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

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We studied a dipstick assay for the detection of *Leptospira*-specific immunoglobulin M (IgM) antibodies in human serum samples. A high degree of concordance was observed between the results of the dipstick assay and an IgM enzyme-linked immunosorbent assay (ELISA). Application of the dipstick assay for the detection of acute leptospirosis enabled the accurate identification, early in the disease, of a high proportion of the cases of leptospirosis. Analysis of a second serum sample is recommended, in order to determine seroconversion or increased staining intensity. All serum samples from the patients who were confirmed to be positive for leptospirosis by either a positive microscopic agglutination test or a positive culture but were found to be negative by the dipstick assay were also judged to be negative by the IgM ELISA or revealed borderline titers by the IgM ELISA. Some cross-reactivity was observed for sera from patients with diseases other than leptospirosis, and this should be taken into account in the interpretation of test results. The dipstick assay is easy to perform, can be performed quickly, and requires no electricity or special equipment, and the assay components, a dipstick and a staining reagent, can be stored for a prolonged period without a loss of reactivity, even at elevated temperatures.

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 submitted to the reference center for performance of the laboratory diagnosis of leptospirosis during the years 1992, 1993, and 1994 to confirm the clinical diagnosis, because leptospirosis was suspected or because leptospirosis was in the differential diagnosis. We further refer to this group of patients as Dutch patients. The selected sera consisted of two sets. One set of sera was taken from patients who either had a positive culture or whose serum samples (one or more serum samples were tested) were judged to be positive ($≥1:160$) by MAT performed with a panel of 14 live strains. This first group of patients confirmed to be positive for leptospirosis were considered to be leptospirosis case patients in the evaluation of the dipstick assay. The second set of sera was taken from patients who had a negative culture and from whom all available sera were negative in the MAT. These nonleptospirosis patients were considered controls, but it is admitted that some of these control patients potentially could have suffered from leptospirosis but that the only serum sample that they submitted was negative. Because the dipstick assay aims at the detection of *Leptospira*-specific IgM antibodies, the results of the IgM ELISA, which is normally performed along with culture and MAT in the laboratory diagnosis of leptospirosis, were not taken into account when selecting the sera from the case patients and the controls. Inclusion of the results of the IgM ELISA could have biased for the presence or absence of specific IgM antibodies in these two sets of sera, respectively.

The first set of serum samples ($n = 284$) 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 (3 patients, 6 serum samples), Autumnalis (1 patient, 4 serum samples), Bataviae (2 patients, 5 serum samples), Canicola (1 patient, 2 serum samples), Celledoni (1 patient, 2 serum samples), Grippotyphosa (21 patients, 56 serum samples), Icterohaemorrhagiae (33 patients, 84 serum samples), Pomona (13 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

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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 mainly consisting of IgM antibodies first starts to develop and seroconversion predominantly occurs; a second group of sera (128 serum samples) was collected between days 10 and 30, when IgM antibody production usually peaks; and a third group of sera (89 serum samples) was from convalescent 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 ($n = 10$), hepatitis B virus infection ($n = 10$), syphilis ($n = 20$), malaria ($n = 20$), toxoplasmosis ($n = 10$), Hanta virus infection ($n = 10$), meningococcal meningitis ($n = 20$), or an autoimmune disease (rheumatoid arthritis, $n = 10$; systemic lupus erythematosus, $n = 20$) were used to screen for cross-reactivity.

(iv) Sera from suspected leptospirosis patients from countries outside The Netherlands, notably, Benin ($n = 14$ serum samples), India ($n = 75$), Portugal ($n = 36$), Suriname ($n = 19$), Sweden ($n = 5$), and Thailand ($n = 20$), were used to determine the reactivities of sera from patients with infections with strains of serogroups which are uncommon in Dutch patients. This group of sera is further referred to as sera from foreign patients. Leptospirosis in these patients was confirmed by MAT.

