EVALUATION OF TWO ENZYME-LINKED IMMUNOSORBENT ASSAY METHODS FOR DETECTION OF IMMUNOGLOBULIN M ANTIBODIES IN ACUTE LEPTOSPIROSIS

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Abstract. Leptospirosis is a common zoonosis of worldwide distribution. Diagnosis of leptospirosis is usually accomplished by serology, but the microscopic agglutination test (MAT) generally requires paired sera for detection of seroconversion and is considered too complex for routine use. A number of rapid assays have been developed in recent years. In the present study, 2 immunoglobulin (Ig) M enzyme-linked immunosorbent assay (ELISA) methods were evaluated for the early diagnosis of acute leptospirosis in Barbados. A total of 103 patients admitted to the Queen Elizabeth Hospital for diagnosis of suspected leptospirosis were investigated. A case of leptospirosis was confirmed by a 4-fold rise in titer between 2 sera tested by MAT, an initial titer of ≥ 800 in the MAT, or by isolation of leptospirae from blood or urine. A total of 48 cases of leptospirosis were confirmed. In 33 cases, both commercial assays were positive in the first sample, taken at admission, a mean of 6.7 days after onset of symptoms, whereas seroconversion was detected in a further 9 cases. Both assays were negative in 5 cases, and the remaining case gave discordant results in the 2 assays. False-positive IgM results were detected in 4 patients without leptospirosis. The sensitivity of the 2 assays was 89.6 and 97.5%, respectively, and specificities were 92.7 and 96.4%, respectively. The positive predictive values were 87.8 and 95.5%, and the negative predictive values were 90.7 and 89.5%, respectively. Either of these assays can be used for early diagnosis of leptospirosis, particularly in laboratories that cannot perform more specialized leptospiral serology.

INTRODUCTION

Leptospirosis is a common zoonosis throughout the world. The burden of disease is heaviest in tropical developing countries, but large outbreaks have occurred in temperate regions. Because the symptoms of leptospirosis may resemble those of dengue and other tropical diseases, laboratory diagnosis is essential for optimum treatment. Severe leptospirosis may be associated with high mortality if not treated adequately.

The definitive diagnosis of leptospirosis may be accomplished by isolation of the infecting spirochete from blood, urine, or tissues, but this requires special media and prolonged incubation and is not often performed. Serologic diagnosis by the microscopic agglutination test (MAT) is time-consuming, requires significant expertise that is available in few laboratories, and requires paired sera for confirmation of seroconversion. There is a need for simpler serologic tests that can be used in nonreference laboratories. A wide variety of screening tests have been developed.

Detection of immunoglobulin (Ig) M antibodies by enzyme-linked immunosorbent assay (ELISA) was described a number of years ago, but until recently, there were no assays available commercially. We describe the evaluation of 2 commercial IgM-ELISA assays intended for presumptive serologic diagnosis of acute leptospirosis.

MATERIALS AND METHODS

Specimens. Samples were obtained from patients admitted to the Queen Elizabeth Hospital, Bridgetown, Barbados, with a history and clinical manifestations suggestive of leptospirosis, including fever, jaundice, anorexia, headache, conjunctival suffusion, myalgia, and abdominal pain, by means of the protocol described previously. The study protocol was approved by the Medical Research Advisory Committee of the Ministry of Health. Blood samples for serology were collected on the day of admission (A1 sample) and on the fourth day after admission (A2 sample). For some patients, a convalescent sample was taken before discharge from the hospital or at a follow-up visit to the outpatient clinic. Blood cultures were made on the day of admission by inoculating 3 drops of blood into 10 mL polysorbate medium at the patient’s bedside (EMJH; Difco Laboratories, Detroit, MI). Urine samples from patients who were not anuric at the fourth day of their admission were centrifuged at 1500 × g for 30 minutes. The pellet was resuspended in 1 mL of sterile phosphate-buffered saline and inoculated into the same medium within 2 hours of collection.

In-house ELISA. Immunoglobulin G and IgM titers were determined by ELISA with the strain Patoc I (serovar patoc) as antigen in order to detect antibodies against a wide range of leptospires. An IgM titer ≥ 160 was regarded as positive.

Microscopic agglutination test. Sera were examined by the MAT; we used a battery of 22 serovars to establish seroconversion or a rise in titer. The antigens used included both reference strains and locally prevalent serovars of the following serogroups (serovars in parentheses): Australis (bajan, barbadensis, bratislava), Autumnalis (him, forbragg), Ballum (arborea, ballum), Bataviae (bavatiae, brasilensis), Canicola (canicola), Cynopteri (cynopteri), Grippotyphosa (grippotyphosa), Icterohaemorrhagiae (copenhagenii), Mini (georgia), Panama (mangus, panama), Pomona (pomona), Pyrogenes (pyrogenes), Tarassovi (tarassovi), Sejroe (hardjo, sejroe), and L. biflexa Semarang (patoc).

Case definition. The diagnosis of leptospirosis was confirmed by a 4-fold rise in titer between 2 sera tested by MAT, an initial titer of ≥ 800 in the MAT, or by isolation of leptospires from blood or urine.

