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VIRULENCE-LINKED COLONIAL AND MORPHOLOGICAL VARIATION IN *LEPTOSPIRA*

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ABSTRACT

FAINE, S. (University of Sydney, Sydney, Australia), AND J. VAN DER HOEDEN. Virulence-linked colonial and morphological variation in *Leptospira*. J. Bacteriol. **88**:1493-1496. 1964.—Large-colony typical hooked *Leptospira icterohaemorrhagiae* was virulent for hamsters and guinea pigs. On cultivation, it was gradually replaced by a serologically identical small-colony avirulent straight mutant. The hooked virulent form was selected in vivo.

The genus *Leptospira* comprises coiled organisms with either one or both ends of the cells bent into a semicircular hook when observed in liquid media (Breed, Murray, and Smith, 1957). However, nonhooked, straight organisms are known to occur, as in the Jackson strain of *L. icterohaemorrhagiae* (Alston and Broom, 1958) and in the strains of *L. celledoni* and *L. hyos* studied by Simpson and White (1964). Alston and Broom (1958) stated that the Jackson strain became straight during laboratory cultivation, but whether or not other strains of straight leptospires were previously hooked is not recorded.

The Jackson strain is avirulent, but information is not available about the virulence of other straight leptospires. Since the Jackson strain was presumably virulent in the host from which it was isolated, loss of virulence in this strain of straight leptospires was accompanied by loss of hooks.

Stalheim and Wilson (1963) described variation in colonial morphology but did not find any relationship between colonial form and virulence, and did not comment on the morphology of leptospires of different colonial types. The strain of *L. icterohaemorrhagiae* to be described showed a variation from hooked to straight forms, each of a different order of virulence, growing in a characteristic colony.

MATERIALS AND METHODS

Leptospires. The leptospiral strain (GP) described here was isolated from the kidneys and urine of a guinea pig (GP IV) which had been inoculated 1 month previously with material from the kidneys of a rat suspected to be infected with leptospirae. The parent cultures were typical *L. icterohaemorrhagiae* (AB) which agglutinated to full titer with reference antiserum. At the outset of the study, a dose of 10^8 leptospires was lethal in 5 days for guinea pigs weighing 200 to 250 g; 10^3 leptospires killed adult gerbils (*Meriones shawi*) in 7 days.

Media. The leptospires were grown at 28 ± 1 C in stationary cultures in modified Korthof medium containing 10% rabbit serum and 10 μg/ml of thiamine. Solid media were prepared by adding a final concentration of 1 g per 100 ml of agar (Difco) to the liquid medium; sometimes 2,6-dichlorophenol-indophenol was added to a final concentration of 1:50,000 (Kirschner and Graham, 1959), with no apparent effect on the results obtained. Tubes of solid media were inoculated either by surface drops or stabs; similar results were obtained in either instance. Petri dishes were inoculated by streaking or dropping diluted cultures. Colonies were viewed by transmitted light and photographed with the apparatus described by Rudge (1960).

Serological methods. Rabbits were immunized with one intravenous injection of 2 to 5 ml of live fluid culture 7 to 10 days old, and were bled 11 to 17 days later. International standard reference antiserum (*L. icterohaemorrhagiae*, AB) was prepared by B. Babudieri, Istituto Superiore di Sanitá, Rome, Italy, and was distributed by the World Health Organization.

Standard agglutination-lysis and cross-absorption procedures (Wolff, 1954) were followed.

RESULTS

Colonial variation. The parent (GP) strain plated on solid medium was found to be a mixture of large-colony (LOH) and small-colony (SOD) types, respectively similar to the LOH and SOD types described by Stalheim and Wilson (1963) except that the LOH type did not show the tendency to change to LOD → LTD. Each type was isolated (Fig. 1). Suspensions of fluid cultures made from each type of colony reproduced the characteristic colonial type when replated on solid medium.

Each of the colonial types was compared with the other and with the parent GP strain growing in tubes of solid medium, inoculated either by stab or surface drop methods. Similar results were obtained in all cultures; typical Dinger discs (Dinger, 1932) of growth were situated, in order of density, 12, 21, and 16 mm below the surface in tubes of GP and LOH, and 16 and 19 mm in SOD.

Morphological variation. Dark-field microscopy showed that the LOH colonies, and cultures derived from them, were typical leptospires, with hooks at both ends and vigorous translational and rotational motility. In contrast, the SOD colonies, and cultures derived from them, were straight leptospires with rotational motility comparable with that of the hooked type, but with barely discernible translational motility, at most, approximately one-tenth to one-twentieth that of hooked leptospires (Fig. 2). During successive subcultures in fluid medium, the straight leptospires showed a tendency to grow in chains, although usual densities of approximately 5×10^7 to 5×10^8 leptospires per ml were achieved, comparable with those of the hooked leptospires. This trend was especially noticeable in cultures incubated at 37 C; 70 to 90% of the population of the GP strain and its subcultures were typically straight and slowly motile, and tended to grow in chains. During successive subcultures of hooked leptospires, an increasing proportion of straight leptospires appeared, with a correspondingly increased proportion of small colonies among the large when plated on solid media.

