

## Surface Colonies of *Leptospira interrogans*

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Observations of colonial growth of *Leptospira interrogans* serovar *pomona*, BJ, on soft-agar (1% agar) plates revealed the presence of both surface and subsurface colonies. The organisms from both types of colonies appeared to be similar in motility, morphology, antigenic composition, and pathogenicity. Passage in vivo tended to produce a higher incidence of surface colonies, whereas passage in vitro tended to produce a higher incidence of subsurface colonies, suggesting the importance of environmental factors in determining the type of colony produced.

Although colonial growth of leptospires was described as early as 1927 by Fukushima (9) and 1938 by Sugimoto (17), these observations received little attention until the report of Cox and Larson in 1957 (6). Subsequently, numerous reports on the colonial growth of leptospires were published (7, 8, 14-16, 18, 19). Larson et al. (13) demonstrated that leptospiral colonies were derived from the growth of single cells. It was observed that a sequential change in colonial morphology may occur as the colonies mature (16) and that colonial heterogeneity exists in some serovars of *Leptospira* (14, 16). Studies of three stable and distinct colonial forms of serovar *autumnalis* provided evidence that no differences existed in their antigenicity or virulence (16). Faine and Van der Hoeden (7) reported virulence-linked morphological variation in serovar *icterohaemorrhagiae*. Upon cultivation, the actively motile, virulent, typically hooked form was replaced by a serologically identical, weakly motile, avirulent, unhooked form. In this situation colonial morphology could be associated with virulence. The avirulent, weakly motile, unhooked leptospires formed small colonies, whereas the virulent, motile, hooked leptospires formed large colonies (7). Similar results were obtained by Petrov and Chernukha (14) with serovar *mozdok*.

One of the characteristics of leptospires is that, when inoculated on the surface of soft agar (1%), the cells grow into the medium, forming subsurface colonies. In this report we describe the colonial growth of some leptospires that occurs on the surface of soft agar.

### MATERIALS AND METHODS

**Maintenance of organisms.** Serovars of *Leptospira interrogans* used in this study were maintained at 30°C in liquid medium or by hamster passage. The primary liquid medium used was a Tween 80-albumin medium (2, 10), with the occasional use of a 10%-rabbit

serum medium (10) containing 100 µg of sodium pyruvate per ml (11). Hamster passage of the virulent strains was accomplished by intraperitoneal injection, and isolation of the leptospires from blood was obtained by cardiac puncture from moribund animals.

Growth in liquid medium was monitored by nephelometry. Direct counts were performed with a Petroff-Hausser chamber and correlated with the turbidity of the culture.

**Solid media.** Solid medium was prepared by the incorporation of 1% (wt/vol) agar in liquid medium. Each petri dish (15 by 100 mm) contained approximately 30 ml of medium. The solid medium was inoculated by spreading a suspension of cells evenly over the surface of the medium with a bent glass rod. The inoculated plates were sealed with tape and incubated at 30°C. Colonies of leptospires were suspended in liquid medium before their transfer to other agar plates.

**Antisera preparation.** Rabbits 3 to 4 months of age were immunized by four weekly intravenous injections of 0.5, 1.0, 2.0, and 4.0 ml of 10<sup>8</sup>/ml of live leptospires. The serum was collected 14 days after the fourth injection.

**Microscopic agglutination test.** The microscopic agglutination test was performed as described by Cole et al. (5).

### RESULTS

Leptospires were isolated by R. F. Bey from the urine of a cow in a herd experiencing problems with abortion. After 10 transfers in liquid medium, the isolate was grown on soft agar (1% agar) medium, and a well-isolated colony was the source of leptospires for further studies. The colonial growth obtained after 10 subcultures in liquid medium was of the subsurface type, which is characteristic of members of the genus *Leptospira* when cultivated on soft agar medium. This isolate was identified as *Leptospira interrogans* serovar *pomona* by the Leptospirosis Reference Laboratory, Center for Disease Control, Atlanta, Ga. It will be referred to as strain BJ.

The original isolate initiated growth well in liquid medium, but ceased growing when cell numbers of approximately  $10^8$ /ml were achieved. After six to eight transfers in liquid medium, cell yields of  $10^9$ /ml were realized. The possibility that the change in growth pattern might be reflected in a change in colonial morphology was investigated. The 1st subculture (which had been stored in liquid nitrogen) and the 20th subculture, (which had been stored in liquid medium) of the original isolate were spread on the surface of Tween 80-bovine serum albumin medium containing 1% agar. After 14 days of incubation at 30°C, only the typical subsurface colonies were formed by cells from the 20th subculture. In contrast, the agar plates inoculated with cells from the first subculture appeared devoid of growth. However, upon careful inspection of the surface of the plates with reflected light, small, dewdrop-like surface colonies were seen. The surface of the agar plate in Fig. 1 contains predominantly surface colonies with a few subsurface colonies. When the surface colonies first appear, they are small, with diameters of 0.5 to 2 mm, and transparent to slightly hazy in appearance, with even margins (Fig. 2). As the colonies mature, there is a small increase in size to diameters of 2 to 4 mm, and concentric rings appear on the surface of the colonies (Fig. 3). The colonies remained as either surface or subsurface types during incubation periods of 90 days. Generally, 3 to 4 additional days were