PanBio Leptospira IgM ELISA. PanBio Leptospira IgM ELISA reagents were obtained from the manufacturer (PanBio, Queensland, Australia). Sera to be tested were diluted 1:100 in serum diluent supplied by the manufacturer, and 100 μL of the resulting dilutions were added to antigen-coated microwells. Positive and negative controls were included in the test.
each test run, together with a calibrator serum run in triplicate. Plates were incubated at 37°C for 30 min before washing with an automated plate washer and completion of the protocol supplied by the manufacturer, using an anti-human IgM conjugate. The total incubation time for this assay was 1 hour, 10 minutes. Absorbance was read at 450 nm/600 nm. A PanBio unit was calculated for each sample by dividing the absorbance of the sample by the mean absorbance of the calibrator sample replicates and multiplying by 10. A score of < 9 units indicated a negative result, 9–11 units an equivocal result, and > 11 units a positive result, indicating the presence of leptospira-specific IgM antibodies.

**InDx IVD Microwell ELISA.** The InDx IVD microwell ELISA was obtained from the manufacturer (Integrated Diagnostics, Baltimore, MD). Sera to be tested were diluted 1:40 in the dilution buffer supplied. To 100 µL diluted sample was added 40 µL of goat anti-human IgG absorbent (ProSorbG). After mixing and incubating for 10 min, the entire 140 µL was transferred to antigen-coated microwells. Positive and negative controls were included in each test run and were treated with ProSorbG in the same way as test sera. Plates were incubated at 25°C for 10 min, before completion of the protocol supplied by the manufacturer. The total incubation time for this assay was 35 minutes. Absorbance was read at 450 nm/600 nm. A negative result was defined as an absorbance of 0.0–0.3 optical density (OD) units, an equivocal result as 0.5 to ≤ 1.0 OD units, and a positive result as > 1.0 OD units.

**Data analysis.** The Pearson correlation coefficient was calculated by Prism version 3.02 (GraphPad Software, San Diego, CA). The degree of agreement between the 2 assays was estimated by the kappa test.22

**RESULTS**

A total of 103 patients admitted to the Queen Elizabeth hospital for diagnosis of suspected leptospirosis were investigated. A diagnosis of leptospirosis was confirmed in 48 patients (46.6%). Leptospires were isolated from 8 (16.7%) of 48 cases; in one, the diagnosis was made only by culture. The isolates were identified as *Leptospira kirschneri* serovar bim (4), *L. interrogans* serovar copenhageni (2) and *L. borgpetersenii* serovar arboarea (2), consistent with the distribution of serovars in this population.8,23 The remaining cases were confirmed by serologic investigations; a total of 47 cases (98%) were positive in the MAT.

In 33 of 48 cases, both commercial assays were positive in the first sample, taken at admission, a mean of 6.7 days after onset of symptoms. In a further 9 cases, seroconversion was detected between the A1 and A2 samples by both commercial ELISA assays. The commercial assays were both negative in 5 cases, whereas the remaining case gave discordant results. False-positive IgM results were detected in 4 patients who did not have leptospirosis by the InDx IVD ELISA, and in 2 patients by the PanBio ELISA. The sensitivity and specificity of the InDx IVD ELISA were 89.6 and 92.7%, respectively, and for the PanBio ELISA, they were 87.5 and 96.4%, respectively. The positive predictive value and negative predictive values were 87.8 and 90.7%, respectively, for the InDx IVD ELISA and 95.5 and 89.5%, respectively, for the PanBio ELISA.

Of the 8 cases from whom leptospires were isolated, one remained seronegative, whereas 5 were positive by all serologic tests in the A1 sample; in the remaining 2 cases, seroconversion occurred between the A1 and A2 samples in all assays.

**Results on A1 samples.** The distribution of test results on A1 samples from leptospirosis patients is shown in Table 1. Seroconversion occurred by MAT in all but one patient. The A1 sample was negative in 20 (41.7%) of 48 patients, whose samples were taken a mean of 4.8 days after onset. A total of 14 (29.2%) of the A1 samples gave an MAT titer ≥ 800; these samples were taken a mean of 8.1 days after onset of symptoms. In one patient, seroconversion did not occur until the convalescent sample, taken 24 days after the onset of symptoms. In the remaining patients, there was a 4-fold rise in titer between the A1 and A2 samples, taken 4 days apart.

In 12 cases, IgM antibodies were detected in first samples by the in-house ELISA, but there was no detectable agglutinating antibody. The mean intervals between onset of symptoms and positive and negative results in the respective tests are shown in Table 2. The sensitivity of diagnosis based on a positive A1 sample was 29% for MAT, 73% for the in-house IgM-ELISA, 70% for the PanBio ELISA, and 69% for the InDx IVD ELISA.

In the A1 samples from 55 patients who did not have leptospirosis, the InDx IVD ELISA and the PanBio ELISA were each positive in 2 samples (3.6%) and negative in 53 (96.4%) cases. In A2 samples from these patients, the InDx IVD ELISA was positive in a further 2 patients.