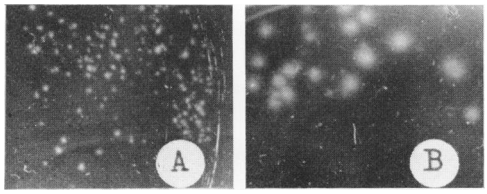

FIG. 1. *Small (SOD-type) colonies (A) and large (LOH-type) colonies (B) of Leptospira icterohaemorrhagiae (×2). A few small colonies may be seen among the large colonies.*

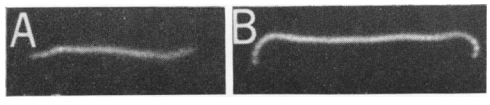

FIG. 2. *Straight (A) and hooked (B) leptospires corresponding to forms in SOD and LOH colonies, respectively. Dark-field illumination (×1,200).*

TABLE 1. *Cross-absorption titers**

Antiserum	Agglutinating suspension			
	Hooked	Straight	Wijnberg	Kantarowicz
Hooked unabsorbed	10,240	10,240	10,240	5,120
Hooked absorbed with straight	40	<40	<40	<40
Straight unabsorbed	5,120	5,120	5,120	2,560
Straight absorbed with hooked	0	0	0	0

* Antisera against hooked (LOH) and straight (SOD) types of *Leptospira icterohaemorrhagiae* titrated against homologous, heterologous, and reference strains ("complete biotype, AB," Wijnberg and "incomplete biotype A," Kantarowicz) before and after absorption with homologous and heterologous leptospires.

Serological comparison. Antisera against hooked leptospires cross-agglutinated straight leptospires to full titer, and vice versa. Each type was agglutinated to full titer by World Health Organization reference antiserum. A series of cross-absorptions with the use of antisera against the homologous strain, the "complete biotype" (*L. icterohaemorrhagiae* AB, Wijnberg), and the "incomplete biotype" (*L. icterohaemorrhagiae* A, Kantarowicz) showed similar results in all tests. A typical result (Table 1) showed that homologous, "AB," and "A" titers were all reduced by an equivalent amount after absorption with cultures of either hooked or straight leptospires.

Comparison of virulence. Virulence of each type was compared by titration of lethality in

groups of five hamsters of an average weight of 95 g, and in pairs of guinea pigs weighing approximately 700 g. The GP cultures used for the hamster titrations comprised 95% straight forms; those used for the guinea-pig titrations comprised 70% straight and 30% hooked shapes. The results (Table 2) indicate that the GP and hooked (LOH) cultures were virulent, but that the straight (SOD) cultures were not. Two guinea pigs injected intraperitoneally with 2×10^9 straight leptospires became febrile and were jaundiced on the third day after infection, but did not die. Pure cultures of straight leptospires were isolated from heart-blood cultures from these guinea pigs. By contrast, a pure population of hooked leptospires was seen in the blood and in tissue suspensions of guinea pigs dying from infection with small doses of cultured hooked or GP type leptospires 5 to 6 days after infection, and in blood cultures from their heart-blood. These findings indicated that the virulent hooked leptospires survived selectively in vivo during infection. As the blood cultures grew and were subcultured, straight shapes appeared, so that within one subculture, estimated at 20 generations, the population in various experiments comprised 50 to 90% straight shapes and the rest hooked shapes.

TABLE 2. *Virulence of variants of Leptospira icterohaemorrhagiae for hamsters, guinea pigs, and mice**

Leptospiral type	Hamster (95 g) LD_{50}	Guinea pig (700 g) LD_{50}	Mouse (12 g) ID_{50}†
GP	10^7	10^4	5×10^6
LOH (hooked)	$<10^3$	10^5	—
SOD (straight)	$>10^7$	$>2 \times 10^9$	—

* All cultures underwent the same number of subcultures between their last animal passage and a testing for virulence in hamsters and guinea pigs.

† Carriers 6 weeks.

DISCUSSION

Although colonial variation in leptospires has been described by Stalheim and Wilson (1963), and although the straight morphological variant has been noted (Simpson and White, 1964), the association of morphology with colonial forms is a new observation. A reasonable explanation would be that the relatively nonmotile straight leptospires tend to proliferate locally, whereas the more motile hooked leptospires travel further in the medium, and produce larger colonies. There may be a nutritional or metabolic difference between the straight and the hooked leptospires, because the straight leptospires tend to form chains characteristic of growth in nutritionally deficient medium, especially at 37 C, and because there are differences in the appearances of discs in cultures in tubes of solid media. The causes of these differences have not been investigated further, but appear to be important. On the basis of electron microscopy, Simpson and White (1964) concluded that there were basic morphological differences between the hooked and straight leptospires, although they were comparing different serological types, one of them nonpathogenic. It is unlikely however, that the basis of virulence lies in the hooked shape of virulent leptospires, for many avirulent leptospires are hooked. Rather, this strain investigated is an example of a coincident simultaneous variation in virulence and visible shape.

The results of the comparison of virulence show that virulent hooked leptospires grow selectively in vivo, so that they were isolated in pure cultures from guinea pigs dying from infection with mixed populations, such as the GP strain or subcultures of the LOH type. The observation that a pure blood culture of straight leptospires was obtained from guinea pigs on the third day of nonlethal infection with straight SOD leptospires is attributed to prolonged initial leptospiraemia following the very large dose. In previous studies of virulence in leptospires, it was suggested that virulent leptospires might be selected by their ability to grow in vivo, and that loss of virulence might be the result of growth in vitro of an avirulent mutant better adapted to cultural conditions (Faine, 1957). Straight shape and SOD type colonies are visible markers of the changes to avirulence in vitro in the strain described here, giving additional evidence to support the above hypothesis.

ADDENDUM

The Taxonomic Subcommittee on Leptospira of the International Committee on Bacteriological Nomenclature has recommended that the former "complete biotype, AB" should be known as *L. icterohaemorrhagiae icterohaemorrhagiae*, and

the former "incomplete biotype, A" as *L. icterohaemorrhagiae incompleta.*

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