required for the surface colonies to develop as compared with the subsurface colonies. When agar plates contained large numbers of colonies of both types, the colonial types were not interspersed. Instead, sections of the agar plate would contain predominantly either surface or subsurface colonies, suggesting that one colonial type may have an antagonistic effect on the development of the other type.

Examination of material from the colonial types by dark-field microscopy revealed spirochetes with the gross morphological characteristics of leptospire. Electron microscopic studies indicated that cells from the two colonial types possessed the ultrastructure of leptospire. Both cell types displayed translational motility when placed in liquid plus 3% methylcellulose (0.3 poise [ca.  $0.3 \times 10^{-1}$  Pa·s]). Since the colonial forms were so different, the possibility that the urine isolate contained two antigenic types of leptospire was investigated. The titers obtained with the microscopic agglutination test with antisera to serovar *pomona*, strain Pomona was: 1:25,600 with *pomona*, Pomona; 1:12,800 with surface colony cells; and 1:12,800 with subsurface colony cells. Because the titers did not differ from one another by more than one dilution, it provides presumptive identification of the two colonial types as serovar *pomona*.

Two solid media were evaluated for their capacity to support the growth of surface colonies. The T80-bovine serum albumin solid medium

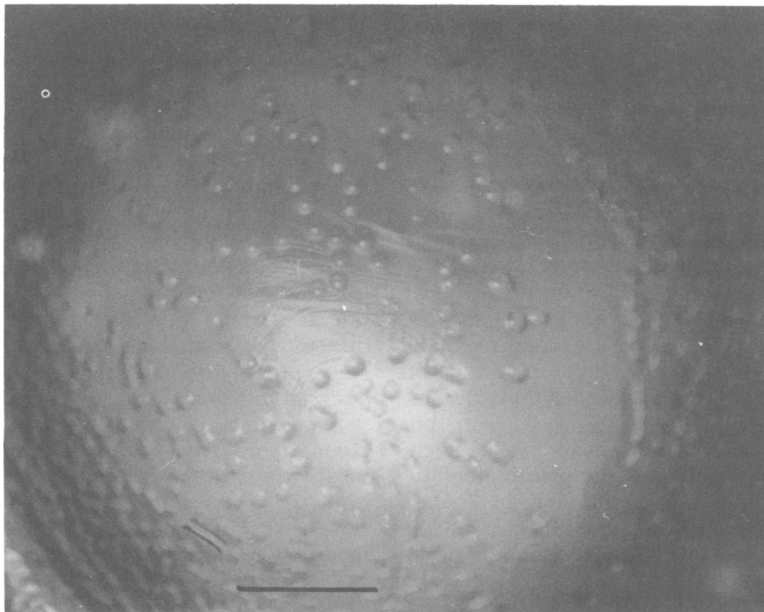


FIG. 1. Surface and subsurface colonies on serovar *pomona*, BJ. Bar = 10 mm.

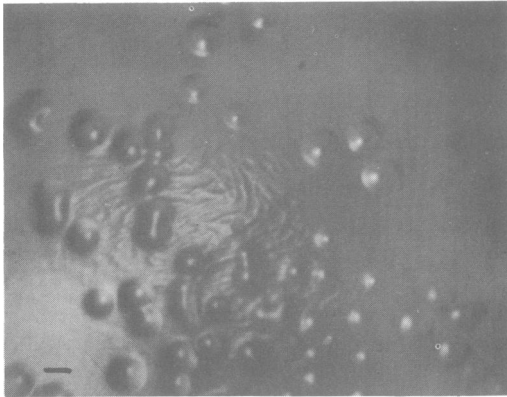


FIG. 2. Surface colonies of serovar *pomona*, BJ. Bar = 1 mm.

