

INTRODUCTION OF A RAPID DIPSTICK ASSAY FOR THE DETECTION OF LEPTOSPIRA-SPECIFIC IMMUNOGLOBULIN M ANTIBODIES IN THE LABORATORY DIAGNOSIS OF LEPTOSPIROSIS IN A HOSPITAL IN MAKASSAR, INDONESIA

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Abstract. An easy, rapid and robust dipstick assay for detection of leptospira-specific immunoglobulin M (IgM) antibodies was evaluated on 403 patients admitted for hospitalization because of fever. The clinical symptoms and signs of 35 patients were consistent with leptospirosis. The final diagnosis for the remaining patients was as follows: 136 with typhoid fever, 82 with hepatitis, 74 with malaria, 48 with infections of the respiratory tract, and 20 with fever of unknown origin. The clinical diagnosis of leptospirosis was confirmed for 24 (68.6%) patients by the combined results of the microscopic agglutination test (MAT), the reference test for leptospirosis, and of IgM ELISA, a standard laboratory test for the serodiagnosis of leptospirosis. In addition, serum specimens from 8 (2.2%) patients with a final clinical diagnosis other than leptospirosis were found to be positive in MAT and/or IgM ELISA. Compared with the results of MAT and IgM ELISA a sensitivity of 91.6% and specificity of 93.6% was calculated for the dipstick assay. Most of the serum samples from the laboratory confirmed patients gave a moderate to strong staining intensity of the antigen band of the dipstick and were easy to read. The results demonstrate that the dipstick assay is convenient to use and allows the rapid and accurate confirmation of patients with clinical suspicion of leptospirosis in areas where the disease is endemic.

INTRODUCTION

Leptospirosis is caused by infection with spirochaetal microorganisms of the genus *Leptospira* of which more than 200 pathogenic strains have been described (Farr, 1995). Natural hosts of pathogenic strains, which may cause infection in man, include wild animals, livestock and pets. Urine of infected animals is the main source of infection and humans may become infected through wounded skin, mucous membranes and the conjunctivae. Exact incidence rates are not known for most regions of the world. The incidence of leptospirosis in wet tropical countries can be as high as 5-20/100,000 per year.

Leptospirosis may manifest with relatively mild flue-like symptoms or as a severe disease called Weil's syndrome and symptoms may include renal failure, liver impairment, meningitis and (lung) hemorrhages. The fatality rate of severe disease is high. The clinical diagnosis of acute leptospirosis

may easily be missed or the disease may be confused with other major infectious diseases including influenza, malaria, hepatitis, bacterial meningitis and viral hemorrhagic fever. Therefore, laboratory tests are important to make the diagnosis of leptospirosis.

Special techniques are required to come to a valid laboratory diagnosis. Definite proof of leptospirosis is obtained when leptospire are cultured from a clinical specimen. Culturing, however, may take weeks to months and hence does not contribute to an early diagnosis. Besides, the sensitivity of culture is low even during the first few days of the infection when leptospire are present in the blood. The laboratory diagnosis thus mainly depends on serology. Commonly used serological assays are the microscopic agglutination test (MAT) (Terpstra *et al*, 1980; Turner, 1968), the enzyme linked immune sorbent assay (ELISA) (Adler *et al*, 1980; Terpstra *et al*, 1985; Zichowski *et al*, 1987) and the immuno-fluorescent antibody test (IFAT) (Torten *et al*, 1966). These tests are however relatively complicated and therefore are not always available. The direct microscopic observation of leptospire in urine (leptouria test) is sometimes applied. However this test has a low speci-

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ficity since the presence of fibrin and protein in the urine samples can be mistaken for leptospires.

We recently have developed an easy and robust dipstick assay, for the quick screening of patients with clinical suspicion of leptospirosis (Gussenhoven *et al*, 1997). The assay is aimed at the detection of *Leptospira*-specific immunoglobulin M (IgM) antibodies in serum samples. The highly stable ingredients allow the use of the assay under a wide range of conditions. Evaluation of the assay under different epidemiological and clinical conditions has demonstrated a high sensitivity, specificity and predictive values (Gussenhoven *et al*, 1997; Sehgal *et al*, 1999; Smits *et al*, 1999). The aim of this study was to determine the clinical utility of the test when applied in a hospital in a tropical country where the disease is endemic.