All sera were randomly arranged, and the identity was blinded for the investigators when performing and interpreting the dipstick assay, MAT, and IgM ELISA.

Preparation of antigen for the LEPTO 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 Ellinghausen 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 (8, 11), with minor modifications. After heating, the antigen was concentrated eight times by centrifugation. Antigens were stored at $-20°C$ until use. Insoluble residues were pelleted by centrifugation just before use.

Preparation of dipsticks. Antigens were bound to nitrocellulose strips by incubation of a 30-cm strip with 0.6 ml of antigen. Unbound antigen was removed by brief washing 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 internal control, anti-human IgM antibodies were coated onto the 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 conjugated to a colloidal dye by a patented method described by Gribnau and coworkers (4, 5) and Snowden and Hommel (7). The monoclonal antibody was coated with a washed suspension of palanil red in 10 mM phosphate buffer containing 2.7 mM NaCl. The concentration of the dye suspension used for coating was adjusted such that a 1/500 dilution had a spectrophotometric A_{520} of 1. The conjugate was subsequently blocked with 30% bovine serum albumin in 5 mM NaCl. After blocking, the conjugate was suspended in a 32.3 mM phosphate buffer containing 125 mM NaCl, 6% trehalose, and 1.67% bovine serum albumin. Finally, the suspension was lyophilized for preservation. The optimal concentration of antibody was determined by performing the procedure with a serial dilution.

Execution of the human LEPTO dipstick assay. Dilutions of serum (1:50) were made directly in the reconstituted detection reagent. Dipsticks were wetted in distilled water for 15 s to prevent adsorption of the detection reagent to the solid support and were then incubated for 3 h in a reaction vial containing 0.3 ml of the detection reagent and the diluted serum sample. At the end of the incubation period the dipsticks were rinsed with water, and after removal of excess liquid with a cotton swab, they were air-dried. A reddish-stained antigen band indicated a positive reaction. Staining of various intensities was observed, and these were subjectively scored from 1+ to 4+. In the evaluation of the dipstick assay a staining intensity of ≥ + 1 was considered a positive result.

ELISA. The ELISA for the detection of *Leptospira*-specific IgM antibodies (IgM ELISA) was performed with antigen prepared from strain Wijnberg as described elsewhere (8, 9). Sera with a titer of ≥1:40 were considered positive.

MAT. MAT was performed with a panel of live leptospires as described previously (8). The panel consisted of the following 14 strains of 12 serovars:

TABLE 1. Detection of sera by LEPTO dipstick assay, IgM ELISA, and MAT

Subject and test	No. of serum samples positive/ total no. tested	% Positive
Case patients		
LEPTO Dipstick	217/284	76.4
IgM ELISA	218/284	76.8
MAT	231/284	81.3
Controls		
LEPTO Dipstick	20/274	7.2
IgM ELISA	16/274	6.2
MAT	2/274	0.7

strain Jez Bratislava (serovar *bratislava*, serogroup Australis), strain Mus 127 (serovar *ballum*, serogroup Ballum), strain Hond Utrecht IV (serovar *canicola*, serogroup Canicola), strains Duyster and Mandemakers (serovar *grippotyphosa*, serogroup Grippotyphosa), strain Hebdomadis (serovar *hebdomadis*, serogroup Hebdomadis), strain Kantorowicz (serovar *icterohaemorrhagiae*, serogroup Icterohaemorrhagiae), strain Wijnberg (serovar *copenhageni*, serogroup Icterohaemorrhagiae), strain Poi (serovar *poi*, serogroup Javanica), strain Pomona (serovar *pomona*, serogroup Pomona), strain 1161 U (serovar *proechimys*, serogroup Pomona), strains Hardjoprajitno and Lely 607 (serovar *hardjo*, serogroup Sejroe), and strain M 84 (serovar *sejroe*, serogroup Sejroe). The sera from the foreign patients were additionally examined by using a second panel of strains representative of global leptospires, including strain Swart (serovar *bataviae*, serogroup Bataviae) and strain celledoni (serovar *celledoni*, serogroup Celledoni). Agglutination was performed in microtiter plates with serial twofold dilutions of the sera starting at 1:20 (5 μl of serum, 45 μl of phosphate-buffered saline, and 50 μl 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 dipsticks 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 the incubation time was reduced to 2 h (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 dipsticks 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 LEPTO 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

