

Evaluation of the Indirect Hemagglutination Assay for Diagnosis of Acute Leptospirosis

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Serology plays an important role in the diagnosis of leptospirosis. Few laboratories have the resources and expertise to perform the microscopic agglutination test. There is a need for rapid and simple serological tests which facilitate the early diagnosis of leptospirosis, while antibiotic therapy may be most effective. A commercially available indirect hemagglutination assay (IHA; MRL Diagnostics, Cypress, Calif.) was evaluated with multiple serum specimens from 107 patients being investigated for leptospirosis. By using a combination of enzyme-linked immunosorbent assay (ELISA) methods for immunoglobulin M (IgM) and IgG antibodies and the microscopic agglutination test, 54 patients were found to have leptospirosis and 53 were found not to have leptospirosis. The sensitivity of IHA for the detection of acute leptospirosis was 100%, the specificity was 94%, the positive predictive value was 95%, and the negative predictive value was 100%. IHA was negative when 13 antinuclear antibody-positive sera, 24 serum specimens from patients with syphilis, and 16 serum specimens false positive by the Venereal Disease Research Laboratory test were tested. IHA was shown to detect both IgM and IgG classes of antibodies in human sera. Serum specimens from 27 dogs investigated for leptospirosis were studied: 3 samples gave nonspecific hemagglutination, but for all remaining samples, the results of IHA and an IgM ELISA were concordant. Performance of IHA was simple, and IHA requires no specialized equipment. It represents a useful assay for laboratories which require a leptospiral diagnostic capability but lack the expertise to perform specialist investigations.

Leptospirosis is a common zoonosis in most tropical countries (8). In temperate climates the risk of acquiring the disease is strongly associated with occupational or recreational exposures, whereas in tropical countries and subtropical regions the risk of infection is more widespread and occurs through indirect contact with the urine of infected host animals (5). Leptospirosis is thus a common cause of acute febrile disease in tropical climates and must be differentiated from typhoid, malaria, dengue, viral hepatitis, and hantavirus infections when these diseases are present in the population. Early diagnosis of leptospirosis is important, since the mortality rate is high among patients with the most severe presentations (6). However, clinical diagnosis is difficult during the early stages of the disease, when it may be confused with many other common febrile illnesses, such as dengue fever, malaria, typhoid, and viral hepatitis. Diagnosis of leptospirosis is often made by serological tests, since culture is both slow and expensive. Performance of the reference serological test, the microscopic agglutination test (MAT), requires significant expertise, and MAT is rarely performed by routine diagnostic laboratories (7). It remains useful however, for epidemiological investigations. Several alternative serological methods for the early diagnosis of leptospirosis have been described, including the slide agglutination assay (9), indirect hemagglutination assay (IHA) (17), microcapsule agglutination tests (3), immunofluorescence (2), and enzyme-linked immunosorbent assay (ELISA) methods for immunoglobulin M (IgM) antibodies (1, 12–15, 19, 20). We evaluated a commercially available IHA for the early detection of leptospirosis. IHA has not previously

been compared with the detection of IgM antibodies for diagnosis.

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MATERIALS AND METHODS

Sera. Serum samples were obtained from patients admitted to the Queen Elizabeth Hospital, Bridgetown, Barbados, with a history and clinical manifestations suggestive of leptospirosis. Blood samples for serology were collected on the day of admission and on the 4th day after admission, and for some patients a convalescent-phase blood sample was taken before discharge from the hospital or at a follow-up visit to the outpatient clinic.

A panel of 13 serum samples from patients positive for antinuclear antibodies, 24 serum samples from patients with syphilis, confirmed by a positive Venereal Disease Research Laboratory (VDRL) test result and a positive *Treponema pallidum* hemagglutination assay or fluorescent treponemal test result, and 16 serum samples which gave false-positive VDRL test reactions was included in this study. Specimens from dogs investigated for leptospirosis were also studied; paired serum samples from 8 dogs and single serum samples from a further 19 dogs were available.

ELISA. IgG and IgM titers were determined by ELISA (19) by using strain Patoc I (*Leptospira interrogans* serovar *patoc*) as the antigen. An IgM titer of ≥ 160 was regarded as positive. Canine sera were tested by a modified ELISA method (21). In the canine ELISA an IgM titer of ≥ 320 was considered positive.

