

TWO SIMPLE IMMUNOASSAYS USING ENDEMIC LEPTOSPIRAL ANTIGENS FOR SERODIAGNOSIS OF HUMAN LEPTOSPIROSIS

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Abstract. Two simple enzyme immunoassays, a conventional microplate and dot-ELISA, were developed to detect specific IgM antibodies using pool sonicated antigen prepared from three of the most reactive serovars of *Leptospira* associated with disease in Thailand. Both assays were evaluated and compared with the standard microscopic agglutination test (MAT) performed with 343 serum samples. A battery of 16 pathogenic serovars of *L. interrogans* were used as antigens in the MAT assay. The result of MAT at serum titers $\geq 1:400$ showed three pathogenic serovars of *leptospira*, Bratislava (71.88%), Sejroe (63.54%) and Pyrogenes (36.46%), were among the most commonly reacted serovars and they were selected for preparation of pool sonicated antigen for both IgM ELISA tests. The microplate IgM-ELISA, performed with sera at 1:80 dilution using the cutoff OD of 0.60, demonstrated sensitivity, specificity and efficiency of 87.50, 97.57, and 94.75%, respectively. The same values for IgM dot-ELISA performed with sera at 1:160 dilution were 98.96, 93.93, and 95.33%, respectively. Both ELISA methods showed results with statistically significant differences from MAT ($p < 0.05$). The agreement rate of IgM dot-ELISA compared with microplate IgM-ELISA was 0.85 by Kappa analysis. Both assays offered relatively high negative predictive values (95.26-99.57%), thus making the assays ideally suited for rapid screening. Future applications of the IgM dot-ELISA as a test kit would be suitable for use at the peripheral level as a rapid screening test for human leptospirosis.

INTRODUCTION

Leptospirosis is a reemerging zoonosis caused by pathogenic spirochetes of the genus *Leptospira*. Although traditionally considered an occupational risk among persons exposed to infected animal urine or contaminated water (Faine *et al*, 1999), leptospirosis is becoming an important cause of febrile illness in tropical environments worldwide (Farr, 1995). Resurgent interest in leptospirosis has resulted from large outbreaks associated with recreational exposures (CDC, 1998, 2000), and epidemics of leptospirosis-associated severe pulmonary hemorrhage syndrome (Zaki *et al*, 1996; Yersin *et al*, 2000). In Thailand, cases of leptospirosis were

rarely diagnosed. During the period 1972-1981, which is the first 10 years of the National Disease Surveillance, only 10-20 cases were reported annually. An outbreak in Thailand started in 1996, when a total of 398 cases were reported to the Public Health Ministry of Thailand; the majority were reported from the northeastern region of the country. Average annual reported cases in 1996-2001 ranged between 398-11,309 (Division of Epidemiology, 1996-2001).

Due to the complexity of the clinical symptoms and signs, which vary greatly from a mild flu-like form to lethal pulmonary hemorrhage or hepatorenal failure (Levett, 2001), rapid and appropriate laboratory diagnostic tests are needed to aid clinical case identification and to facilitate the implementation of rapid outbreak investigations for prompt and proper treatment. To date, serological testing has been most frequently used to confirm the clinical diagnosis and to conduct epidemiological studies. The standard

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serologic test, the microscopic agglutination test (MAT), is specific and provides useful epidemiologic data in the form of presumptive serogroup (Cumberland *et al*, 1999). However, this assay is inadequate for rapid case identification, since it is technically demanding, costly, and requires the maintenance of live, hazardous stock serovar cultures; it also requires analyses of paired sera to verify the seroconversion, which delays diagnosis (Faine *et al*, 1999). To overcome this problem, some potentially useful screening tests for use in all routine laboratories have been proposed. Among these serologic approaches, ELISA for both IgG and IgM-leptospiral antibodies have been developed using a number of different antigen preparations and assay protocols (Adler *et al*, 1980; Pappas *et al*, 1985).

IgM ELISA-reactive antibodies appear a day or so earlier than those detected by MAT; therefore, the demonstration of specific IgM can help in the rapid diagnosis of disease. This is particularly important in areas where leptospirosis is widespread and residual antibodies of past infection are found in a large part of the population (Adler *et al*, 1980). Though several commercial ELISA test kits are available for diagnosing systemic leptospirosis, most use broadly reactive *Leptospira* antigen prepared from nonpathogenic *L. biflexa* serovar Patoc, which may affect the sensitivity of testing in some regions where different leptospiral serovars predominate, which do not induce antibodies that crossreact with serovar Patoc (Vinetz, 2001). In this study, two enzyme immunoassays, a conventional microplate and dot-ELISA were developed to detect specific IgM antibodies using broadly reactive Leptospiral antigens prepared from predominant reactive serovars currently in endemic areas. Both assays were evaluated and compared with the standard MAT for serodiagnosis of acute leptospirosis.