Duration of disease (days)	LEPTO dipstick assay		IgM ELISA	
	No. of serum samples positive/total no. tested	Sensitivity (% [95% CI])	No. of serum samples positive/total no. tested	Sensitivity (% [95% CI])
<10	34/54	63.0 (49–76)	29/54	53.7 (40–67)
10–30	78/91	85.7 (77–92)	81/91	89.0 (81–95)
30–100	50/64	78.1 (66–88)	52/64	81.3 (70–90)

1:160 and 1:320). Again, none of the blood bank sera were positive.

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. 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 day 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
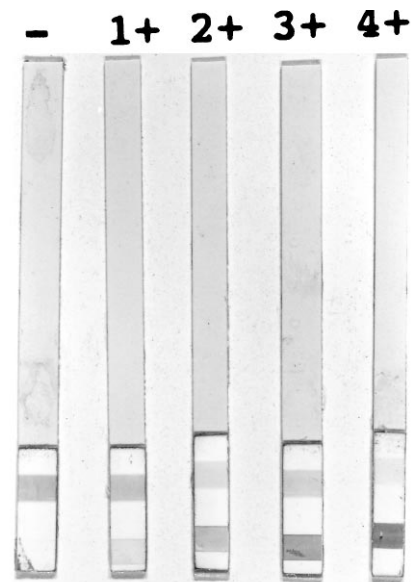

FIG. 1. Staining intensity of the LEPTO dipstick assay. The staining intensity of the antigen band (lower band) is rated zero (−) for a negative reaction or according to the intensity of the staining (1+ to 4+). The internal control band (upper band) should stain in all cases.

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

Serum group and period of disease (days) or MAT result	% Sera with the following staining intensity:					No. of serum samples
	0	1+	2+	3+	4+	
Dutch patients confirmed to have leptospirosis						
<10 days	38	16	20	23	3	54
10–30 days	13	7	13	37	29	91
30–100 days	25	13	23	33	6	64
Negative controls	96	3	1			274
Patients with diseases other than leptospirosis	94	4	2			180
Foreign patients suspected of having leptospirosis						
MAT positive	7	26	8	32	24	84
MAT negative	94	4	2			85

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).

Seroconversion and LEPTO dipstick assay-negative patients. Seroconversion was observed by the dipstick assay in 17 (14.9%) of the 114 patients confirmed to have leptospirosis (Table 4), 14 of whom also showed seroconversion by the IgM ELISA. For all but two of the patients showing seroconversion by the dipstick assay, the first dipstick assay-negative serum was collected during the first 9 days of the disease. Seroconversion determined by the dipstick assay occurred around day 7, when the detection rate increased from 45 to 83% of the sera (data not shown). By the IgM ELISA, seroconversion was detected in 24 patients (Table 4).

All serum samples (i.e., a total of 34 serum samples, of which 26 were collected after day 10) from 14 of the 114 leptospirosis patients were dipstick negative (Table 4). For 6 of these 14 patients, leptospirosis also was not detected by the IgM ELISA. With the exception of one of the serum samples from the other eight patients, which had a titer of 1:10,240, all other samples from those eight patients were either negative by the IgM ELISA or had borderline titers of 1:40 and 1:80. Two of the 14 dipstick assay-negative patients also scored negative by MAT. These two patients were judged to be positive for leptospirosis on the basis of a positive culture result.

