LEPTO dipstick, a dipstick assay for detection of Leptospira-specific immunoglobulin M antibodies in human sera


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LEPTO Dipstick, a Dipstick Assay for Detection of *Leptospira*-Specific Immunoglobulin M Antibodies in Human Sera

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Leptospirosis, a zoonosis with a worldwide distribution, is an acute febrile illness caused by microorganisms of the genus *Leptospira* (3). Currently, more than 200 pathogenic *Leptospira* serovars have been described. Leptospires are present in the blood until they are cleared after 4 to 7 days following the production of *Leptospira*-specific antibodies, initially mainly on the immunoglobulin M (IgM) class. The laboratory diagnosis of leptospirosis is mainly based on serological methods. Several methods are available for this purpose. The microscopic agglutination test (MAT) is the cornerstone of the serodiagnosis of leptospirosis, because this assay has a high sensitivity and allows for the detection of group-specific antibodies (8, 11). Unfortunately, the performance of MAT is restricted to laboratories that are capable of maintaining strains for the preparation of live antigens. Enzyme-linked immunosorbent assays (ELISAs) (1, 8, 9, 12) or indirect fluorescent-antibody tests (10) are useful alternative or additional tests by virtue of their capacity to detect with a high sensitivity specific IgM antibodies as a sign of current or recent leptospirosis. Because of the relative complexity and costs of these assays, they can only be performed in relatively specialized laboratories, thus hampering the diagnosis and appropriate treatment of leptospirosis in many tropical countries. We argue that a simplified version of the ELISA giving either a positive or a negative indicative result would suffice as a screening test for current or recent leptospirosis in situations where appropriate diagnostic facilities are lacking. Therefore, we developed a dipstick assay using a broadly reactive leptospiral antigen fixed onto a solid support in combination with a stabilized and stained anti-IgM conjugate as a signal system instead of the usual enzyme-based detection system and a single serum dilution giving a qualitative result. Here, we describe the evaluation of this simple dipstick assay for the detection of *Leptospira*-specific IgM antibodies in human serum samples.

**MATERIALS AND METHODS**

Sera. The following four groups of sera were used. (i) One group of sera (n = 558), which was used to calculate the sensitivity and specificity of the dipstick assay, was taken from the serum bank of the Leptospirosis Reference Centre of the Royal Tropical Institute. The sera were confirmed by MAT and culture to have been from patients with a clinical diagnosis of leptospirosis. (ii) The second group was derived from 114 patients. The serology results by MAT for these patients were suggestive of an infection with a strain of the serogroups Australis (5 patients, 6 serum samples), Autumnalis (1 patient, 4 serum samples), Bataviae (2 patients, 5 serum samples), Canicola (1 patient, 2 serum samples), Celleonidi (1 patient, 2 serum samples), Grippotyphosa (21 patients, 56 serum samples), Icterohaemorrhagiae (33 patients, 84 serum samples), Pomona (15 patients, 22 serum samples), and Sejroe (19 patients, 48 serum samples). For 20 patients (55 serum samples) the causative serogroup could not be classified. The second set of control sera (n = 274) was derived from 274 patients. For evaluation of the dipstick assay, MAT and the IgM ELISA were repeated for all sera from the case patients and the controls, and the evaluation was based on these test results. The mean agglutination titer...
of the MAT-positive sera from the case patients ranged from 1:320 to 1:1,280 for the different antigens (data not shown). Upon retesting, two serum samples from the controls were borderline by MAT, i.e., a titer of 1:160 or 1:320. None of the case patients became negative upon retesting. The mean titer measured by the IgM ELISA of the IgM ELISA-positive sera from the patients was 1:320. Among the controls, 16 serum samples were positive by the IgM ELISA. Two of the 16 serum samples from the control group of leptospirosis-negative patients which were positive in the IgM ELISA had titers of 1:160 and 1:640. The other 14 serum samples had borderline titers of 1:40 or 1:80. From 109 of the 114 case patients, two or more serum samples taken at different days after the onset of the disease were tested. The sera were divided into three groups as follows. One group consisted of sera that were collected during the first 9 days of the disease (67 serum samples), when the immune response was mainly supported by IgM antibodies. The second group (28 serum samples) was collected between days 10 and 30, and when IgM antibody production usually peaks; and a third group of sera (89 serum samples) was obtained after patients collected 30 days to 1 year after the onset of disease, when the antibody level is declining. The 274 serum samples from the controls were equally divided into a set from the first 10 days of the disease and a set from the period between days 10 and 30.