MATERIALS AND METHODS

Leptospiral serovars

A battery of 16 pathogenic serovars, representing 11 serogroups of *L. interrogans*, were used as antigens in the MAT assay. Of these different serovars, 15 represented the locally circulating strains causing disease, *ie* Sejroe, Wolffi, Bratislava, Bangkoki, Ballico, Pyrogenes, Icterohaemorrhagiae, Bataviae, Hebdomadis,

Javanica, Poi, Pomona, Canicola, Akiyami, and Hyos; and one serovar Autumnalis was a recent isolate from rodents in Nakhon Ratchasima Province in 1998. Leptospire were grown in EMJH medium (Difco) and incubated for 5-7 days at 30°C. Fresh subcultures were made by inoculating 0.5 ml from the last transfer of each serovar into 2 tubes. At the same time, a loopful of the culture was examined by darkfield microscopy to confirm the presence of viable leptospire and the absence of contamination.

Serum samples

A total of 343 serum samples were studied, including case group sera from patients with leptospirosis, and control sera from healthy individuals and patients with other diseases. Serum samples from the acute phase of leptospirosis were randomly selected from a bank of samples from cases identified at Maharat Nakhon Ratchasima Hospital during active hospital-based surveillance from 1998-1999 for rural epidemics of leptospirosis in northeastern Thai villages (Tangkanakul *et al*, 1999, 2000). They were collected from 96 patients (50 males and 46 females) aged 12-50 years (mean age 30 years) during hospital admission, a median of 7 days (range 3 to 14 days) after the onset of symptoms. They were judged to be positive by MAT at serum titer \geq 1:400. Control serum samples were obtained from 192 apparently healthy individuals (both endemic and nonendemic areas) with no clinical or epidemiological history of leptospirosis, and from 55 patients with diseases other than leptospirosis, *ie* syphilis (8), hemorrhagic fever (8), hepatitis B and C (20), scrub typhus (10), and murine typhus (9). None of these 247 individuals reacted in the MAT at serum titer \geq 1:400.

Microscopic agglutination test (MAT)

The test was performed using the microtechnique described by Cole *et al* (1973) with a panel of 16 serovars of *L. interrogans* as live antigens. Briefly, an optimal dilution of antigen was mixed with serum dilution, 50 μ l each, in microtiter plate wells (Nunc) and incubated for 2 hours at room temperature in the dark, then examined for agglutination using a dark-field microscope. Each serum was first screened at 1:100 dilution in PBS. Sera showing positive reactions were then retested against the respective serovars to determine the endpoint titer,

which was the highest dilution giving $\geq 50\%$ agglutination. Patients with a titer $\geq 1:400$ were considered leptospirosis case patients in this study.

Preparation of sonicated antigen

Sonicated antigen was obtained from cultures of three serovars, *ie* Bratislava, Sejroe and Pyrogenes, because they are among the most reactive serovars used in the MAT in this study. The antigen was used as a pool and prepared as follows. The organism was cultivated in EMJH medium (Difco). One-liter flasks containing 500 ml of the medium were inoculated with 50 ml of fresh culture containing 10^7 - 10^8 cells/ml and incubated at 30°C with shaking for 7 days to yield a cell density of about 10^8 cells/ml. The organisms were killed with 0.5 mg/l sodium azide for 30 minutes. After being frozen at -20°C for 7 days, they were centrifuged at $10,000g$ for 30 minutes at 4°C . The pellet was washed twice with 0.01 M PBS (pH 7.2) and resuspended to 25% of the original volume in PBS. After disruption by sonication at 20 kHz for 3 periods of 3 minutes each, the three sonicated leptospiral suspensions were mixed in equal proportions of protein contents (w/v; pool antigen), determined according to Bradford (1976). Pool sonicated antigen was stored at -20°C in small aliquots until used.

Microplate IgM ELISA

The IgM ELISA was optimized in a flat-bottomed polystyrene microplate (Polysorb, Nunc, USA) by varying the concentrations of pool sonicated antigen (ranging from 0.125 to 6.0 $\mu\text{g/ml}$), and anti-human IgM-HRP conjugate (1:2,500-1:10,000). Optimization was performed by checkerboard titration with 6 positive and 7 negative control sera serially 2-fold diluted at 1:10-1:5,120. After many trials with various conditions, the following conditions were found to be optimal. The ELISA plates were coated with 100 μl of pool antigen (2 $\mu\text{g protein ml}^{-1}$) in coating buffer (0.05 M carbonate buffer, pH 9.6) and incubated for 2 hours in a moist chamber at 37°C , then washed three times with PBS buffer (0.02 M phosphate, 0.13 M NaCl, pH 7.2) and blocked with 100 μl of PBS-0.05% Tween 20 (PBS-T) containing 1% BSA for 1 hour at 37°C , and washed five times with PBS-T. After the wash, 100 μl of each serum dilution (serially di-