There was a strong correlation between results obtained with the 2 commercial ELISA assays (Figure 1). The correlation coefficient was 0.889. The kappa value was 0.86, showing excellent agreement between the 2 assays.

**DISCUSSION**

Traditional methods for diagnosis of leptospirosis include culture in polysorbate-containing media and detection of agglutinating antibodies by the MAT.8 Both of these approaches have limitations. Culture is slow and yields a retrospective result, and it is insensitive, yielding isolates from < 50% of cases of leptospirosis20; if cultures are not collected at appropriate times during the illness, sensitivity will be much lower. Similarly, the MAT requires significant expertise to perform and interpret.22 Both acute and convalescent sera are usually required for serodiagnosis by using the MAT, to allow for the detection of rising antibody titers. Presumptive diagnosis based on a high initial MAT titer is possible, but the

**TABLE 1**

Distribution of test results in A1 samples from 48 cases of leptospirosis

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>MAT titer</th>
<th>PanBio ELISA</th>
<th>InDx ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>9</td>
<td>Neg</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>3</td>
<td>100–400</td>
<td>Neg</td>
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<td>11</td>
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<td>1</td>
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<td>Pos</td>
</tr>
<tr>
<td>13</td>
<td>≥ 800</td>
<td>Pos</td>
<td>Pos</td>
</tr>
</tbody>
</table>

*ELISA = enzyme-linked immunosorbent assay; MAT = microscopic agglutination test; neg = negative; pos = positive.*
threshold titer will depend on the prevalence of leptospirosis within the population and may vary from 1:200 to 1:1,600. Interpretation is further complicated by persistence of antibodies. In this study, we evaluated the performance characteristics of some assays have varied. Detection of specific IgM antibodies by ELISA has been shown to be more sensitive than the detection of agglutinating antibodies, in some cases for many years.27,28

Detection of specific IgM antibodies by ELISA has been shown to be more sensitive than the detection of agglutinating antibodies. In this study, we evaluated the performance of 2 commercial microtiter IgM-ELISA assays in a series of patients with severe, acute illness thought to be leptospirosis. Both assays were of approximately equal sensitivity and showed excellent concordance, evidenced by the high correlation coefficient and the high kappa value. The performance of these assays was equivalent to that of the in-house IgM-ELISA method we have used for several years. The commercial assays both used the same antigen (serovar patoc) as the in-house assay, but in the InDx IVD ELISA, IgG antibodies were removed from serum samples by use of an anti-IgG antibody before addition of diluted sample to the antigen-coated microwells, and the total time required for completion of the assay was considerably less.

The specificity of the PanBio ELISA was slightly higher than that of the InDx IVD ELISA, and this was reflected in the higher predictive value of a positive test in the population studied. Our findings confirm those of the lone previous study, which was conducted in Australia, where the range of serovars causing disease is quite different, reflecting occupational exposure to serovars not found in Barbados. In addition, since the previous study was published, the protocol for performance and interpretation of the assay has changed (PanBio manufacturer’s package insert). Evaluation of new serologic assays in different populations is important because performance characteristics of some assays have varied. Moreover, some assays may not detect infections caused by all serovars. For this reason, the nonpathogenic serovar patoc is widely used as an antigen in serologic tests for leptospirosis: it detects broadly cross-reactive antibodies.

Both IgM-ELISA assays were more sensitive than MAT for detection of cases early in the acute illness. The main reason for seeking an early diagnosis of leptospirosis is to facilitate appropriate treatment, particularly if the choice of appropriate antibiotic treatment is to be guided by diagnostic test results. Other common infectious diseases that must be included in the differential diagnosis of leptospirosis include dengue fever, typhoid, and malaria, particularly in endemic regions or in travelers returning from the tropics. Because the specific treatment of these infections differs, rapid and accurate diagnosis is of clinical significance.

A limitation to the use of single serum samples for serodiagnosis is the persistence of antibodies, as noted above. Antileptospiral IgM antibodies are also persistent, but the rate of decline shows marked variation. Thus, a single IgM-positive sample taken during an acute febrile illness with symptoms suggestive of leptospirosis is presumptive evidence of infection, but this finding requires confirmation by testing a convalescent sample, preferably by the use of an alternative method. Either of these assays can be used for early diagnosis of leptospirosis, but a positive result will require confirmation by further testing.

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REFERENCES
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Table 2
Time between onset of symptoms and positive and negative tests in 48 patients with acute leptospirosis

<table>
<thead>
<tr>
<th>Method</th>
<th>Negative tests (mean days after onset)</th>
<th>Positive tests (mean days after onset)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT (titer ≥ 800)</td>
<td>5.7</td>
<td>9</td>
</tr>
<tr>
<td>IgM-ELISA (titer ≥ 160)</td>
<td>5.4</td>
<td>6.8</td>
</tr>
<tr>
<td>PanBio IgM ELISA</td>
<td>6.1</td>
<td>6.8</td>
</tr>
<tr>
<td>InDx IgM ELISA</td>
<td>6.3</td>
<td>7.0</td>
</tr>
</tbody>
</table>

*ELISA = enzyme-linked immunosorbent assay; MAT = microscopic agglutination test.


