospirae included in this study were isolated from the kidneys of opossums trapped on two plantations in Baker and Dougherty Counties, Ga., in September and October 1955. These were designated LT79, LT81, LT82, and LT85. Two additional cultures, designated LT95 and LT102, were isolated from the kidneys of two raccoons trapped on a plantation in Decatur County, Ga. All cultures were tested first against immune serums of *L. autumnalis*, *L. ballum*, *L. canicola*, *L. icterohemorrhagiae*, *L. pomona*, and *L. sejroe*, the usual battery used to test leptospiral isolations received from the Communicable Disease Center field station in Newton, Ga., and then with immune serums representative of the remaining 14 serogroups.

Procedures

Preparation of antiserums. Immune serums were prepared by intravenous inoculation of normal rabbits weighing 8–9 lbs. with successive doses of 1.0-ml., 4.0-ml., 4.0-ml., and 6.0-ml. amounts of a 4- to 6-day-old culture of each leptospiral strain grown in Stuart's medium (*16*) and killed by the addition of 0.3 percent formalin. The 4 injections were given at 5- and 7-day intervals. Seven days after the last inoculation the rabbits were bled from the heart. Serum yield averaged 75 ml. per rabbit. After the addition of 50 percent glycerine as a preservative, the serums were stored at 40° F.

Preparation of antigens. Leptospiral strains used for antigen production were maintained in

Stuart's medium and transferred twice weekly. Such actively growing seed cultures were used in approximately 1:10 to inoculate the desired amount of Stuart's medium in screw-capped prescription bottles. The inoculated bottles were incubated 4–5 days at 28°–30° C. and then examined by dark-ground microscopy for density and smoothness. If the antigens appeared satisfactory, 0.3 percent formalin was added, and they were allowed to stand overnight at room temperature. They were centrifuged for 10 minutes at a speed of 1,500×gravity (about 3,000 r.p.m. on a No. 1 International Centrifuge) to remove debris or precipitate. The supernatant was decanted and was then ready for use.

Agglutination test procedure. Serial twofold dilutions of serum in 0.85 percent saline, starting with 1:8 through 1:32,768, in a final volume of 0.2 ml. were prepared. To each serum dilution, 0.2 ml. of antigen was added. The tubes were shaken, incubated in a water bath at 52° C. for 2 hours, and then refrigerated for 1 hour. A drop from each tube was examined by dark-ground microscopy using low-power objective, and 10× oculars without a coverslip. The degree of agglutination was read as 1 plus, 2 plus, 3 plus, 4 plus, or negative. A reaction was recorded as 4 plus when all leptospires appeared clumped, 3 plus when approximately 75 percent of the organisms were agglutinated, 2 plus with 50 percent agglutinated, and 1 plus with 25 percent agglutinated. The end point was taken as the last dilution showing a 1 plus reaction.

Agglutinin absorption procedure. Antigens for absorption studies were prepared from 5- to 6-day-old cultures grown in Stuart's medium and killed by the addition of 0.3 percent formalin. After standing at room temperature overnight, the cultures were centrifuged for 10 minutes at 1,500 × gravity to remove extraneous material. Ten to twenty milliliters of the supernatant were put aside to be used later as antigen for testing the absorbed serum. The remainder of the supernatant was centrifuged for 15 minutes at 9,000 × gravity in a Servall. The supernatant was discarded, and the desired amount of a 1:20 dilution of serum was added to the packed cells. The cells were resuspended using a 2.0-ml. Cornwall pipette with a 4-inch, 17-gauge needle. After incubation of the serum-cell mixture in a 50° C. water bath for 2 hours and overnight at 28°–30° C., the cells were removed by centrifugation and the serums absorbed a second time by the same procedure but without overnight incubation. If necessary, a third absorption was carried out. Absorptions were considered complete when agglutinins were completely removed by the homologous antigen. Microscopic agglutination tests with the absorbed serums were performed by the procedure described above except that the initial dilution was 1:40.

Findings

None of the cultures in this study agglutinated when tested against the usual battery of leptospiral immune serums.

