A card test for the serodiagnosis of human leptospirosis

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SYNOPSIS A macroscopic agglutination test for detecting leptosporal antibodies in human sera is described. The test utilizes a stained preparation of non-pathogenic leptospires and is performed on the Brewer diagnostic cards used for bovine brucellosis screening. The agglutination of the stained antigen is more easily observed than the current macroscopic slide test using unstained leptosporal antigens. The non-pathogenic serotype *patoc* is agglutinated by sera from humans infected with serotypes *pomona*, *hardjo*, *ballum*, and *copenhageni* with a sensitivity of 94% in comparison with the microscopic agglutination test.

The laboratory diagnosis of recent leptosporal infections in man may be established either by isolating the infecting organism or by demonstrating a rise in the specific antibody titre. The isolation of the organism has been shown to be feasible, at least for the New Zealand serotypes (Christmas *et al.*, 1974), and is of undoubted epidemiological importance. However, the time taken to culture and identify the organism allows confirmation of the diagnosis in retrospect only. For the detection of specific antibodies the macroscopic agglutination test (MAT) (World Health Organization, 1967) is still the standard reference test for leptospirosis diagnosis. However, the disadvantages of maintaining a battery of live cultures for the performance of the MAT are well known.

A large number of preliminary tests using genus specific antigens or pools of antigens have been developed. Those involving agglutination of the non-pathogenic serotype *patoc* (Combiescu *et al.*, 1958), haemagglutination (Palit and Gulasekharam, 1973; Sulzer and Jones, 1973), haemagglutination-lysis (Cox *et al.*, 1957), complement fixation (Elian and Nicoara, 1964; Turner, 1968), and immunofluorescence (Torten *et al.*, 1966) all require specialized equipment, and some involve the growth of large volumes of leptospires for the extraction of antigen.

A simple macroscopic slide test reported by Galton *et al.* (1958) is the basis of the commercial screening antigens which are used by some local laboratories in New Zealand. This report describes an improvement of this test and the use of a single non-pathogenic (serotype *patoc*) antigen as a screening test for human leptospirosis.

**Material and Methods**

**Organisms**

*Leptospira interrogans*, serotypes *pomona*, *hardjo*, and *ballum*, were isolated from human cases of leptospirosis in New Zealand. Serotype *copenhageni* was a New Zealand bovine isolate obtained from D. R. Ris, Wallaceville Animal Research Centre. *Leptospira biflexa* serotype *patoc* was obtained from the World Health Organization, Leptospira Reference Laboratory, Brisbane, Australia, where all the strains were serotyped. The leptospires were grown and maintained in synthetic Tween 80-albumin medium (EMJH).

**Rabbit Immunization**

All leptospires for immunization of rabbits were grown in modified Vervoort's medium containing 10% rabbit serum and supplemented with vitamin B_{12} (Wolff, 1954). Immune rabbit sera were prepared in New Zealand White rabbits by giving an intramuscular injection of the leptospires centrifuged from approximately 20 ml of culture, and emulsified in 2 ml of a mixture of equal parts of saline and incomplete Freund's adjuvant. When necessary, booster injections of 5 ml of culture were given intraperitoneally to obtain homologous MAT titres of at least 1:6400.
HUMAN SERA
Human sera were obtained from culturally proven cases of leptospirosis either in New Zealand, or in Denmark in the case of copenhageni, or were selected at random from specimens received for leptospiral serology at the National Health Institute.

ANTIGEN PREPARATION
Leptospires were grown in 50 ml volumes of medium. Well-grown cultures were killed by the addition of 1:10 000 thiomersal (Merthiolate, Lilly), centrifuged, washed twice in normal saline, and resuspended in 1% (w/v) Ponceau S in 0.01M phosphate buffered saline, pH 7.3 (PBS). After standing overnight at 5°C leptospires were washed in PBS until the supernatant was colourless. The staining procedure was repeated when necessary to produce a deep red-brown stained antigen. Stained leptospires were resuspended in PBS to a concentration of 5% (v/v) for the test. For the preparation of antigen pools equal volumes of the 5% suspensions of the different serotypes were mixed. Three preparations were made: (a) a pool of the four New Zealand isolates, serotypes pomona, hardjo, ballum, and copenhageni; (b) a pool of the four New Zealand isolates plus serotype patoc; (c) serotype patoc alone.

Thiomersal 1:10 000 was added to all final preparations. The antigens have been stored at 5°C for up to six months with no variation in activity.

To prepare formalized antigens the leptospires were resuspended in 0.25% formol saline for 3 hours, washed, and then stained with 1% Ponceau S in PBS.

PROCEDURE
The test was performed on Brewer diagnostic cards used for the brucellosis card test. However, similar results were obtained using glass slides viewed against a white background. 0.025 ml each of antigen and of serum were mixed, and tilted to and fro either manually or on an automatic card rocker for 4 minutes at room temperature. The test was read immediately and the positive results were graded from 1+ to 4+, depending upon the amount of agglutination. A negative control serum was included in each test, and care was taken not to mistake drying around the edge of the drop for agglutination.

Results

RABBIT ANTISERA
The stained antigen preparations reacted well with their homologous rabbit antisera. There were also considerable cross-reactions among the serotypes tested. The individual results are shown in table I. Rabbit antisera also reacted with the pool of the four pathogens to the same extent as with their homologous antigens. The agglutination of the stained antigens was more easily observed than the agglutination of the unstained commercial antigens (Difco). Formalinizing the leptospires before staining had no adverse effect.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pomona</td>
</tr>
<tr>
<td></td>
<td>4+</td>
</tr>
<tr>
<td></td>
<td>4+</td>
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<tr>
<td></td>
<td>4+</td>
</tr>
<tr>
<td></td>
<td>4+</td>
</tr>
<tr>
<td></td>
<td>1+</td>
</tr>
</tbody>
</table>

Table I Reactions of leptospiral stained antigens with rabbit antisera
Note 1+ to 4+ denotes degree of agglutination

HUMAN SERA
Fourteen sera representing serial specimens from four patients with culturally proven leptospirosis were tested with the three stained antigen preparations. The results in table II show that the inclusion of patoc produced agglutination to higher titres than the pool of pathogens alone, and that when the sera were tested against patoc alone an even greater degree of agglutination resulted.