MAT. Sera were examined by MAT with a battery of 22 serovars to establish seroconversion or a rise in titer (7). The antigens used included both reference strains and locally prevalent serovars of the following serogroups (serovars are given in parentheses): Australis (*bajan*, *barbadensis*, *bratislava*), Autumnalis (*bim*, *fortbragg*), Ballum (*arborea*, *ballum*), Bataviae (*bataviae*, *brasiliensis*), Canicola (*canicola*), Cynopteri (*cynopteri*), Grippityphosa (*grippityphosa*), Ictero-haemorrhagiae (*copenhageni*), Mini (*georgia*), Panama (*mangus*, *panama*), Pomona (*pomona*), Pyrogenes (*pyrogenes*), Tarassovi (*tarassovi*), and Sejroe (*hardjo*, *sejroe*). *Leptospira biflexa* serogroup Semarang serovar *patoc* was also tested.

The diagnosis of leptospirosis was confirmed by a fourfold rise in titer between two serum samples tested by the same method, an initial titer of ≥ 800 by MAT, an IgM titer of ≥ 160 by ELISA, or any combination of the three.

IHA. A commercially available IHA was obtained from MRL Diagnostics and was performed on the basis of the method described previously (17, 18). A total of 50 μ l of a 1:50 dilution of each serum specimen was mixed with 25 μ l of either antigen-coated test cells or uncoated control cells in the wells of a U-bottom

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TABLE 1. Serology results for follow-up specimens from four patients

| Patient no. | Wk since onset of symptoms | IHA result | ELISA titer | | MAT titer (serogroup) |
|-------------|----------------------------|------------|-------------|--------|---------------------------------------|
| | | | IgM | IgG | |
| 14 | 14 | — | 160 | 160 | 3,200 (Icterohaemorrhagiae) |
| 19 | 19 | — | 80 | 10 | 400 (Ballum) |
| 31 | 22 | ++++ | 160 | 80 | 200 (Ballum/Autumnalis ^a) |
| 33 | 11 | ++++ | 320 | 10,240 | 3200 (Autumnalis) |

^a Ballum and Autumnalis serogroups reacted at the same titer.

microtiter tray. Plates were incubated at 25°C for 1 h. Hemagglutination was read on a scale of from 0 to +++++. Positive and negative control sera were tested each time that the test was performed.

IgG removal. Samples were retested after removal of IgG by diluting 10 µl of serum in 40 µl of a goat anti-human IgG absorbent (proSorb G; Integrated Diagnostics, Baltimore, Md.). After dilution the samples were centrifuged in a microcentrifuge at 17,000 × g for 3 min before further dilution prior to testing by IHA.

RESULTS

Diagnostic samples. A total of 241 specimens from 107 patients were investigated by a protocol for the diagnosis of leptospirosis. Fifty-four patients were found to have leptospirosis and 53 were found not to have leptospirosis. IHA detected all 54 cases of leptospirosis; three patients had false-positive IHA results (the diagnoses for these patients included dengue fever, viral hepatitis, and an undiagnosed pyrexia of unknown origin). The sensitivity of IHA for the detection of acute leptospirosis was 100%, the specificity was 94%, the positive predictive value was 95%, and the negative predictive value was 100%.

A total of 141 serum specimens from 54 patients diagnosed with leptospirosis were examined. First acute-phase (A1) samples were taken on the day of admission to the hospital for all but one patient, who first presented to a polyclinic and was then admitted to the hospital. The mean time from the onset of symptoms to the collection of the A1 sample was 5.1 days (range, 1 to 14 days). The date of onset of symptoms was unknown for six patients. Second acute-phase (A2) samples were taken 4 days after admission to the hospital, and convalescent-phase samples were available from 25 patients and

were taken a mean of 11.5 days after admission. Follow-up samples from four patients were studied; these were taken a mean of 16 weeks (range, 11 to 21 weeks) after the onset of symptoms. One hundred specimens from 53 patients whose diagnosis was not leptospirosis were examined.