The mitis-hyos serogroup strains. Culture LT79 was agglutinated by *Leptospira mitis* Johnson antiserum to 25 percent of the titer, by *Leptospira hyos* antiserum to 6 percent of the titer, by *L. bataviae* antiserum to 3 percent of the titer, and by *L. pyrogenes* antiserum to less than 1 percent of the titer. An antiserum prepared against strain LT79 agglutinated to the homologous titer *L. mitis* and *L. hyos* antigens. Cross agglutinin absorption tests performed with *L. mitis* and *L. hyos* indicated that LT79 is closely related to both strains but not antigenically identical with either, as shown in table 1. (A subculture was sent to Col. Maurice Hale, Division of Veterinary Medicine, Walter Reed Army Institute of Research, Washington, D. C., who confirmed our findings.) This new serotype is tentatively designated *Leptospira bakeri.*

According to Wolff (*17*), the retention of at least 10 percent of the homologous titer is the criterion for heterologous strains. Alexander and his co-workers (*18*) modified Wolff's scheme slightly to conform to their dilution scheme and considered 6.2 percent (1/16 of homologous titer) as the breakpoint for a heterologous serotype.

Culture LT85 also was agglutinated to 25 percent of the titer by *L. mitis* antiserum. It reacted to the homologous titer with LT79 antiserum, and in absorption tests it completely removed the agglutinins for LT79. Absorption

Table 1. Results of cross agglutinin absorption tests with LT79, Leptospira hyos, and Leptospira mitis Johnson [1]

Antiserum	Antigen		
	L. hyos	*L. mitis*	LT79
Leptospira hyos			
Unabsorbed	16, 768	16, 768	1, 024
Absorbed with:			
L. hyos	(2)	(2)	(2)
L. mitis	640	(2)	(2)
LT79	5, 120	5, 120	(2)
Leptospira mitis			
Unabsorbed	4, 096	4, 096	1, 024
Absorbed with:			
L. hyos	(2)	(2)	(2)
L. mitis	(2)	(2)	(2)
LT79	640	640	(2)
LT79			
Unabsorbed	4, 096	4, 096	4, 096
Absorbed with:			
L. hyos	(2)	(2)	80
L. mitis	(2)	(2)	40
LT79	(2)	(2)	(2)

[1] Titer expressed as reciprocal of serum dilution.
[2] No reaction in a 1:40 dilution.

studies indicate that LT79 and LT85 are homologous serotypes.

The other two cultures in this group, LT81 and LT82, appear to be related to the *mitis-hyos* serogroup and to the two new isolates but are not identical with either, as shown in table 2. When LT79 antiserum was absorbed with LT81 and LT82 antigens, these antigens failed to remove the homologous agglutinins by 15 percent. In the initial agglutination tests LT81 reacted to 25 percent of the titer of *L. mitis* antiserum, but LT82 reacted to only 6 percent. Thus, further serologic study is needed to determine the exact relationship of these two strains.

The australis A serogroup strains. Cultures LT95 and LT102 were agglutinated to the homologous titer with *Leptospira australis* A Ballico antiserum. An antiserum prepared against LT95 agglutinated the Ballico strain to 12½ percent of its homologous titer. As shown in table 3, absorption studies revealed that Ballico serum retained 2 percent of its titer when absorbed with LT95 cells, and LT95 serum retained only 1 percent of its titer when absorbed with Ballico cells. Thus, except for minor differences, LT95 is indistinguishable from *L. australis* A Ballico.

Comment

L. mitis was isolated first by Johnson (*19*) in 1940 in Australia from humans with benign leptospirosis. All patients had been in contact with pigs or cattle. It has been isolated since from pigs (*20*) in Australia, and serologic evidence suggests that infections occur in cattle. Clinically and epidemiologically, *L. mitis* infection closely resembles *L. pomona* infection.