luted with PBS containing 1% BSA) were added, and the plates were incubated in a moist chamber at 37°C for 1 hour. After washing five times with PBS-T, 100 μl of goat anti-human IgM (μ -chain specific) horseradish peroxidase (HRP) conjugate (Sigma), diluted to 1:7,500 in PBS (pH 7.2) containing 1% BSA, were added. The plates were incubated for 1 hour at 37°C . After five washes with PBS-T, 50 μl of O-phenylenediamine dihydrochloride (OPD, Sigma), 4 mg/10 ml of 0.1 M citrate phosphate buffer pH 5 plus 20 μl of 3% H_2O_2 , was added, and the plates were incubated for 30 minutes at 37°C . The reaction was stopped by adding 25 μl of 2M H_2SO_4 to each well, and the A_{490} was measured in a Biorad ELISA reader. The titer was the last dilution giving an absorbance of more than half the value of a positive control serum diluted to 1:10.

All sera from patients with leptospirosis and those from the control group were tested in duplicate on each of two different plates, and all testing was performed under code. The same positive and negative control sera were tested simultaneously with all unknown samples. To determine a cutoff point, 192 sera from healthy individuals with no clinical or epidemiological history of leptospirosis were tested. The OD mean plus two standard deviations was used to clarify the lowest level at which specific IgM was considered to be present.

IgM dot-ELISA

Optimization was performed by checkerboard titration with varying concentrations of the antigenic pool (0.125-6.0 $\mu\text{g/ml}$), and anti-human IgM-HRP conjugate (1:2,500-1:10,000). The dot-ELISA was tested with 4 positive and 4 negative standard sera serially diluted with PBS containing 1% BSA at 1:10-1:640, and the following conditions were found to be optimal. Antigen dots were prepared by dispensing 100 μl of 3 $\mu\text{g/ml}$ of the antigenic pool diluted in 0.05 M carbonate buffer (pH 9.6), onto nitrocellulose (NC) membrane (Hybond-C extra, Amersham) by using a dot blot apparatus (Hybridot Manifold, Life Technology, USA). After incubation at 37°C for 30 minutes, the membrane was washed with PBS, and the remaining reactive sites were blocked with 100 μl of blocking buffer (PBS-0.1% Tween 20, pH 7.2 containing 5% BSA) for 30 minutes at room temperature with gentle shak-

ing. Then, 100 µl of each serum sample (dilution of 1:160) were added to antigen coated membrane and incubated for 30 minutes at room temperature with gentle shaking. The membrane was removed from the apparatus, and washed with PBS-T for 15 minutes, 2 times. After blocking in blocking buffer for 15 minutes, 10 ml of anti-human IgM-HRP conjugate diluted in blocking buffer were added at a dilution of 1:2,500. After incubation for 15 minutes at room temperature, the membrane was washed two times with PBS-T for 15 minutes, and then soaked in freshly prepared chromogen solution containing 3-amino-9-ethyl carbazole (AEC, Sigma), prepared by mixing 1 ml AEC (0.1 g in 25 ml DMF) plus 10 µl of 3% (vol/vol) H₂O₂. The NC membrane was incubated for 15 minutes at room temperature with gentle shaking, and the reaction was stopped by rinsing the membrane with PBS. Positive results were indicated visually by the development of the distinct red-pink on spotted sites, whereas negative reactions showed no color on spotted sites. The dot-ELISA was performed with all 343 serum samples (diluted 1:160) under the optimal condition set. Positive and negative control sera were tested simultaneously with all unknown samples.

Statistical analysis

The validities of the conventional IgM ELISA and IgM dot-ELISA were determined by using the results of MAT as the 'gold standard'. Statistical comparison was performed by using *t*-test analysis; *p* < 0.05 was considered significant. The agreement rate of both ELISA assays was determined by Kappa analysis.

RESULTS

Number and percentage of sera at MAT titers ≥1:400 regarding to leptospiral serovars

None of the 247 samples from the control group reacted to MAT at serum titer 1:100, and 96 samples from the patient group with leptospirosis reacted to MAT at serum titers ≥ 1:400 with more than 1 serovar (2 to 4 serovars). Among 16 pathogenic serovars tested, three were recorded as the most commonly positive reacting by MAT; *ie* Bratislava in 69 samples (71.88%), Sejroe in 61 samples (63.54%) and Pyrogenes in 35 samples (36.46%), while other serovars were less detected (Table 1).