MATERIALS AND METHODS

Sera

Single serum samples of all (n=403) patients admitted to the Department of Internal Medicine, Hasanuddin University Hospital, Makassar, Indonesia between March 1996 and February 1998 with fever of 38.5°C and with clinical suspicion of leptospirosis, typhoid fever, hepatitis, malaria or respiratory tract infection were included in this study. Of these patients 35 had a final clinical diagnosis of leptospirosis, 136 typhoid fever, 82 hepatitis, 74 malaria, 48 upper respiratory tract infection, and 20 pyrexia of unknown origin. Eight patients died before a final diagnosis could be made. A group of 194 serum samples collected from school children was taken as negative controls.

Diagnostic criteria

The final diagnosis of leptospirosis was based on clinical signs, including acute febrile illness, myalgia, conjunctival suffusion, icterus, and or hepatic disturbance, on a reported contact with animals or exposure to an environment contaminated with animal urine, and on the microscopic observation of leptospires in a urine sample. The final diagnosis of typhoid fever was based on clinical signs including fever, anorexia, lethargy, malaise, on positive results (titer O antigen $\geq 1:160$) in the widal test for 3 days continuously, and/or culture. The final diagnosis of hepatitis was based on fever with icterus, hepatomegaly, anorexia, nausea, vomiting and Hbs antigen and anti Hbs positivity. The

final diagnosis of malaria was based on clinically symptoms including periodicity of fever with rigor, sweating, anemia, enlarged spleen and on blood smear positivity. Respiratory disease was diagnosed by clinical symptoms including fever, cough, dyspnoea, X-ray and routine blood examination.

Microscopic examination of leptospires in urine

For the microscopic examination of leptospires in urine (leptouria test) 5 ml urine was centrifuged at 1,500 rpm for 5 minutes and the sediment was examined at low magnification (10 x 40).

Serological testing of leptospirosis

IgM ELISA using antigen prepared from strain Wijnberg was performed as described elsewhere according to routine diagnostic procedures (Terpstra *et al*, 1985). A titer of $\geq 1:80$ was considered consistent with leptospirosis. MAT (Terpstra *et al*, 1985) was performed using a panel of 26 pathogenic strains as antigen. A titer of $\geq 1:160$ with any strain was considered consistent with leptospirosis. The following strains were used as antigen: strain CH 11 (serovar *andamana*; serogroup Andaman), strain Ballico (serovar *australis*; serogroup Australis), strain Jez Bratislava (serovar *bratislava*; serogroup Australis), strain Rachmat (serovar *rachmati*; serogroup Autumnalis), strain Mus 127 (serovar *ballum*; serogroup Ballum), strain Swart (serovar *bataviae*; serogroup Bataviae), strain Hond Utrecht IV (serovar *canicola*; serogroup Canicola), strain Celledoni (serovar *celledoni*; serogroup Celledoni), strain 3522 C (serovar *cynopteri*; serogroup Cynopteri), strains Duyster and Mandemakers (serovar *grippotyphosa*; serogroup Grippotyphosa), strain Hebdomadis (serovar *hebdomadis*; serogroup Hebdomadis), strain Poi (serovar *poi*; serogroup Javanica), strain Sari (serovar *mini*; serogroup Mini), strain Kantorowicz (serovar *icterohaemorrhagiae*; serogroup Icterohaemorrhagiae), strain Wijnberg (serovar *copenhageni*; serogroup Icterohaemorrhagiae), strain CZ 214 K (serovar *panama*; serogroup Panama), strain Salinem (serovar *pyrogenes*; serogroup Pyrogenes), strain Pomona (serovar *pomona*; serogroup Pomona), strain 1161 U (serovar *proechimys*; serogroup Pomona), strains Hardjoprajitno and Lely 607 (serovar *hardjo*; serogroup Sejroe), strain Mus 24 (serovar *saxkoebing*; serogroup Sejroe) strain M 84 (serovar *sejroe*; serogroup Sejroe), strain 1342 K (serovar *shermani*; serogroup Shermani) and strain Perepelicin (serovar *tarassovi*; serogroup Tarassovi).

