Evaluation of a Hemagglutination Test for Human Leptospirosis

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An indirect hemagglutination test for the diagnosis of leptospirosis is described; the test uses a soluble antigen from serotype patoc to sensitize sheep erythrocytes which are then fixed with glutaraldehyde. Evaluation of this procedure indicates that it is more reliable than the conventional macroscopic agglutination test and, in contrast with both microscopic and macroscopic agglutination tests, is positive only with sera from persons with current leptospirosis illness. The test is simple and convenient and sensitized fixed cells may be stored for at least a year. In comparison with the macroscopic and microscopic tests, only a single antigen is required.

It is becoming increasingly evident that a new screening test involving fewer serotypes than presently used in the macroscopic agglutination test is needed for the detection of human leptospiral antibodies (9, 10). The macroscopic agglutination procedure gives variable results which are often misleading; at best the results should only be regarded as presumptive. Occasionally, the results of the test are negative when those of the confirmatory microscopic agglutination test are positive.

Many tests have been evaluated for use in the diagnosis of human leptospirosis; two of these are the sensitized erythrocyte lysis test, sometimes known as the hemolytic test (1, 4, 5), and the complement-fixation test (9, 10). Although these appear to be useful tests, they require the use of labile reagents (e.g., complement, hemolysin, fresh sheep erythrocytes) and are time consuming and difficult to perform. There is a clear need for a simple sensitive test with stable reagents. In searching for a good presumptive test to replace the macroscopic (slide) test (7) and compare with the microscopic test during illness, we tried using a soluble antigen from serotype patoc to sensitize erythrocytes which are then fixed with glutaraldehyde as was first done by Cox et al. (1, 4, 5). The result is a stable antigen preparation that can be conveniently used in passive hemagglutination (HA) tests (1, 4, 5). We present here an evaluation of this procedure and a comparison of the results with those obtained by the standard macroscopic and microscopic agglutination tests using only human diagnostic sera.

MATERIALS AND METHODS

One-liter amounts of an 8- to 9-day-old serotype patoc culture grown in a medium of bovine albumin polysorbate 80 (6) were centrifuged live at 5,000 to 10,000 × g for 1 h. The precipitate was then resuspended to 1/4th of the original volume in 0.15 M NaCl. An equal volume of cold (4°C) absolute alcohol was then added to bring the concentration of alcohol to 50%. The mixture was refrigerated overnight at 4°C. The next day the mixture was centrifuged as above.

The supernatant fluid was saved and measured; cold absolute alcohol was then added to bring the final alcohol concentration to 90%, and then the mixture was refrigerated at 4°C for 6 to 9 days or until a white-grayish precipitate had formed. At this time, the mixture was centrifuged again at the same speed as above. Cold 0.15 M NaCl was added to resuspend the precipitate to 1/4th of the original volume. This was the stock material to sensitize sheep red blood cells (SRBC) (3–5).

The optimum concentration of antigen used to sensitize SRBC was determined by a block titration of homologous serum in twofold dilutions to 1:10,000 each against twofold dilutions of antigen up to 1:1,042 in Kent buffer (4). The 10% fresh SRBC suspension was predetermined with a Coleman spectrophotometer. One volume of fresh 10% SRBC was sensitized with 10 volumes of each antigen dilution and incubated for 1 h in a 37°C water bath with occasional shaking (1, 5). The cells were then centrifuged (5,000 to 10,000 × g), washed once, and resuspended to 1% for performance of the test.

To each 0.4 ml of a serum dilution, 0.1 ml of each antigen dilution was added. Controls were made with nonsensitized cells only in the 1:25 serum dilution. A control of sensitized cells was also made with the buffer. A known negative serum (normal rabbit serum) was also tested to be certain that our results
with the homologous serum were true reactions. All sera were inactivated in a 56°C water bath for 1 h and absorbed before testing.

A parallel block titration of the same dilutions of antiserum and antigen with complement was also used. Sixteen units of antigen reacting with four units of antiserum at a 1:40 dilution was the concentration used to sensitize SRBC. This closely paralleled the 4+ (4 units) reaction we received with the highest serum dilution when no complement was used. In both of these instances, an antigen dilution of 1:32 was the concentration of antigen needed to sensitize the SRBC. A large volume of fresh 1% concentration of SRBC was then sensitized with 1:32 dilution of patoc-soluble antigen for 1 h in a 37°C water bath and then washed three times. They were resuspended to 1% for fixation with a 1% final concentration of glutaraldehyde and allowed to stand for 2 h at room temperature with occasional shaking. The fixed cells were then centrifuged at the same speed as before, washed three times, and resuspended to 10 times the concentration for storage (4). Merthiolate was added to the bulk antigen to a final concentration of 1:10,000. Then it was stored in 5-ml amounts in sealed screw-cap bottles and placed in a 4°C refrigerator.

