A PROSPECTIVE EVALUATION OF FOUR IMMUNODIAGNOTIC ASSAYS FOR HUMAN LEPTOSPIROSIS

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Abstract. This was a prospective evaluation of four immunodiagnostic assays for human leptospirosis, including the indirect immunofluorescence test (IFA), the microscopic agglutination test (MAT), the LEPTO dipstick, and the latex agglutination (LA) test. Four hundred ninety-two serum samples were collected from 348 patients who presented with acute fever without localizing signs. The sensitivities of the IFA, MAT, Dipstick, and LA were 91.9, 76.6, 77.4, and 83.1%, respectively. The specificities of these assays were 100.0, 100.0, 89.3, and 83.5, respectively. Even though IFA showed the highest overall sensitivity and specificity, when acute sera were considered, the LA was the most sensitive (28.7%). All 3 genus specific antibody assays had broad reactivity against various serogroups. The MAT is best suited for the reference laboratory, where it can be maintained with the battery of live antigens; the IFA is suited for a laboratory with sophisticated equipment and technical expertise; the Dipstick and LA are suitable for peripheral laboratories which lack expensive equipment and expertise.

INTRODUCTION

Laboratory diagnosis of human leptospirosis relies on either the isolation of the etiologic organism from the specimens or the demonstration of a rise in specific serum antibodies (Winslow *et al*, 1997). Isolation is laborious, time-consuming, expensive, and low sensitivity (Jeandel *et al*,1984; Levette and Whittington, 1998). Direct demonstration of leptospires in preparations from clinical specimens by dark-field microscopy, direct immunofluorescence and immunoperoxidase staining have been hampered by lack of specificity, due to the nonspecific background (Saengjaruk *et al*, 2002). The microscopic agglutination test (MAT) is a serological reference method for the diagnosis and detection of antibodies specific for the infecting serovar and serogroup (Levette, 2001). This test is time-consuming, requires technical expertise and requires maintaining a stock of live cultures, creating a risk for laboratory-acquired infection (Effler *et al*, 2000). The MAT is rarely performed for routine diagnosis. It remains useful for epidemiological investigations.

Several alternative serological methods for the early diagnosis of leptospirosis have been described, including the slide agglutination assay (Galton *et al*, 1958), the indirect hemagglutination assay (Levette and Whittington, 1998), the indirect immunofluorescence test (Appassakij *et al*, 1995), the indirect enzyme-linked immunosorbent assay (ELISA) for immunoglobulin M (IgM) antibodies (Winslow *et al*, 1997), the latex agglutination test (Smits *et al*, 2000), and the LEPTO dipstick (Gussenhoven *et al*, 1997). These methods detect genus-specific antibodies and many of them are currently available commercially.

Our study prospectively evaluated a locally developed latex agglutination (LA) test by the National Institute of Health (Thailand), a commercially available LEPTO dipstick, an indirect immunofluorescence test (IFA) and the microscopic agglutination test (MAT).

MATERIALS AND METHODS

Study population

The study was conducted at Songklanagarind Hospital, Songkhla, Thailand, a university hospital, from 1 to 31 December 2000. A to-

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tal of 492 serum samples from 348 patients (127 pairs and 221 single samples) were collected prospectively from patients who presented with acute fever >38ºC for more than 1 day but not exceeding 3 weeks. Exclusion criteria were the presence of profuse rhinorrhea, exudative pharyngitis, pneumonia, urethritis, and diarrhea.

Diagnostic criteria for leptospirosis

Due to the lack of sensitivity with the MAT, from it's slow antibody response, and is limited by slow response of antibody, the need for getting convalescent serum, along with the fact that the IFA has a higher sensitivity with a low false positive rate (Appassakij *et al*, 1995; Naigowit *et al*, 2001), we decided to use as a definitive diagnosis a positive result with either the MAT or the IFA test (a titer of ≥ 1:400 in a single serum or a four-fold increase in the antibody titer in paired serum for both tests).

Microscopic agglutination test (MAT)

The MAT followed a modified Galton technique (Galton *et al*, 1965). All twenty-three serovars of *Leptospira interrogans* were used as an antigen: Australis, Ballico, Bratislava, Akayami, Rachamati, Bataviae, Canicola, Cellidoni, Djasiman, Grippotyphosa, Hebdomadis, Hyos, Tarasovi, Icterohemorrhagiae, Copenhageni, Javanica, Saigon, Pomona, Pyrogenes, Sejroe, Hardjo, Wolffi, and Andamana. Twenty-five μl of serum diluted to 1:50 with phosphate buffer saline (PBS) was added into 23 wells of a microtiter plate. Twenty-five μl of each live leptospires serovar was added to each well. The specimen was mixed gently. After leaving for 2-3 hours at room temperature, 3 μl of the suspension was dropped on a slide. Agglutination was observed under a dark field microscope (OLYMPUS model BH-2) at a final magnification of 100x. Any serum specimen with a positive reaction was then retested against the respective serovar to determine the endpoint titer which gave the highest dilution giving more than 50% agglutination of the leptospires. The serovar giving the highest titer was considered to be the infecting serovar.