Results for A1 samples. The IHA result was positive for 21 of 26 (81%) of the A1 samples positive by the IgM ELISA (titer, ≥160). The geometric mean titers (GMTs) for A1 samples from the patients were 312 (IgM), 106 (IgG), and 81 (MAT). For five patients whose A1 sample was IgM positive but IHA negative, there was a mean delay of 4 days before a sample was IHA positive. The IHA result was negative for 18 of 19 (95%) samples which were IgM negative (titer, ≤80) and 6 of 7 (86%) samples that were weakly positive by ELISA (titer, 160). The GMT of IgM in IHA-negative samples was 61, while in IHA-positive samples, the GMT was 1,947. The mean time since the onset of symptoms for patients with negative IgM ELISA results (titer, ≤80) was 3.9 days, while for those patients with positive IgM ELISA results it was 6.2 days. For IHA the corresponding times were 4.0 and 6.8 days, respectively.

Leptospirosis was confirmed by an MAT titer of ≥800 for the A1 sample for 14 of 52 (27%) patients and by a fourfold rise in titers between two specimens for the remainder of the patients.

Convalescent-phase and follow-up samples. The IHA result was positive for convalescent-phase samples from 25 patients with leptospirosis; these samples were taken a mean of 11.5 days after admission to the hospital. A total of 22 of 25 (88%) samples were strongly positive by IHA (+++ or +++++). All 25 samples were also positive by ELISA, with IgM titers of from 160 to 20,480 (GMT, 4,335). IgG titers were slightly lower, with a GMT of 3,453. Twenty-four samples had MAT titers in the range of 800 to ≥51,200 (GMT, 6,223), but one sample had a MAT titer of 100.

Follow-up samples from four patients were studied (Table 1). Samples were collected at outpatient visits between 11 and 20 weeks after admission to the hospital. Two samples remained strongly reactive by the IHA, whereas three samples were weakly positive by the IgM ELISA.

Samples retested after IgG removal. Ten samples were retested by IHA after removal of IgG (Table 2). Three acute-

TABLE 2. Results for sera retested after IgG removal

| Patient no. | Specimen ^a | Time after admission to hospital [time [days] since onset of symptoms) | IHA result | IHA result after IgG removal | ELISA titer | | MAT titer (serogroup) |
|-------------|-----------------------|--|------------|------------------------------|-------------|--------|--|
| | | | | | IgM | IgG | |
| 23 | A1 | 1 day (5) | — | — | 1,280 | 320 | Negative |
| 24 | A1 | 1 day (10) | +++ | + | 20,480 | 5,120 | 25,600 (Icterohaemorrhagiae) |
| 27 | A1 | 1 day (3) | — | — | 160 | 40 | 400 (Icterohaemorrhagiae) |
| 46 | A2 | 4 days (14) | ++++ | + | 640 | 20,480 | 6,400 (Autumnalis/Icterohaemorrhagiae/Australis ^b) |
| 48 | A1 | 1 day (6) | — | — | 2,560 | 640 | 400 (Autumnalis) |
| 50 | A1 | 1 day (4) | ++++ | ++ | 10,240 | 20,480 | 3,200 (Autumnalis) |
| 50 | A2 | 4 days (7) | +++ | ++ | 10,240 | 20,480 | 12,800 (Autumnalis) |
| 31 | C2 | 5 mo | ++++ | — | 160 | 80 | 200 (Ballum) |
| 33 | C | 27 days (32) | ++++ | — | 20,480 | 40,960 | 25,600 (Autumnalis) |
| 33 | C2 | 11 wk | ++++ | — | 320 | 10,240 | 3,200 (Autumnalis) |

^a C2, follow-up serum; C, convalescent-phase serum; A1 and A2 are defined in the text.

^b Autumnalis, Icterohaemorrhagiae, and Australis serogroups reacted at the same titer.

phase samples initially negative by IHA remained so after IgG removal. Four acute-phase samples which were strongly reactive by IHA showed a reduction in the intensity of hemagglutination, consistent with the presence of high IgG titers. One convalescent-phase and two follow-up samples from two patients were examined; these remained reactive by IHA between 1 and 5 months after the onset of symptoms (Table 1). After IgG removal, these three specimens were negative by IHA.

Other human samples. Five of 24 samples from patients with recently diagnosed syphilis were positive for IgM by ELISA, giving titers of 160. Four of 16 serum samples with false-positive VDRL test results gave titers of 160 or 320 in the IgM ELISA. No VDRL test-positive sera were positive by IHA. Thirteen serum samples containing antinuclear antibodies were negative by IHA and by ELISA for both IgM and IgG antibodies.