LEPTO Dipstick assay

The dipstick (Gussenhoven *et al*, 1997) contains two horizontal bands: an antigen band consisting of broadly reactive specific antigen (lower band) and an internal control consisting of anti-human IgM antibodies (upper band). The assay is based on the binding of leptospira specific IgM antibodies to the antigen. Bound IgM antibodies are specifically detected in a non-enzymatic reaction with a stabilized anti-human IgM dye conjugate. The assay is performed by making a serum dilutions (1:50) in 200 µl detection reagent and incubating a wetted dipstick in this mixture for 3 hours. At the end of the incubation the dipstick is rinsed with tap water and dried. A reddish stained antigen band indicated a positive reaction. The staining intensity of the antigen band was quantified by comparison with a colored reference strip and was scored negative (no visible staining), weak (1+), moderate (2+ to 3+) or strong (4+).

RESULTS

Of the 403 patients included in this study 35 had a final clinical diagnosis of leptospirosis. Of these 35 patients 24 (68.6%) had a positive leptouria test, 3 tested negative and 8 were not tested. Of the 368 patients with a final diagnosis other than leptospirosis 184 patients also were tested in the leptouria test and of these 11 (6%) were judged positive.

Examination in the dipstick assay of single serum samples collected from the group of 35 patients with clinical evidence of leptospirosis revealed the presence of leptospira-specific IgM antibodies in the samples from 24 (68.6%) patients. All but one of the dipstick positive samples gave a moderate (2+) to strong (4+) staining. A positive result in the dipstick assay with a $\geq 2+$ staining intensity was obtained for only 7 (1.9%) of the 368 patients with a final diagnosis other than leptospirosis. Twenty-one (5.7%) other patients of the later group stained weakly (1+). Four of the patients with a final diagnosis other than leptospirosis who gave a $\geq 2+$ staining intensity in the dipstick assay had a final diagnosis of typhoid fever of whom one was culture positive, two had a final diagnosis of hepatitis, and one patient died before a final diagnosis could be made. The samples of eight (4.1%) individuals of the healthy control group gave a 1+ staining.

For laboratory confirmation of leptospirosis by standard serological tests serum specimens were sent to the Department of Biomedical Research of the Royal Tropical Institute. Confirmatory testing was done by IgM ELISA, an assay routinely used in the serodiagnosis of leptospirosis, and by MAT, the reference test for leptospirosis, performed with a panel of 26 strains as antigen. IgM ELISA was performed on all serum specimens included in this study. MAT was performed on a selected panel of samples to confirm the results of ELISA.

The results of IgM ELISA agreed well (91.3% agreement) with the results of the dipstick assay. A discordant result for the group of patients with clinical leptospirosis was only obtained for two patients who tested positive in the dipstick and negative in ELISA. Of this group of patients thus in total 22 tested positive in ELISA and 13 negative. For the group of 368 patients with a final diagnosis other than leptospirosis a discordant result was obtained for the samples of 6 patients which tested positive with $\geq 2+$ staining intensity in the dipstick and negative in ELISA and for the sample of one patient which tested negative in the dipstick assay and positive in ELISA. Also all serum specimens which stained weakly in the dipstick assay were negative in ELISA or gave low, not significant titers ($< 1:80$). Of this group of patients thus only two patients tested positive in ELISA and 366 negative.