To evaluate the suitability of the patoc antigen card test as a single preliminary test for the serodiagnosis of leptospirosis, a further 70 sera from 23 culturally proven cases of human leptospirosis (10 pomona, 9 hardjo, 4 copenhageni) were tested. In all 23 cases there was a rise in the MAT titre after an initial negative (less than 1:100) or low titre serum taken at the same time as the blood culture. Unfortunately, five of the initial sera were unavailable for card tests. Two of the second serial specimens failed to react in the card test; however, in both cases, the third serial specimen showed a rise in the MAT titre and agglutinated the patoc card test antigen. The results of the reactions of the previous 14 sera are included with these results in table III.

One hundred and eighty human sera were then selected at random from the specimens received at the National Health Institute for leptospiral serology. Seventy-eight of these were positive in the MAT and 102 were negative (less than 1:100). The results of card tests using the patoc antigen are recorded in table IV. All of the five ‘false negative’ sera in the card test belonged to series of sera taken from five different patients.
Table II  Reactions of human sera with three stained antigen preparations

<table>
<thead>
<tr>
<th>Time after Onset of Illness</th>
<th>Number of Sera</th>
<th>Microscopic Agglutination Test (MAT)</th>
<th>Card Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive (&gt;1:100)</td>
<td>Negative (&lt;1:100)</td>
</tr>
<tr>
<td>0-7 days</td>
<td>22</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>8-21 days</td>
<td>26</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>22-49 days</td>
<td>20</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>3-6 months</td>
<td>16</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>Totals</td>
<td>84</td>
<td>61</td>
<td>23</td>
</tr>
</tbody>
</table>

Table III  Reactions of 84 human sera from 27 culturally proven cases of leptospirosis

15 sera unavailable

Table IV  Reactions of human sera with patoc card test antigen

In the two cases where the MAT titre was 400 the card test was positive for the next serial specimen taken three to four weeks later. In the other three cases, subsequent sera taken four to five weeks later were negative in the MAT, indicating that the sera giving the anomalous results were taken in the later stages of the infection.

If all these results are combined, 264 sera were tested with the patoc card test antigen. The results in table V show that there is a type I error (false positive) of 45% and a type II error (false negative) of 5-6%. The sensitivity, therefore, is 94% and if in the three cases where the sera were taken in the later stages of the infection can be discounted, the sensitivity is better than 96%. The specificity is 55%.

Discussion

The species of animal being studied influences the suitability of any particular leptospiral serological test, especially for establishing the infecting serotype. The broad cross reactivity of rabbit immune sera...
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has been demonstrated in complement fixation tests (Palit and Sharma, 1971; Robertson and Boulanger, 1963), haemagglutination tests (Palit and Sharma, 1971), haemagglutination-lysis tests (Cox, 1955), and in the macroscopic slide test (Galton et al., 1958), while bovine sera show very little cross-reactivity. Cross reactions have been observed in most leptospiroserological tests when human sera have been examined, the frequent reaction with serotype patoc leading to the development of many genus-specific antigens (Combescu et al., 1958; Cox, 1957; Elian and Nicoa, 1964; Palit and Gulasekham, 1973).

Our results show that in the card test human sera agglutinate the patoc antigen more strongly than they agglutinate a pool of antigens containing the infecting strain. The cross-reactions of the human sera were the same as those of the rabbit immune sera but with a much lower degree of agglutination. The card test is therefore of little value for determining the infecting serotype and there is no advantage in using either pools of pathogenic leptospires or individual antigens. Since the treatment for leptospirosis is not in general influenced by the infecting serotype, a rapid diagnosis of leptospirosis using a genus-specific antigen is adequate.

The patoc card test compares favourably in sensitivity to the MAT. Provided the serum was taken at least 8-10 days after the onset of illness a negative card test can reasonably be taken to preclude a diagnosis of leptospirosis. While the specificity is rather low this is not a disadvantage in a preliminary test as all reactive sera should be referred for MAT titres. The false positives could be either sera taken early in the infection before the MAT has risen to 1:100 or a reflection of natural antibody (Faine and Carter, 1968). A preliminary study of 'normal' human sera indicates a high incidence of low levels of antibody to patoc; however, these reactions are eliminated by diluting the serum 8-10 fold before testing in the card test. It is suggested, therefore, that all sera are tested undiluted, and that those that are reactive are re-tested in serial dilutions. As with the MAT, it is still necessary to demonstrate a rise in antibody titre in two serial specimens in order to confirm a diagnosis of leptospirosis.

The patoc antigen has the advantage of being non-pathogenic and more easily and cheaply grown than pathogenic strains, and it may detect antibodies to previously unsuspected strains. The test performed on cards with the stained antigen is more easily read than the macroscopic slide test; it is simple and reproducible and could be performed by unskilled personnel provided adequate controls were always included. It is suitable for field investigations where laboratory facilities are not available and where a rapid screening test is required. Card tests for bovine brucellosis (Joint FAO/WHO, 1971; Pilet et al., 1972) and for syphilis (World Health Organization, 1970) are well established and have been used satisfactorily for many years.

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References