(ii) Sera (n = 25) from healthy blood bank donors were obtained from the local blood bank and served as negative controls.

(iii) Sera from patients with Lyme borreliosis (n = 20 serum samples), human immunodeficiency virus (HIV) infection (n = 20), hepatitis A virus infection (n = 20), hepatitis B virus infection (n = 20), hepatitis C virus infection (n = 20), syphilis (n = 20), malaria (n = 20), toxoplasmosis (n = 10), Hanta virus infection (n = 20), meningococcal meningitis (n = 20), or an autoimmune disease (rheumatoid arthritis, n = 10; systemic lupus erythematosus, n = 20) were used to screen for cross-reactivity.

Preparation of antigen for the LEPTOD dipstick assay. Heat-stable antigen was prepared from a culture of Leptospira biflexa. The leptospires were grown in bovine albumin polysorbate medium (EMJH medium), as described by Ellingham and McCullough (2) and modified by Johnson and Harris (6). Antigens were prepared by heating well-grown cultures (10^9 ml^-1) as described for the ELISA (6, 11), with minor modifications. After heating, the antigen was concentrated eight times by centrifugation. Antigens were stored at -20°C until use.

Preparation of dipssticks. Antigens were bound to nitrocellulose strips by incubation of a 30-cm strip with 0.6 ml of antigen. Unbound antigen was removed by two washes with phosphate-buffered saline. After blocking with 3% skim milk, the membranes were allowed to dry at room temperature. Nitrocellulose strips containing the antigen were then adhered to a plastic support with tape that was sticky on both sides and that was cut into 2.5-mm-wide sticks. To obtain an antigen band, the human IgM antibody was coated onto this strip using nitrocellulose as a separate band. The optimal concentrations of antigen and antibody were determined by applying serial dilutions.

Preparation of the detection reagent. A monoclonal anti-human IgM antibody was prepared from mouse ascitic fluid by pepsin digestion (30). After blocking with 3% skim milk, the membranes were allowed to dry at room temperature. Nitrocellulose strips containing the antibody were then adhered to a plastic support with tape using 150 g of pressure and coated with 3% skim milk. After blocking, the antigen was detected using a 1/500 dilution of a horseradish peroxidase-labeled antibody which was made up in phosphate-buffered saline and 50% of antigen. Agglutination was examined by dark-field microscopy. The titer was defined as the highest dilution giving 50% agglutination in comparison with that of the negative control. Sera were considered positive when a titer of ≥1:160 was obtained with at least one strain.

Statistical evaluation. The intermethod variation between the dipstick assay and the IgM ELISA was determined by calculating kappa values with 95% confidence intervals (CIs). Kappa values express the agreement beyond chance. Generally, a kappa value of >0.80 represents almost perfect agreement beyond chance. Values below 0.40 represent slight agreement, and values between 0.40 and 0.80 represent fair to good agreement.

Internal control. To facilitate interpretation of the assay results and to control for the integrity of the ingredients and the performance of the assay, an anti-human IgM antibody was coated as a separate band on the dipstick. The coated antibody binds IgM molecules from the serum which subsequently are stained by the detection reagent. The staining intensity of this internal control band was rated 2+ for most of the sera tested.

Test and storage conditions. In all these experiments the dipssticks were incubated for 3 h at room temperature (20 to 24°C). When testing a selection of these sera by incubation at a higher temperature (37°C), the same result was obtained when an incubation time of 2 h was used (data not shown). Some development of background was noted when the incubation was performed for a longer period at this temperature. The lyophilized detection reagent was stored for 1 year at various temperatures (i.e., 4, 20, 37, and 56°C), without a loss of activity (data not shown). When the dipssticks were stored at these temperatures for up to 3 months, some background development was noticed at the higher temperatures. Reconstituted detection reagent could be stored at 4, 20, and 37°C for at least 3 months without a loss of activity. When stored at 56°C, activity was lost within a month.