Confirmatory testing by MAT was done for all 35 patients with a final clinical diagnosis of leptospirosis, for the 7 patients with a final diagnosis other than leptospirosis and a positive ($\geq 2+$) result in the dipstick assay, and on the specimens from 42 randomly selected patients with a final diagnosis other than leptospirosis and a negative result in the dipstick assay. Thus in total 84 specimens were tested in MAT. A positive result in MAT was obtained for 24 patients with a clinical diagnosis of leptospirosis and 7 patients with a final diagnosis other than leptospirosis. The 24 MAT positive patients with clinical leptospirosis included all 22 patients with a positive result in ELISA and two patients with a negative result in MAT. MAT confirmed the presence of leptospira specific antibodies as determined by ELISA in the sample from only one of the two patients from the group with a final diagnosis other than leptospirosis and a positive result in IgM ELISA. The samples of 6 patients of this group which tested positive in MAT had a negative result in ELISA. Leptospira-specific antibodies thus were detected in either

MAT or IgM ELISA in 24 patients with a clinical diagnosis of leptospirosis and in 8 patients with a final diagnosis other than leptospirosis.

The results of the dipstick assay for the members of the group of patients with clinical leptospirosis and for the members of the patient group with a final diagnosis other than leptospirosis stratified according to the combined results of IgM ELISA and MAT is given in Table 1. We calculated the sensitivity of the dipstick assay based on the results obtained for the members of the group of patients with a clinical diagnosis of leptospirosis confirmed by the results of MAT and IgM ELISA. The specificity was calculated based on the results obtained for the members of the group with a final diagnosis other than leptospirosis and a negative result in MAT and IgM ELISA. The sensitivity was calculated to be 91.6%. The specificity was 93.6%. Compared with the results of MAT and IgM ELISA the results of the leptouria test indicated a sen-

sitivity of 94.1% and a specificity of 94.3% (Table 2).

DISCUSSION

In this study we validated the introduction of an easy, rapid and robust dipstick assay for the detection of leptospira-specific IgM antibodies in the diagnosis of leptospirosis. The results of MAT and IgM ELISA confirmed the clinical diagnosis of leptospirosis for 24 out of 35 patients. In addition MAT and or IgM ELISA were positive for 8 out of 368 patients with a final diagnosis other than leptospirosis. Compared with the results of MAT and IgM ELISA the dipstick assay had a sensitivity of 91.6% and a specificity of 93.3% for results with a staining intensity $\geq 1+$. The relatively high sensitivity and specificity of the dipstick assay further was illustrated by the fact that whereas most of the dipstick positive serum specimens

Table 1

Results of dipstick assay for patients with and without clinical leptospirosis stratified according to the results of IgM ELISA and MAT.

Dipstick	Patients with clinical leptospirosis		Patients with a final diagnosis other than leptospirosis	
	IgM ELISA/MAT		IgM ELISA/MAT	
	Negative	Positive	Negative	Positive
Negative	10	2	336	4
1+	1		21	
2+		3	3	1
3+		6		
4+		13		3
Total	11	24	360	8
Sensitivity		91.36%		
Specificity			93.6%	

Table 2

Results of leptouria for patients with and without clinical leptospirosis stratified according to the results of IgM ELISA and MAT.

Leptouria	Patients with clinical leptospirosis		Patients with a final diagnosis other than leptospirosis	
	IgM ELISA/MAT		IgM ELISA/MAT	
	Negative	Positive	Negative	Positive
Negative	2	1	167	6
Positive	8	16	10	1
Total	10	17	177	7