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 Icterohaemorrhagiae, 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

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

Test (patient group)	No. (%) of patients				
	Negative	Patients who seroconverted	Patients with first serum sample positive	All positive patients	Total no. of patients tested
LEPTO dipstick assay (all patients)	14 (12.3)	17 (14.9)	83 (72.8)	100 (87.7)	114 (100)
LEPTO dipstick assay (patients with a serum sample collected before day 10)	3 (5.9)	16 (33.3)	31 (60.8)	48 (94.1)	51 (100)
IgM ELISA (all patients)	10 (8.7)	24 (21.1)	80 (70.2)	104 (91.2)	114 (100)

TABLE 5. Detection of different serogroups by
LEPTO dipstick assay

Serogroup	No. of serum samples positive/ total no. tested (%)	
	Dutch patients	Foreign patients
Australis	2/3 (66)	32/38 (84.2)
Autumnalis	1/1 (100)	12/12 (100)
Bataviae	2/2 (100)	1/1 (100)
Ballum		1/2 (50)
Cannicola	1/1 (100)	
Celledoni	1/1 (100)	6/6 (100)
Grippothyphosa	19/21 (90.4)	2/2 (100)
Icterohaemorrhagiae	31/33 (93.9)	9/9 (100)
Javanica		2/2 (100)
Louisiana		1/1 (100)
Pomona	11/13 (84.6)	
Pyrogenes		2/2 (100)
Sejroe	14/19 (73.8)	1/1 (100)
Unknown	17/20 (85)	6/6 (100)

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 boreliosis; 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 individuals in present study group, 72.8% of the patients confirmed to have leptospirosis were found to be positive by analyzing the first serum sample, whereas 86.8% were found to be positive when a second sample was also analyzed. For comparison, testing of a second serum sample by the IgM ELISA increased the percentage of patients found to be positive, from 70.2 to 92.1%. Analysis of a second serum sample also may be advisable when weak staining is observed with the first serum sample. Paired serum samples mostly showed a rise in staining intensity. Examination of paired sera may help to distinguish between patients with a present infection and patients with a past infection or cross-reactivity with sera from patients with diseases other than leptospirosis.

A slightly lower number of patients were found to be positive by the dipstick assay than by the IgM ELISA. However, all but one of the patients found to be negative by the dipstick assay but positive by the IgM ELISA had low borderline IgM titers of 1:40 or 1:80. Two of these patients also scored negative by MAT. These two patients had positive culture results.

The assay has been evaluated as well against serogroups which are common in The Netherlands, such as Icterohaemorrhagiae, Grippotyphosa, Sejroe, and Pomona, compared with serogroups such as Autumnalis and Australis, which may more often be observed in other countries. The broad reactivity of the dipstick assay suggests that it is suitable for the detection of most if not all leptospires. However, since the evaluation of the assay did not include representative serovars of all serogroups, complete coverage cannot be claimed. For instance, one patient showed a high titer by the IgM ELISA but was negative by the dipstick assay. The causative strain, however, could not be classified for this patient.

In summary, we have developed an easy-to-perform dipstick method for use by clinical and field laboratories as a valuable addition to the armaments for the serodiagnosis of leptospirosis. The assay is performed by preparing a 1:50 dilution of serum in detection reagent, followed by incubation of the dipstick for 3 h. The highly stable reagents and the simple performance make the method suitable for use in tropical countries. The assay corresponds well with the IgM ELISA in terms of sensitivity and specificity. Application of the dipstick assay instead of the IgM ELISA is thus likely to give similar results. Performance of the dipstick assay is useful for a single serum specimen but is recommended for use with paired serum samples because besides strong staining, seroconversion or a rise in staining intensity are consistent with current leptospirosis. An internal control validates the performance of the assay, and the staining intensity of the internal control band, which usually is rated 2+, facilitates the interpretation of the assay result. The numerous practical advantages of the dipstick assay may well contribute to an improved diagnosis.

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