RESULTS AND DISCUSSION

One hundred forty-two sera were tested by the three tests: macroscopic, microscopic (10, 11), and HA. Some of the sera tested were collected late in the course of illness. In Table 1 each serum specimen is shown as an individual sample regardless of the number of paired sera tested. Table 1 also shows how many sera were positive and negative and the number of times that each test had positive and negative results. In Table 2 are listed the 30 individuals from whom paired sera were available (shown as single sera in Table 1) and the number of paired sera which showed a significant rise or fall in titer.

These sera were sent in sets of two, three, and four or more samples per individual. Sixty of the 142 serum samples sent into our laboratory were paired sera.

We noted a 70.42% agreement (100 of 142) between the three tests. With all controls acceptable at the time of testing, there were nine sera that gave negative results by the HA test but positive results by the other two tests (Table 1, column 3). This could mean that these individuals were no longer ill at the time the serum samples were drawn because it is believed that the HA test detects antibodies as early as 4 days after infection. Titers are thought to decline rapidly after the person has recovered from the illness. By using coded paired sera from 14 patients with known cases of leptospirosis, we are pursuing the theory that the HA test does detect antibodies early in the course of the illness. Twelve of these 14 sera which were positive only by the HA test are the early acute sera from individuals who sent in late convalescent samples that then proved to be positive.

Table 1. Agreement of 142 diagnostic sera by three tests: macroscopic agglutination (slide), microscopic agglutination, and the indirect HA tests

<table>
<thead>
<tr>
<th>Agglutination</th>
<th>HA +</th>
<th>HA -</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macro +</td>
<td>61</td>
<td>9</td>
</tr>
<tr>
<td>Micro +</td>
<td>42.96%</td>
<td>6.34%</td>
</tr>
<tr>
<td>Macro -</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Micro +</td>
<td>2.82%</td>
<td>2.11%</td>
</tr>
<tr>
<td>Macro -</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Micro -</td>
<td>5.63%</td>
<td>2.82%</td>
</tr>
<tr>
<td>Macro -</td>
<td>14</td>
<td>39</td>
</tr>
<tr>
<td>Micro -</td>
<td>9.86%</td>
<td>27.46%</td>
</tr>
</tbody>
</table>

* These include 30 paired sera, as well as single sera counted as one each which were later positive by both the macroscopic and the microscopic agglutination tests, with the exception of two (2) which were positive by the three tests but had no second or third samples sent in to be tested.

Table 2. Paired sets of sera which showed significant change in titer by microscopic agglutination MA and HA tests

<table>
<thead>
<tr>
<th>Positive by both tests</th>
<th>Positive by HA only</th>
<th>Positive by MA only</th>
<th>Negative by both tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>19*</td>
<td>2</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>63.3%</td>
<td>6.66%</td>
<td>6.66%</td>
<td>23.3%</td>
</tr>
</tbody>
</table>

* Significant change: at least a fourfold rise or fall in titer in at least one of the paired sera sent in for testing.

Of the 19 sera positive by both tests, there were 10 that showed a rise in titer by both tests (MA and HA). In nine sera the HA showed two- to fourfold and greater drop in titers, yet the HA was still positive on these paired sera in which (when tested by 2-mercaptoethanol) some of the antibody response of the second seven samples remained after treatment, whereas on the first sample of these individual sera, the antibody response was removed completely after treatment.

The treatment with 2-mercaptoethanol on these paired sera removed all antibody response when serology was done.

* These pairs of sera showed no removal of antibody when treated with 2-mercaptoethanol when tested in serology.
be positive by the other two tests as well, whereas 7 of these showed decreasing titers by HA and 5 were negative by HA. This would indicate that the HA test is detecting immunoglobulin M antibody since this is thought to be the first globulin response after infection with the leptospiral organism. Our results with 2-mercaptoethanol treatment of the sera bear this out. Since, with the early sera, the antibodies were removed by 2-mercaptoethanol and, in the late convalescent sera, part of the antibody response remained with the 7 sera showing decreased titers with HA and the remaining 5 of the 12 sera, the antibody response remained.

Much more work is needed to support this hypothesis. The HA test detects antibodies for leptospirosis in more instances than does the macroscopic test; therefore, it appears to be much more sensitive with early serum samples than the macroscopic or the microscopic in the course of the illness. An added advantage of the HA test is that only one antigen is required; the macroscopic test requires the preparation of 12 serotypes (antigens) that must be checked at regular intervals (10, 11). The HA antigen is easy to extract (from leptospires grown in 1-liter amounts of Ellinghausen medium from 8 to 9 days [6]) and evaluate and is found to be stable when stored at 4°C for at least 1 year. The antigen is easy to prepare, and the test may be easier to perform than the one advocated by Imamura et al. (8).

This method, although very similar to that of Chang et al. (2) in preparation and testing, is different in three major respects: (i) we use only an alcoholic-extracted soluble substance from one serotype; (ii) it is stable for at least 1 year because the sensitized SRBC are fixed with glutaraldehyde; and (iii) no previous number of leptospires need be determined, and the actual testing need not be done in a 37°C water bath but may be left overnight at room temperature.

**LITERATURE CITED**