RESULTS

Performance of the LEPTOD dipstick assay. By the dipstick assay and the IgM ELISA, similar numbers of serum samples were found to be positive for the group of sera from the case patients. The numbers of serum samples from the controls that scored positive by the two assays were also comparable (Table 1). None of the blood bank sera from healthy controls were found to be positive by either the dipstick assay or the IgM ELISA. In comparison with these assays, the numbers of serum samples found positive by MAT were slightly higher for the group of sera from the case patients and slightly lower for those from the controls (Table 1). Most positive sera agglutinated two or more strains. The two MAT-positive serum samples from the control group were borderline positive (titers,
TABLE 2. Sensitivities of LEPTO dipstick assay and IgM ELISA for sera from patients at different stages of disease

<table>
<thead>
<tr>
<th>Duration of disease (days)</th>
<th>LEPTO dipstick assay</th>
<th>IgM ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of serum samples positive/total no. tested</td>
<td>Sensitivity [% [95% CI]]</td>
</tr>
<tr>
<td>&lt;10</td>
<td>34/54</td>
<td>63.0 (49–76)</td>
</tr>
<tr>
<td>10–30</td>
<td>78/91</td>
<td>85.7 (77–92)</td>
</tr>
<tr>
<td>30–100</td>
<td>50/64</td>
<td>78.1 (66–88)</td>
</tr>
</tbody>
</table>

The results of MAT obtained with sera from Dutch patients suspected of having leptospirosis were used to calculate the sensitivity and specificity of the dipstick assay. The calculated overall sensitivity of the dipstick assay was 86.8% (95% CI = 81 to 93%) and the specificity was 92.7% (95% CI = 89 to 96%). The values for the IgM ELISA were slightly higher: 88.5% (95% CI = 83 to 94%) and 94.2% (95% CI = 91 to 97%), respectively. The 95% CIs, however, overlapped. The calculated overall sensitivity of the dipstick assay was 86.8% (95% CI = 81 to 93%) and the specificity was 92.7% (95% CI = 89 to 96%). The values for the IgM ELISA were slightly higher: 88.5% (95% CI = 83 to 94%) and 94.2% (95% CI = 91 to 97%), respectively. The 95% CIs, however, overlapped. As described above, more than one serum sample was analyzed from most patients. The calculations were therefore based on the results either for the first serum sample collected between days 10 and 30 or for the serum sample collected nearest this period for those patients from whom no sample was collected during that period. By the dipstick assay, the numbers of true-positive, false-positive, false-negative, and true-negative results were 99, 20, 15, and 254, respectively. By the IgM ELISA, these numbers were 101, 16, 13, and 258, respectively.

The 284 serum samples from the patients confirmed to have leptospirosis included 67 serum samples collected early in the course of the disease, before significant levels of specific IgM antibodies are generally produced; 89 serum samples from convalescent patients, in whom specific IgM antibody levels are already declining; and 128 serum samples collected between days 10 and 30. The sensitivities of the dipstick assay and the IgM ELISA were calculated for sera from each of these three groups of patients (Table 2). More than one serum sample was collected from a number of patients during one of these three stages of the disease. In the calculation of the sensitivity of the assays, only the serum samples that were collected first were taken into account for the first two groups of sera, whereas for the third group, only the samples that were collected last were taken into account. The sensitivity of the dipstick assay appeared to be 85.7% for sera collected between days 10 and 30, when the IgM response peaks, and 78.1% for sera from convalescent patients. Because antibodies usually become detectable between 5 and 10 days after the onset of the disease, the sensitivity of the assay was accordingly found to be low (63%) when sera collected at an early phase of the disease were tested. For the IgM ELISA, the sensitivity was lower for the first group of sera and slightly higher for the other two groups, but 95% CIs again overlapped (Table 2).

Analysis of the first serum sample from each of the patients allowed for the detection of Leptospira-specific IgM antibodies in 72.8% of the patients. Analysis of a second serum sample increased the detection rate of the dipstick assay to 86.8% for patients confirmed to have leptospirosis. For the 51 patients from whom the first serum sample was collected before day 10, analysis of a second serum sample even improved the detection rate, from 60.8 to 94.1%. For comparison, analysis of a second serum sample by the IgM ELISA increased the number of patients found to be positive from 70.2 to 92.1%.

Comparison of the results of the dipstick assay with the results of the IgM ELISA showed a high degree of agreement between the two assays for the total group of Dutch patients suspected of having leptospirosis. The observed agreement was 88.7%, and the agreement beyond chance (kappa value) was 0.73 (95% CI = 0.65 to 0.80). Ninety-four serum samples were dipstick assay and IgM ELISA positive, 250 serum samples were negative by both assays, 23 serum samples were positive by the dipstick assay and negative by the IgM ELISA, and 21 serum samples were negative by the dipstick assay and positive by the IgM ELISA.