from the laboratory confirmed leptospirosis patients stained moderately to strongly ($\geq 2+$), most of the dipstick positive serum specimens from the patients with a negative result in MAT and IgM ELISA stained weakly (1+). When only considering test results with a moderate to strong staining intensity the specificity of the dipstick was calculated to be 99.2%, while the sensitivity was unaltered. By testing serum samples from laboratory confirmed leptospirosis patients and controls from The Netherlands we previously were able to calculate an overall sensitivity of 86.8% and an overall specificity of 92.7% for the dipstick assay (Gussenhoven *et al*, 1997). The sensitivity ranged from 63.0% for samples collected early in the disease to 85.7% for samples collected 10 to 30 days after the onset of the disease. These values were similar to those obtained for the IgM ELISA. These results were confirmed in a multi-center study involving 13 laboratories in 12 countries including Barbados, Hawaii, India, Kenya, New Zealand, Philippines, Puerto Rico, Russia, Seychelles, Surinam, Thailand and The Netherlands testing in total 2,665 samples from 2,057 patients with clinical suspicion of leptospirosis (Smits *et al*, 1999), and in a study performed on the Andaman Islands, India (Sehgal *et al*, 1999). In these studies we noted that the dipstick assay may give false-positive results at a weak (1+) staining intensity due to cross-reactivity with samples from patients with a number of other diseases including hepatitis, meningitis, malaria and with rheumafactor positive samples. False results also may occur when low levels of persisting antibodies against leptospire are present due to a previous exposure. This in particular may be the case in samples collected from patients living in endemic areas. False results rarely were observed at a moderate to strong staining intensity and hence the positive predictive value of a test result with a moderate to strong staining intensity was calculated to be much higher (91.2%) than the positive predictive value of a test result with a weak staining intensity (47.1%) (Smits *et al*, 1999). In the present study direct examination of urine samples by dark-field microscopy for the presence of leptospire, the leptouria test, was performed on part (n=219) of the samples only. Compared with MAT and IgM ELISA the sensitivity of the leptouria test was 94.1 and a specificity was 94.3% which is similar to that of the dipstick assay. However, compared with the dipstick assay the leptouria test gave a high number of positive results among the group of patients with clinical signs and symptoms of leptospirosis

but with a negative result in the serological tests. This result is in accord with the notion that the leptouria test may give false results due to the presence in the urine of artifacts resembling leptospire (Faine, 1994; Warren and Mahmoud, 1984). The result of the leptouria test may highly depend on the quality of the urine sample, the stage of the disease and the expertise of the microscopist.

The dipstick assay is meant as a rapid screening test for leptospirosis. A high negative predictive value is important as it reduces the risk of misdiagnosis of patients with the disease. The predictive value of a test varies with the prevalence of the disease. From the results presented in this study a negative predictive of 98.6% can be calculated at a prevalence of 8.5% of patients with a laboratory confirmed final diagnosis of leptospirosis among patients submitted with fever. We previously calculated a negative predictive of 98.0% at prevalence of 4.1% of the disease among patients with clinical suspicion of leptospirosis and of 90.8% at a prevalence of 29.5% (Smits *et al*, 1999).

Serological testing for confirmation of clinical suspicion of leptospirosis as for any other disease ideally should be performed on paired serum samples as seroconversion or a rise in titer provides strong evidence of the disease. Unfortunately only single serum samples were available in the present study due to their collection in routine clinical procedures. It is thus possible that seroconversion could have provided evidence of leptospirosis for some of the patients with clinical suspicion of leptospirosis which failed to give a positive result by testing just a single specimen.

Leptospirosis may be caused by a large number of strains, which are grouped in different serovars and serogroups (Kmetz and Dikken, 1993). Application of MAT sometimes allows the characterization of the infecting strain at the serovar level. Genus specific assays such as IgM ELISA and the dipstick assay, which utilize broadly reactive antigen, do not provide information regarding the serovar. Knowledge of the serovar is not important for treatment but may be important for epidemiological investigation and tracing of possible sources of infection. The result of the present study confirms the broad reactivity of the dipstick assay. In this study most samples from the laboratory confirmed leptospirosis patients showed cross-reactivity in MAT with two or more strains from different serovars. The results thus did not allow

typing of the serovar involved. Cross-reactivity was observed with strains belonging to the serovars *andamana*, *australis*, *bataviae*, *celledoni*, *cynopteri*, *grippoty-phosa*, *hebdomadis*, *icterohaemorrhagiae*, *javanico*, *pyrogenis*, *sejroe*, *shermani*, and *tarassovi*. Only samples of three patients gave reacted predominantly with a single strain, eg strains of the serovars *bataviae*, *icterohaemorrhagiae* and *mini*. In conclusions, the present study demonstrates that the dipstick is a rapid and convenient assay for the accurate screening for leptospirosis patients among suspects. A test results with a moderate to strong staining (2+ to 4+) was highly consistent with leptospirosis. Results obtained with the dipstick were concordant (92.3% agreement) with those of MAT and IgM ELISA, tests routinely used in the serodiagnosis of leptospirosis. The assay is quick and simple to perform and can be applied in the field without special equipment or need for refrigeration.

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