Indirect immunofluorescence assay (IFA)

The IFA was performed using a modified Appassakij *et al's* method (1995). Normal yolk sac was added to the 5-7 days culture of *L. interrogans* serovar Bataviae in neopeptone medium to reach a final concentration of 0.5%. The antigen was dotted into the wells of a clean taflon-coated slide and air-dried at room temperature. The slide was then fixed in acetone for 10 minutes, left to air-dry and stored at -70ºC until used.

Ten μl of diluted serum (starting at 1:100) was added to the slide and incubated at 37ºC in a moist chamber for 30 minutes. The slide was washed with phosphate buffer saline pH 7.2 three times, then rinsed once with distilled water and air-dried. Ten μl of optimal dilution fluorescein isothiocyanate (FITC) conjugated rabbit anti-human immunoglobulin (IgG, IgM, IgA) (Dako, Code F200, Denmark) was placed on the slide and incubated at 37ºC in a moist chamber for 30 minutes. The slide was washed with phosphate buffer saline as previously done. The slide was examined under a fluorescent microscope (OLYMPUS model BH-2 with FITC filter and exciter filters at a 400x magnification). All positive sera were further diluted and final titers were determined. The endpoint titer was the highest serum dilution giving a visible fluorescence of the leptospires. Positive and negative reference sera were included in every batch tested.

Latex agglutination test (LA)

The LA test was developed locally by the National Institute of Health, Ministry of Public Health, Thailand as described by Naigowit *et al* (2001). Twenty μl of antigen-coated latex particles was mixed with 20 μl of undiluted serum on a clean black glass slide. The slide was rotated at 100-120 rpm for 5 minutes and then read for the aggregation of latex particles. Agglutination was observed by 2 independent readers in order to avoid subjective results. Positive and negative controls were included in every batch tested.

LEPTO dipstick assay

The LEPTO dipstick assay (Gussenhoven *et al*, 1997) was manufactured by the Royal Tropical Institute (KIT), The Netherlands. The assay was used to detect leptospira-specific IgM antibodies in human sera. To perform the test, 5 μl of serum was added to 250 μl of detection reagent in a reaction vial. The test strip was pre-wetted with dipstick fluid and then placed into the serum mixture for 3 hours at room temperature.

Table 1
Evaluation of four immunodiagnostic assays for human leptospirosis.

Statistic values	IFA	MAT	Dipstick	LA
Sensitivity (%)	91.9	76.6	77.4	83.1
Specificity (%)	100.0	100.0	89.3	83.5
False positive rate (%)	0.0	0.0	10.7	16.5
False negative rate (%)	8.1	23.4	22.6	16.9
Positive predictive value (%)	100.0	100.0	80.0	73.6
Negative predictive value (%)	95.7	88.5	87.7	89.9
Accuracy (%)	97.1	91.7	85.1	83.3
Kappa value	0.94	0.81	0.67	0.65

Table 2
Sensitivity of four immunodiagnostic assays for human leptospirosis using acute sera.

Assays	Sensitivity (No. of positive patients/ Total no. of patients)
IFA	33.1% (41/124)
MAT	25.8% (32/124)
Dipstick	50.0% (62/124)
LA	62.1% (77/124)

After rinsing with distilled water. The strip was air-dried. A reddish-stained antigen band indicated a positive reaction. The strip was read by 2 independent readers. The result was recorded as 1+, 2+, 3+, 4+, based on the intensity of color of the antigen band. The intensity of ≥ 2+ was considered a positive result.

Statistical analysis

The sensitivity, specificity, false positive rate, false negative rate, positive predictive value, and negative predictive value of the assays were calculated according to standard methods (Griner *et al*, 1983). The kappa statistics for agreement between the evaluated test and standard diagnostic criteria were determined using Statistical Packages for the Social Science release 9.05 (SPSS, Chicago, USA)

RESULTS

Four hundred ninety-two serum samples from 348 patients were evaluated for leptospiral antibody using the IFA, the MAT, the Dipstick and the LA tests. One hundred twenty-four patients were considered to have leptospirosis by the criteria stated previously. The results of the assays are summarized in Table 1. Regarding acute sera, the sensitivities of the different assays is shown in Table 2. Ninety-five infecting serovars of *Leptospira interrogans* by the MAT were analysed to determine the sensitivity of the tests against the various serogroups. The results are shown in Table 3.