For the sera from the group of patients with diseases other than leptospirosis, 8.9% (16 of 180) cross-reactivity was observed when using the dipstick assay. With the different groups of sera, no cross-reactivity was found for sera from syphilis, hepatitis B virus-infected, and meningitis patients, but cross-reactivity was found at the following percentages for the other patients: 3% for patients with an autoimmune disease; 10% for each group of patients with HIV, Hanta virus, or toxoplasma infection; 15% for Lyme borreliosis and malaria patients; and 20% for patients with meningococcal meningitis and hepatitis A virus infection. None of the sera from this group of 180 serum samples were positive by the IgM ELISA. The volumes of the serum samples from the patients with Lyme borreliosis, toxoplasmosis, and Hanta virus infections were too small for evaluation by MAT. By MAT, 9.2% (12 of 130) of the sera tested were positive. Agglutination was observed with sera from patients with autoimmune disease (10%), HIV infection (10%), syphilis infection (40%), hepatitis B virus infection (10%), and meningococcal meningitis (10%). Most of these sera had borderline reactions.

When the results of the dipstick assay were rated from 1+ to 4+ according to the intensity of the staining (Fig. 1), most of the dipstick assay-positive sera from the group of leptospirosis patients revealed a high staining intensity (Table 3). The group of sera collected between days 10 and 30 had the highest

![FIG. 1. Staining intensity of the LEPTO dipstick assay. The staining intensity of the antigen band (lower band) is rated zero (−) for a negative reaction or according to the intensity of the staining (1+ to 4+). The internal control band (upper band) should stain in all cases.](image-url)
We have developed a simple dipstick method for the detection of Leptospira-specific IgM antibodies. Evaluation of the assay revealed that the results of the assay are in agreement with the results of an ELISA for the detection of Leptospira-specific IgM antibodies. Nearly the same number of serum samples from case patients and controls were found to be positive by the dipstick assay as by the IgM ELISA, and no differences between the detection rates by each of the assays was seen for sera collected at different stages of the disease. This shows that proportion of sera giving a strong staining intensity, and as expected, the staining intensity of paired serum samples more often revealed an increased staining or seroconversion for sera collected early in the disease than for sera collected at a later phase (data not shown). In contrast, most of the dipstick-positive sera from the negative control patients and the patients with selected diseases other than leptospirosis showed a weak (1+ or 2+) staining intensity only (Table 3).

**Detection of different serovars.** On the basis of the results of MAT and culture, leptospirosis in the Dutch patients was mainly caused by strains of the serogroups Icterohaemor rhagiae, Grippotyphosa, Sejroe, and Pomona. Our results indicate that the dipstick assay allows for the detection of each of these major serovars of leptospires equally well (Table 5). In order to determine the reactivity of the dipstick assay with sera from patients infected with strains of other serogroups, the dipstick assay was used to test 169 serum samples from patients from countries other than The Netherlands. Of these serum samples, 84 were positive by MAT. For 38 patients, the results of MAT were consistent with infections with strains belonging to the serogroup Australis and for 12 patients the results of MAT were consistent with an infection with a strain of the serogroup Autumnalis. The dipstick assay also allowed for the detection of antibodies in these two groups of sera at a high rate (Table 5). These results indicate that the dipstick assay has a broad reactivity for sera from patients infected with different strains. The broad reactivity was further supported by comparison of the results of MAT and the dipstick assay for sera from the group of foreign patients. The dipstick assay detected 78 of the 84 (92.9%) MAT-positive serum samples and 5 of the 85 (5.9%) MAT-negative serum samples. Most of the sera in the group of MAT-positive sera from the foreign patients stained strongly (Table 3).

**Reproducibility.** Performance of the assay with 60 samples (45 serum samples from patients and 15 serum samples from controls) by two different experimentors resulted in an agreement of 96.7% (kappa value = 0.92). Reading of the test results of the assay performed with 120 samples by two different observers showed agreement for 95.8% of the serum samples (kappa value = 0.9). In both cases the differences were due to disagreement in the interpretation of weakly positive (1+) results.

**DISCUSSION**

Due to the lack of simple diagnostic tools, the diagnosis of leptospirosis cannot be easily made in many laboratories. Hence, leptospirosis often is not recognized or is erroneously mistaken for other diseases with similar symptoms. As a consequence, this often serious disease may be either left untreated or treated improperly; besides, information on the prevalence and incidence of leptospirosis may be unreliable.

We have developed a simple dipstick method for the detection of Leptospira-specific IgM antibodies. Evaluation of the assay revealed that the results of the assay are in agreement with the results of an ELISA for the detection of Leptospira-specific IgM antibodies. Nearly the same number of serum samples from case patients and controls were found to be positive by the dipstick assay as by the IgM ELISA, and no differences between the detection rates by each of the assays was seen for sera collected at different stages of the disease. This shows that proportion of sera giving a strong staining intensity, and as expected, the staining intensity of paired serum samples more often revealed an increased staining or seroconversion for sera collected early in the disease than for sera collected at a later phase (data not shown). In contrast, most of the dipstick-positive sera from the negative control patients and the patients with selected diseases other than leptospirosis showed a weak (1+ or 2+) staining intensity only (Table 3).