L. hyos was isolated by Savino and Rennella (*21*) from humans with mild leptospirosis and from swine in Argentina. Babudieri (*22*) studied both the *L. mitis* Johnson and the *L. hyos* strains and reported that they were serologically identical. However, further examination of these strains by A. D. Alexander, Walter Reed Army Institute of Research, showed that *L. hyos* is a complete biotype of *L. mitis* Johnson. The results of the present study substantiate Alexander's observations.

L. australis A was identified by Lumley (*23*) in 1937 from canefield workers in Queensland. Clinical symptoms were reported to be severe. Stagnant water in the canefields yielded the organisms, and a native rat (*Rattus conatus*) was

Table 2. Results of agglutination and cross agglutinin absorption tests with Leptospira mitis Johnson, LT79, LT81, LT82, and LT85 [1]

Antiserum	Antigen			
	LT79	LT81	LT82	LT85
Leptospira mitis				
Unabsorbed	1, 024	1, 024	256	1, 024
LT79				
Unabsorbed	4, 096	256	256	4, 096
Absorbed with:				
LT81	640	(2)	------	------
LT82	640	------	(2)	------
LT85	(2)	------	------	(2)

[1] Titer expressed as reciprocal of serum dilution.
[2] No reaction in a 1:40 dilution.

Table 3. Results of cross agglutinin absorption tests with Leptospira australis A Ballico, LT95 and LT102 [1]

Antiserum	Antigen		
	Ballico	LT95	LT102
Leptospira australis A Ballico			
Unabsorbed	2, 048	2, 048	2, 048
Absorbed with:			
Ballico	(2)	(2)	(2)
LT95	40	40	--------
LT95			
Unabsorbed	1, 024	8, 192	8, 192
Absorbed with:			
Ballico	(2)	80	--------
LT95	(2)	(2)	--------
LT102	--------	(2)	(2)

[1] Titer expressed as reciprocal of serum dilution.
[2] No reaction in a 1:40 dilution.

found to be the principal animal carrier. Recently, two additional strains of the *australis* A serogroup have been found, one in Australia (*24*) and the other in Malaya, according to Alexander. Further studies with these and with the isolations from raccoons will be done to determine the exact relationships.

The identification of these strains in the United States further emphasizes the importance of serotyping all leptospirae isolated. In addition, inclusion of *L. mitis* or *L. hyos* and *L. australis* A cultures in the battery of antigens employed in the agglutination and agglutination lysis tests in diagnostic medical and veterinary laboratories must now be considered.

Summary

Four strains of leptospirae isolated from opossums, belonging to the *mitis-hyos* serogroup, and 2 strains from raccoons, belonging to the *australis* A serogroup, have been identified. Cross agglutinin absorption studies indicate that the opossum strains are not identical with *Leptospira mitis* Johnson or *Leptospira hyos*, but that they represent at least one new serotype, tentatively designated *Leptospira bakeri*, within the serogroup and possibly one other serotype. Further studies are being done to determine the exact relationships.

Two cultures from raccoons were shown by cross agglutinin absorption studies with *Leptospira australis* A Ballico to be within the range considered acceptable for homologous serotypes.

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Since this work was completed, we have learned of a new serotype of the mitis-hyos group. The new leptospiral serotype, designated Kisuba, was isolated in the Belgian Congo (25). The tentative designation of LT79 as Leptospira bakeri is suggested until its relationship with strain Kisuba is established.

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Advisory Committee

Surgeon General Leroy E. Burney has appointed a committee of physicians to advise him on Public Health Service activities related to the practice of medicine. The first meeting of the new Advisory Committee on Medical Practice Relations was held on April 4–5, 1957, in Washington, D. C.

Dr. Burney said that with the growth of medical and related research, it has become increasingly important to work with private physicians as well as with health agencies in applying new knowledge promptly and effectively.

Members of the committee are Dr. Stuart Adler, Albuquerque, N. Mex.; Dr. C. Byron Blaisdell, Asbury Park, N. J.; Dr. Hugh H. Hussey, Washington, D. C.; Dr. W. L. Portteus, Franklin, Ind.; Dr. Julian P. Price, Florence, S. C.; Dr. Stanley R. Truman, Oakland, Calif.; and Dr. William B. Walsh, Washington, D. C.