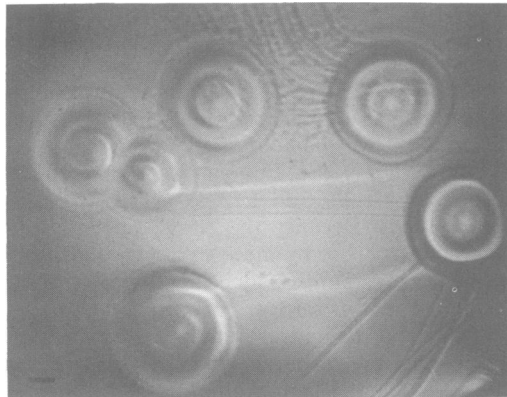


FIG. 3. Surface colonies of serovar *pomona*, BJ, with concentric rings on surface of colonies.

and 1% agar medium containing 10% rabbit serum supported the growth of the surface colonies to approximately the same extent. Subsequent studies were conducted with the T80-bovine serum albumin medium.

The virulence of cells from surface and subsurface colonies was examined. Since the only means of identifying the two cell types is by colonial morphology, it was necessary to use cells grown on soft agar. The surface colonies were scraped from the surface of agar plates, and the subsurface colonies were excised from the agar. Because of the difficulty in obtaining an accurate cell count with these cell preparations, determinations of 50% lethal doses were not attempted. Groups of 10 hamsters were inoculated intraperitoneally with approximately  $10^7$  cells from each colonial type. Within 7 days, both cell types produced lethal infection in 80% of the hamsters, suggesting that no major differences in virulence can be associated with the two colonial types of *pomona*, BJ.

The animal host appears to provide an envi-

ronment that is favorable for the growth of the surface colony-forming cells of *pomona*, BJ. The original urine isolate contained predominantly surface colony cells. When hamsters were infected with surface colonies and the organisms were passed in hamsters four times, mostly surface colonies were present on agar plates inoculated with leptospiremic blood. Also, if hamsters were infected with subsurface colonies, a mixture of cells of the two colonial types could be isolated. In contrast, when cells from either colonial type were continually transferred in liquid medium, the subsurface colony type cells predominated. In addition, the transfer of subsurface colonies to another agar plate resulted in the growth of only subsurface colonies, whereas a mixture of subsurface and surface colonies were obtained when surface colonies were plated on soft agar medium. These observations suggest that the *pomona*, BJ cell possesses the genetic information to form either surface or subsurface colonies, depending on environmental conditions.

Several other serovars of *Leptospira* were tested for their capability to form surface colonies. Only subsurface colonies were observed when the following serovars and strains were cultivated on 1% agar plates: *canicola*, Moulton; *grippityphosa*, 11808; *hardjo*, 11601; *peruviana*, MC20; and *autumnalis*, M7. The first four strains of leptospires were virulent for hamsters, whereas the fifth strain was avirulent. Serovar *icterohaemorrhagiae*, strains CF1 (hamster virulent) and I (hamster avirulent), formed both surface and subsurface colonies on 1% agar, as did 7 of 10 strains of serovar *pomona* tested.

## DISCUSSION

The observation of leptospires growing on the surface of soft agar plates as discrete colonies was quite unexpected, since the colonial growth of leptospires has been studied by a number of investigators who have not reported this colonial form (6-8, 14-16, 18, 19). This delayed observation of surface colonies of leptospires is probably due to a number of reasons. Surface colonies are difficult to visualize because they are small and transparent. Also, they may only be formed by a few strains of leptospires; we were only able to detect surface colony-forming cells in serovars *pomona* and *icterohaemorrhagiae*. In addition, most isolates are not cultivated on solid medium until they have been transferred a number of times in liquid medium, which apparently is favorable to cells that form subsurface colonies.

Presently, the only means we have for identifying surface colony-forming cells is by colonial morphology. Consequently, we have not been

able to correlate any identifying physiological characteristic with the ability to form surface colonies. The cells of these two colonial types probably differ in their oxygen requirements or their ability to penetrate the agar. However, preliminary attempts to visualize differences in translational motility in the two cell types utilizing methylcellulose (0.3 poise) were unsuccessful.

Although cells from both colonial types of *pomona*, BJ were lethal for hamsters, it would appear that the surface colony type cells predominate in the animal host; only surface colonies were formed from the first subculture of our isolate. Moreover, continuous hamster-to-hamster passage is favorable for surface colony-forming cells, whereas subculturing in artificial media results in a predominance of subsurface colony-forming cells.

The *pomona*, BJ cell has the capacity to form either surface or subsurface colonies, depending upon the environmental conditions. When cells from a surface colony are transferred to either 1% agar plates or liquid medium, both types of colonies are formed. Conversely, cells of both colonial forms are observed in the blood of hamsters infected with cells from a subsurface colony.

It is of interest to note that of the serovars investigated, surface colony-forming cells were only detected in *pomona* and *icterohaemorrhagiae*, two serovars also known to possess thermolabile antigens (3, 4, 12). The manifestation of the thermolabile antigens is a variable characteristic of these serovars.

We are presently surveying a number of *Leptospira* serovars and strains to ascertain how widespread the capability to form surface colonies is.

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