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**TABLE 3. Staining intensity of LEPTO dipstick assay and duration of disease**

<table>
<thead>
<tr>
<th>Serum group and period of disease (days) or MAT result</th>
<th>% Sera with the following staining intensity:</th>
<th>No. of serum samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutch patients confirmed to have leptospirosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10 days</td>
<td>38</td>
<td>23 3</td>
</tr>
<tr>
<td>10–30 days</td>
<td>13</td>
<td>29 91</td>
</tr>
<tr>
<td>30–100 days</td>
<td>25</td>
<td>6 64</td>
</tr>
<tr>
<td>Negative controls</td>
<td>96</td>
<td>1 1</td>
</tr>
<tr>
<td>Patients with diseases other than leptospirosis</td>
<td>94</td>
<td>2 180</td>
</tr>
<tr>
<td>Foreign patients suspected of having leptospirosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAT positive</td>
<td>7</td>
<td>26 32 24 84</td>
</tr>
<tr>
<td>MAT negative</td>
<td>94</td>
<td>4 2 85</td>
</tr>
</tbody>
</table>

**TABLE 4. Detection of confirmed leptospirosis patients by LEPTO dipstick assay and IgM ELISA**

<table>
<thead>
<tr>
<th>Test (patient group)</th>
<th>No. (%) of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>LEPTO dipstick assay (all patients)</td>
<td>14 (12.3)</td>
</tr>
<tr>
<td>LEPTO dipstick assay (patients with a serum sample collected before day 10)</td>
<td>3 (5.9)</td>
</tr>
<tr>
<td>IgM ELISA (all patients)</td>
<td>10 (8.7)</td>
</tr>
</tbody>
</table>
the method is well suited for the early diagnosis of leptospirosis. The dipstick method has the advantage, however, that it is easy to perform, is highly reproducible, and depends on neither special equipment nor refrigeration.

The sensitivity and specificity of the dipstick assay and the IgM ELISA appeared to agree well. The sensitivity of the dipstick assay for sera collected between days 10 and 30 of the disease was 86.8%, and that of the IgM ELISA was 88.5% (Table 2). As expected, the sensitivities of the two assays were lower for sera collected early (63.0%) and during convalescence (78.1%). In the Netherlands, many patients are seen relatively early in the course of the disease, before significant antibody levels develop, and they receive prompt treatment, often before confirmation by laboratory diagnosis. Early treatment may interfere with the development of a strong immune response. As a consequence, the sensitivity of the serodiagnosis may be low, especially when serum samples collected at an early phase of the disease, before seroconversion has occurred, are being analyzed. Of the present group of sera from leptospirosis patients from The Netherlands, about 22% were collected in the early seronegative phase of the disease. However, often, the first serum sample submitted for laboratory diagnosis will be collected when patients have been ill long enough for seroconversion to have occurred. The specificity of the dipstick assay was calculated to be 92.7%, and that of the IgM ELISA was 94.2%. All sera from the control group of leptospirosis-negative patients which scored positive in the dipstick assays stained weakly. The dipstick assay revealed cross-reactivity with sera from patients with several other diseases, notably, with sera from patients with HIV, Hanta virus, and toxoplasma infection; Lyme boriolosis; malaria; meningococcal meningitis; or hepatitis A virus infection. However, in most cases the observed staining intensity was weak. Nevertheless, the possibility that cross-reactivity may occur should be taken into account in the interpretation of the assay results. In contrast, no cross-reactivity was observed with these sera in the IgM ELISA and the performance of the dipstick assay thus differs in this aspect from the performance of the IgM ELISA. The higher degree of cross-reactivity of the dipstick assay, however, did not result in a lower specificity when analyzing sera from the group of Dutch patients.

Our results support the advice indicating that a second sample should be analyzed when the first serum sample is negative but the suspicion of leptospirosis remains. Among the individu-
jaard, C. Fijen, and T. van Gool (Academic Medical Centre, Amsterdam, The Netherlands), J. P. Verhave (University of Nijmegen, Nijmegen, The Netherlands), and A. Osterhaus (Erasmus University, Rotterdam, The Netherlands) for gifts of sera.

REFERENCES