NOTES

Comparative Serological Studies Between a Newly Isolated Halophilic *Leptospira* and Two Other *Leptospiras* Isolated from Brackish Water

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A leptospiral strain designated "Muggia" was isolated from seawater. It was found to be halophilic, growing only in media prepared with seawater; otherwise it was characteristic of "saprophytic" (biflexa) leptospiras. By the use of extensive cross-agglutination tests, it was compared with two other brackish-water, but nonhalophilic, leptospiral isolates, 8M and 11M. The three strains were serologically distinct from each other. Strain 8M was found to belong to a new serotype, which was designated *timavo*. Strains Muggia and 11M cross-reacted with various biflexa serotypes but could not be definitively identified with any known serotype. They may also represent new serological types.

The isolation of leptospiras from seawater has been reported a number of times in the past (1-3, 9, 10). However, none of these microorganisms required a medium containing an unusual salt concentration for growth. In the present paper, we report the isolation of a strain of *Leptospira* which shows a unique salt requirement for growth.

For the isolation of this strain, 250 ml of seawater, drawn from the Adriatic Sea near Trieste (Bay of Muggia), was divided into 70 samples. Each sample was inoculated in Zuelzer (10) medium prepared with seawater with a salt concentration of 3.5%. After 15 days of incubation at 20 C, leptospiras were demonstrated in 19 of the 70 samples by dark-field microscopy.

By filtration of the 19 positive samples through a membrane filter (0.22 μ m; Millipore Corp.) and serial passage in Korthof-Babudieri medium in which seawater was substituted for distilled water, a strain of *Leptospira*, which we designated "Muggia," was isolated, apparently in pure culture, from one of the samples. An antiserum prepared against the 6th-passage organisms completely suppressed the growth of the first isolate. This finding provided strong presumptive evidence that the culture was composed of organisms belonging to only one serotype (7). Strain Muggia is resistant to

various growth inhibitors of the pathogenic leptospiras: growth was not inhibited by sodium bicarbonate (1 mg/ml) (8), 8-azaguanine (200 μ g/ml) (6), or copper sulfate (10 μ g/ml) (5). Electron microscopy of strain Muggia showed a morphology typical of members of the genus *Leptospira*. Thin sections (Fig. 1, 2) revealed the presence of: an outer sheath with three layers (two dense and one light); a cell wall-membrane complex, 6 nm wide, with two dense layers and a light, intermediate one; and finally an axial filament 0.01 to 0.02 μ m in diameter.

The new leptospiral strain and two strains not yet classified, 8M and 11M, were compared with the reference strains of all of the *Leptospira* serotypes belonging to the "biflexa" complex by using the standard cross-agglutination and cross-absorption tests (according to the WHO/FAO rules on leptospiras, WHO/FAO Technical Rep. no. 380). Strains 8M and 11M were isolated from brackish water and have no special salt requirement for growth (3). The results of the cross-agglutination tests are given in Table 1, in which only the strains that showed some degree of reactivity with the three strains under study are reported.

The three strains shared no major agglutinogens. Strain 8M had antigenic affinities with serotype *tredici* strain AM13, but was anti-

Fig. 1. Thin section of a cell of strain *Muggia* fixed in 1% OsO_4 . Abbreviations: OS, outer sheath; PC, protoplasmic cylinder; CW, cell-wall membrane complex. $\times 307,800$.

genically distinct from this strain on the basis of cross-agglutinin-adsorption test findings. Accordingly, strain 8 M represents a new serotype, which we designate *timavo* and which can be grouped in the Holland serogroup (comprises

tredici, roma, and holland). Strains Muggia and 11M could not be classified into any of the currently recognized serogroups, and they may represent new serological types.

Strain Muggia proved to be halodependent.

Fig. 2. Thin section of a cell of strain Muggia. Abbreviations: AF, axial filament; OS, outer sheath; PC, protoplasmic cylinder. $\times 239,400$.

TABLE 1. Cross-agglutination tests between strains Muggia, 8M, and 11M and reference strains of leptospiral serotypes having antigens in common

Leptospiral serotype ^a	Strain	Titers ^b (% of homologous titer)					
		Muggia		8M		11M	
		Antigen	Antiserum	Antigen	Antiserum	Antigen	Antiserum
<i>timavo</i>	Muggia	100		5	0.2	1	0.1
	8M	0.2	5	100		1	2
	11M	0.1	1	2	1	100	
<i>tredici</i>	AM13	1	50	50	50	0	10
<i>roma</i>	AM6	0	10	0.5	10	1	100
<i>holland</i>	WaZH	0	2	100	5	2	0.5
<i>pulpudeva</i>	Bulgaria 6	0	0	0	5	0	1
<i>farneti</i>	Farneti	1	0	5	0.1	10	10
<i>rupino</i>	RPE	0.1	0	1	0.2	1	0.1
<i>aurisina</i>	Aurisina	10	0.2	0.5	0	2	0.1
<i>basovizza</i>	Basovizza	0	0	0	0	0.1	0
<i>doberdò</i>	Doberdò 1	0	0	10	0.2	1	0
<i>acquamarcia</i>	AM20	0.2	2	10	10	10	0.2

^aCross-reactions with 17 biflexa serotypes were negative.^bHomologous titers ranged from 1:50,000 to 1:100,000.

TABLE 2. Results of cross-agglutinin-absorption tests between leptospiral strains 8M and AM13

Anti-serum	Treatment	Reciprocal of titer with antigen	
		8M	AM13
8M	None	50,000	25,000
	Adsorbed with 8M	<500	<500
	Adsorbed with AM13	25,000	500
AM13	None	50,000	100,000
	Adsorbed with 8M	<1,000	100,000
	Adsorbed with AM13	<1,000	<1,000

Indeed, the doubling time of a culture of this strain in Korthof-Babudieri seawater medium was about 16 to 18 h, and a maximal concentration of 2×10^8 cells/ml was reached after 10 days of incubation at 30 C. No growth was observed in the same medium prepared with distilled water. On the contrary, an inoculum of 10^7 cells/ml of strain Muggia was lysed in such a medium, as judged by the fact that no cells were observed by dark-field microscopy of the inoculated medium after 48 h of incubation at 30 C. Limited growth was observed in Korthof-Babudieri medium prepared with 50% seawater and 50% distilled water. It has recently been demonstrated that strain Muggia shows a specific NaCl requirement for growth (4).

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REPRINT REQUESTS

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