DISCUSSION

A rapid, accurat, and reliable method for the diagnosis of human leptospirosis is important to both clinicians and patients. Among the four comparative assays, the IFA showed the highest sensitivity at 91.9%, and excellent specificity at 100%. This data confirms that the IFA test is suitable for the detection of leptospiral antibodies in a routine clinical laboratory. The test has some limitations, requiring a fluorescent microscope and technical expertise. Since the MAT was the reference method for the diagnosis of acute leptospirosis, it had a specificity of 100% and a sensitivity of 76.6%. This test is not suited for a routine laboratory because it requirs a battery of live antigens and is time-consuming. The other two alternative assays showed good sensitivity and specificity. Although the Dipstick was more specific than the LA, the LA was slightly more sensitive than the Dipstick. The sensitivity and specificity of the Dipstick were 77.4% and 89.3%, respectively, similar to Sehgal *et al*'s report (1999) which were 78.7% and 88.3%, respectively. The

Table 3
Sensitivity of four immunodiagnostic assays against leptospiral serogroups.

Serogroups	Sensitivity (No. of positive patients/Total no. of patients)			
	IFA	MAT	Dipstick	LA
bataviae	88% (66/75)	100% (75/75)	81.3% (61/75)	89.3% (67/75)
bratislava	83.3% (5/6)	100% (6/6)	66.7% (4/6)	50% (3/6)
hebdomadis	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)
hyos	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)
javanica	100% (4/4)	100% (4/4)	50% (2/4)	100% (4/4)
pomona	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)
pyrogenes	100% (3/3)	100% (3/3)	100% (3/3)	66.7% (2/3)
saigon	100% (1/1)	100% (1/1)	100% (1/1)	100% (1/1)
Undefined[a]	100% (3/3)	100% (3/3)	66.7% (2/3)	66.7% (2/3)
Total	89.5% (85/95)	100% (95/95)	80% (76/95)	86.3% (82/95)

[a]More than one equal titer shown.

Dipstick is simple to perform, does not require special equipment or refrigeration, and is completed within 3 hours. The kappa value for agreement between the two independent observers was 0.79 (data not shown). There was not much difference in reading the strip. However, the kappa value between unskilled and skilled experimenters was 0.4 (data not shown). This indicates that training is needed to perform the Dipstick for reliable results. The advantage of the LA is that it is extremely simple to perform, not requiring any sophisticated laboratory equipment. The LA gives a result within five minutes, compare to one and a half hours for the IFA or three hours for the Dipstick. In this study, the sensitivity and specificity of the LA were 83.1% and 83.5%, respectively, slightly lower than previous reports that showed a sensitivity of 94.7% and a specificity of 93.3% (Naigowit *et al*, 2001). This may be the result of different infecting serogroups. *L. interrogans* serovar Pyrogenes was used as the antigen in the LA assay, but serovars Bataviae and Bratislava are more common in southern Thailand (Sundharagiati, 1968; Pradutkanchana *et al*, 2002) and serovar Pyrogenes is more common in north-eastern Thailand (Montian-arsana *et al*, 1997).

Leptospiral antibodies usually appear within a few day of the onset of clinical symptoms and persist in detectable quantities for many months (Silva *et al*, 1997). Early recognition of the disease is very important for initiating appropriate treatment to avoid severe complications. When acute sera were compared, the LA was the most sensitive, the Dipstick was less sensitive than the LA, and the sensitivities for the IFA and the MAT were lower, at 33.1% and 25.8%, respectively. The insensitivity of all four assays in acute sera is probably because the patients present to the hospital soon after they have fever. The average duration of fever at presentation in the patients of this study was 4 days. This is too soon for antibodies to appear.

The three genus specific assays detected infection with the serogroups bataviae, bratislava, hebdomadis, hyos, javanica, pomona, pyrogenes and saigon, showing that these assays have broad reactivity. The results observed with the other serogroups are not specific due to insufficient numbers. Since the assays did not test against the full range of serogroups, further studies are required to determine the sensitivity of the assays against the other serogroups. Information about the infecting serogroup cannot be obtained from genus-specific assays. Knowledge of the serogroup has no clinical implications, and is mainly of epidemiological interest.

In summary, we evaluated four immunodiagnostic assays for human leptospirosis. Although the IFA was the most sensitive and specific, the need for a fluorescent microscope and technical expertise limits the use of this assay. Both the Dipstick and the LA are highly sensitive and specific. Neither requires specialized equipment,

and can be performed in peripheral laboratories. The LA is rapid and extremely easy to perform while the Dipstick has highly stable reagents. Both are suitable for use in diagnostic laboratories.

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