Plate Assay for Detection of Leptospira interrogans serovar pomona Hemolysin

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A plate assay which utilizes the addition of a blood agar overlay for detection of hemolysin by colonies of Leptospira interrogans serovar pomona is described.

The production of hemolysin by some strains of pathogenic Leptospira interrogans serovars was previously reported by Alexander et al. (1), Russell (9), and Bauer et al. (3). These investigators employed a tube titration assay involving dilutions of supernatant fluids from L. interrogans grown in a rabbit serum medium and a sheep erythrocyte (SRBC) suspension to detect hemolysin. The tube titration assay has proven valuable for the quantitation of hemolysin and has demonstrated that the hemolysin is most active for SRBCs, heat labile (56°C, 10 min), potentiated by a hot and then cold incubation period, and inhibited by a factor present in normal rabbit serum (1, 9). However, the tube titration assay does not allow for detection of hemolysin by individual colonies of L. interrogans. This study was initiated to develop such an assay.

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Three strains of L. interrogans serovar pomona were used for this study. Strains Wickard and Pomona were obtained from R. C. Johnson, University of Minnesota, Minneapolis. Strain DM2H was obtained from R. Nervig, National Animal Research Center, Ames, Iowa. L. interrogans serovar illini strain 3055 (referred to as L. interrogans serovar illini) was also obtained from R. C. Johnson.

All leptospires were grown in the oleate-palmitate albumin (OPA) medium of Charon et al. (6), except the albumin (Scientific Protein Laboratories, Waunakee, Wis.) was not chloroform-methanol extracted. This medium was chosen because the routinely used Tween 80-albumin medium (7) is lytic to erythrocytes. Cultures were maintained in glass culture tubes with stainless steel closures in an incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 30°C. Growth was monitored with a Coleman model 7 photonephelometer by the method of Johnson and Harris (7).

All L. interrogans serovar pomona strains were first tested for hemolysin production by a tube titration assay similar to that of Bauer et al. (3). Sheep blood for the hemolysin assay was collected by venipuncture and preserved in modified Alsever solution (8). To prepare SRBCs for the hemolysin assay, we removed the cells from Alsever solution by centrifugation at 1,500 × g for 10 min at 4°C. Sedimented cells were washed three times with phosphate-buffered saline, pH 7.4 (5). The concentration of SRBCs was adjusted so that a 1:2 dilution, after complete hemolysis by a 1:1,000 saponin solution, gave an absorbance of 0.8 at 545 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). This method produces a 0.7% standardized SRBC suspension (4).

To assay hemolysin activity, we collected hemolysin-containing supernatant fluids from stationary-phase cultures which had been centrifuged at 12,000 × g for 15 min at 4°C. Twofold dilutions of hemolysin were prepared by serial dilution of 2 ml of supernatant fluid with an equal amount of 0.9% NaCl. Each dilution was mixed with an equal volume of 0.7% standardized SRBCs in a glass cuvette. For each assay, a cuvette containing 0.9% NaCl and a cuvette containing a 1:1,000 saponin solution in place of hemolysin were included. Supernatant fluids from these tubes served as the blank and the 100% hemolysis standard, respectively. All tubes were incubated in a water bath at 37°C for 4 h and then refrigerated at 4°C for 16 to 18 h. Cuvettes were centrifuged at 1,000 × g for 10 min, and the optical density of the lysed cells was determined in a Spectronic 20 spectrophotometer. The hemolysin titer was the highest final dilution in which at least 50% of the SRBCs were lysed.

All L. interrogans serovar pomona strains used for this study produced detectable hemolysin by the tube titration assay (Table 1). No activity was detected in the supernatant fluid of L. interrogans serovar illini. In agreement with
the results of Alexander et al. (1) and Russell (9) who employed a rabbit serum growth medium, the hemolytic activity of cells grown in OPA medium was found to be heat labile and potentiated by a hot and then cold incubation period. These results indicate that the OPA growth medium is a suitable medium for the production of *L. interrogans* hemolysin. Moreover, it is superior to rabbit serum medium because it supports more luxuriant growth of *L. interrogans*, is more defined, and presumably lacks the inhibitory factor present in normal rabbit serum.

To perform the hemolysin plate assay, 1-ml samples of exponentially growing cells were appropriately diluted in OPA medium, and 0.1-ml samples were plated on individual OPA-1% Noble agar (Difco Laboratories, Detroit, Mich.) plates. The plates were sealed and incubated aerobically at 30°C until colonies became visible (2 to 3 weeks). A blood agar overlay method was used due to the long incubation period required for obtaining colonies. The OPA-agar plates containing the colonies were overlaid with 20 ml of a sterile, 5% SRBC overlay prepared by the addition of 15 ml of defibrinated sheep blood to a 50°C solution of isotonic agar containing 2.7 g of NaCl and 3.0 g of agar in 285 ml of distilled water. After the overlay solidified, plates were incubated at 30°C until small, clear zones of hemolysis appeared surrounding individual colonies (24 to 48 h). The plates were transferred to the cold (4°C) for 12 h and then reexamined for zones of hemolysis.

The hemolytic activity of colonies of strain Wickard was readily discernible (Fig. 1). Clear, circular zones of beta hemolysis were observed surrounding individual colonies. In agreement with the hemolysin tube assay, zones of hemolysis were visibly enhanced when plates incubated at 30°C were transferred to 4°C. These results correspond with the hemolysin tube assay results of Alexander et al. (1), Russell (9), and Bauer et al. (3), who demonstrated maximal hemolysis after a hot and then cold incubation period. Strains Pomona and DM2H also exhibited beta hemolysis. As expected, control plates with colonies of *L. interrogans* serovar *illini* did not exhibit hemolysis.

The hemolysin plate assay has proven to be both simple and reliable. The sensitivity of the system for detection of hemolysin production is emphasized by the observation that even weak hemolysin-producing *L. interrogans* serovar *pomona* strains demonstrated discernible zones of hemolysis (Table 1). Because of the antigenic similarities between *L. interrogans* serovar *pomona* hemolysin and *L. interrogans* serovar *canicola* hemolysin observed by Alexander et al. (2), we expect the hemolysin plate assay to be applicable to other serovars which produce hemolysin. This procedure will be valuable to investigators interested in isolation of nonhemolytic mutants, in correlation of in vitro hemolysin production with virulence, and in isolation of hemolytic and nonhemolytic serovars from mixed *L. interrogans* infections.

**TABLE 1. Results of hemolysin assays for *L. interrogans* serovar *pomona* strains and *L. interrogans* serovar *illini* 3055**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hemolysin tube assay titer</th>
<th>Hemolysin plate assay</th>
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<tbody>
<tr>
<td><em>L. interrogans</em> serovar <em>pomona</em> Wickard</td>
<td>1:8</td>
<td>+^a</td>
</tr>
<tr>
<td><em>L. interrogans</em> serovar <em>pomona</em> Pomona</td>
<td>1:8</td>
<td>+</td>
</tr>
<tr>
<td><em>L. interrogans</em> serovar <em>pomona</em> DM2H</td>
<td>+^b</td>
<td>+</td>
</tr>
<tr>
<td><em>L. interrogans</em> serovar <em>illini</em> 3055</td>
<td>--^c</td>
<td>--</td>
</tr>
</tbody>
</table>

^a Exhibits clear zones of hemolysis around colonies.
^b Exhibits 27% hemolysis at a 1:2 dilution.
^c Exhibits no hemolysis over medium control at a 1:2 dilution.

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