Canine sera. Paired serum samples from eight dogs investigated for leptospirosis were studied. Seven animals were confirmed as having leptospirosis, and the remaining one was seronegative by all methods. The IHA result was negative for two specimens with IgM titers of 640, but became positive for the second samples from the two animals. Specimens from one dog consistently hemagglutinated both test and control erythrocytes and were considered unreadable by IHA.

Single serum samples from 19 dogs were tested. For one animal the serological results were equivocal and the diagnosis of leptospirosis was not confirmed. Sera from two animals gave unreadable results due to nonspecific hemagglutination. The remaining 16 samples were all positive by ELISA and IHA.

Sera which hemagglutinated control cells were retested after absorption with the control cells, as recommended by the manufacturer. After absorption these four serum samples were all negative by IHA.

DISCUSSION

The diagnosis of leptospirosis is complex. The definitive serological test is MAT, which requires both significant expertise and the maintenance of a panel of live antigens. Moreover, the diagnosis of acute leptospirosis by MAT often requires paired serum samples, thus delaying the diagnosis. MAT remains useful, however, because of the epidemiological information that it can provide about the leptospiral serogroups present in a population and also because the same methodology can be applied to sera from different animal species.

Several methods have been developed for use in the diagnosis of leptospirosis during the early stages of the disease, when treatment is most likely to be effective. Those which remain in use are of two types: agglutination tests (3, 17) and ELISAs for the detection of IgM (1, 10, 14, 16, 19). These methods all detect genus-specific antibodies. The major difference between the various ELISA techniques lies in the antigen used, whether it is a single serovar of pathogenic leptospire, a nonpathogenic serovar such as the *L. biflexa* serovar *patoc*, or a mixture of several serovars.

IHA was previously found to have a sensitivity of 92% and a specificity of 95% when compared to the results of MAT (17). In our study IHA detected all cases of leptospirosis, while the specificity was 94%. In comparison with IgM detection by ELISA, IHA detected 81% of cases of disease with the A1 specimen, taken a mean of 6.8 days after the onset of symptoms, while the remaining cases of disease were detected with the A2 sample, taken a mean of 8 days after the onset of symptoms. The A1 sample from one patient was IgM negative but IHA positive.

IHA and IgM ELISA remained strongly reactive with convalescent-phase samples, usually taken just before discharge from the hospital (a mean of 11.5 days after admission). Our data confirm for the first time that IHA detects both IgM and IgG antibodies. At this time most patients have MAT titers which indicate recent infection, but there are some exceptions, that is, patients who exhibit late seroconversion. In the population studied, late seroconversion by MAT and low MAT titers are almost invariably associated with infection by serovar *arborea* of serogroup Ballum.

According to the results for follow-up samples taken 3 to 5 months after recovery from the acute infection, some patients remained strongly reactive by IHA. This strong reactivity was mediated by IgG and was removed by pretreatment with anti-human IgG. Thus, a strongly reactive IHA persisting after recovery may be due to IgG antibodies. MAT titers may be retained for many years (4, 11). Nonspecific reactions with sera from patients with other clinical conditions were not observed by IHA.

A potential advantage of agglutination tests over ELISA methods is the ability to apply them, without modification, to the detection of antibodies from several animal species. We applied the IHA to the diagnosis of acute leptospirosis in dogs, diagnosed by MAT and an ELISA technique modified to facilitate the detection of canine IgM or IgG (21).

Sera from 3 of 27 dogs were unreadable by IHA because of nonspecific hemagglutination. With these exceptions, IHA and the detection of IgM by ELISA gave identical results. Further studies on the use of IHA for the diagnosis of acute leptospirosis in dogs or other animal species are warranted. Absorption with unsensitized human group O erythrocytes may be helpful in reducing the occurrence of nonspecific hemagglutination, but this technique requires further evaluation.

In this study we have confirmed the utility of IHA as an initial screening test for the investigation of hospitalized patients clinically suspected of having acute leptospirosis. We did not titrate sera which reacted in the IHA, although this is recommended by the manufacturer. Used in this way, the IHA has a low cost and requires no specialized equipment or incubation conditions. These factors make it ideal for laboratories in which leptospirosis is not a frequent diagnosis and also for use in clinical diagnostic laboratories in which resources are limited.

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