Table 1

Number and percentage of serum samples from the patient group with leptospirosis (n=96) showing positive results to MAT at serum titers ≥ 1:400 regarding to leptospiral serovars.

Leptospiral serovars	Number of samples (%) showing positive results with serovars tested
Bratislava	69 (71.88)
Sejroe	61 (63.54)
Pyrogenes	35 (36.46)
Balico	26 (27.08)
Bangkoki	17 (17.71)
Hebdomadis	11 (11.46)
Javanica	3 (3.13)
Pomona	3 (3.13)

Standardization of IgM-ELISA (microplate format)

Among various concentrations tested, 1 and 2 µg/ml were the two lowest antigen concentrations that gave a clear cut between positive- and negative-group sera. The reciprocals of IgM titer in the positive-group sera ranged from 160 to 2,560 with a geometric mean (GM) titer of 640 at 1 µg/ml; and from 160 to 5,120 (GM titer; 1,140) at 2 µg/ml; while those in the negative-group sera ranged from 10 to 40 (GM titer; 16) at 1 µg/ml; from 20 to 40 (GM titer; 26) at 2 µg/ml antigen concentration (data not shown). Fig 1 shows the results of IgM ELISA at different serum dilutions and pool sonicated antigen concentrations. Results are shown for assays with three selected serum dilutions, 1:40, 1:80, and 1:160. For each point, a mean absorbance value at OD₄₉₀ was plotted for sera from six patients with confirmed leptospirosis and seven healthy controls.

From the results, the use of the antigenic-pool at either 1 or 2 µg/ml distinguish most clearly the positive- and negative-group sera. However, the positive-group sera mostly gave higher titers tested at 2 µg/ml than those tested at 1 µg/ml (GM titers; 1,140 vs 640), while the titers in the negative-group sera were not much different when tested at either 2 or 1 µg/ml (GM titers; 26 vs 16). Therefore, the 2 µg/ml antigenic-pool was selected for all subsequent testing; the result also indicated an optimal conjugate at

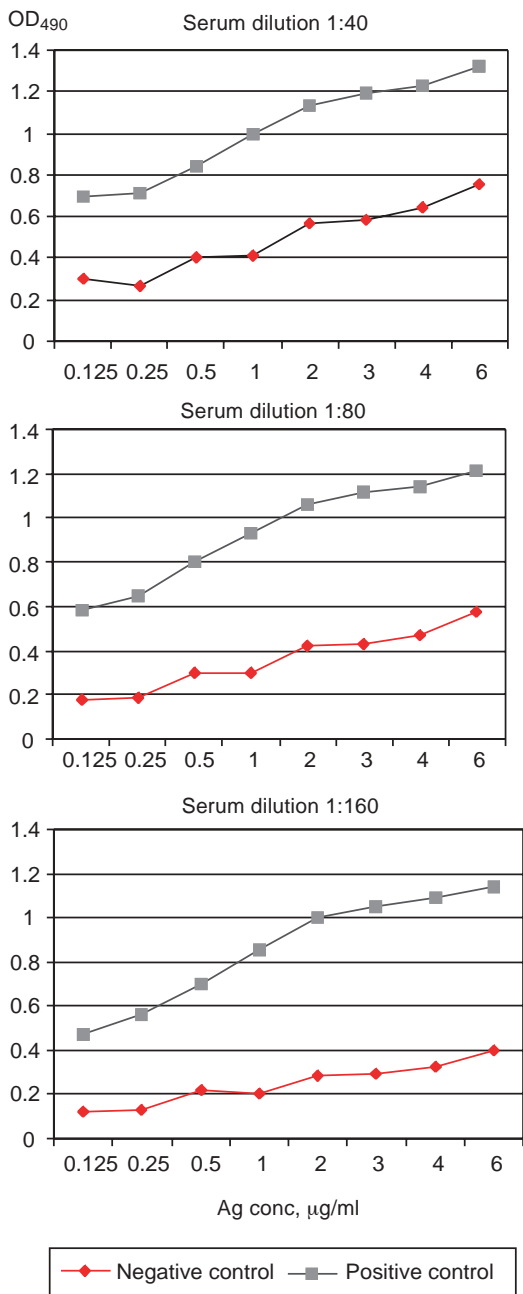


Fig 1—Performance of IgM ELISA at different serum sample dilutions and pool sonicated antigen concentrations. Results are shown for assays with three selected serum sample dilutions 1:40, 1:80 and 1:160. For each point, a mean absorbance value at OD₄₉₀ (on the vertical axis) were plotted for serum sample from six patients with confirmed leptospirosis and seven healthy control individuals. Reactions for wells were coated with 0.125, 0.25, 0.5, 1, 2, 3, 4 and 6 µg/ml of pool sonicated antigen.

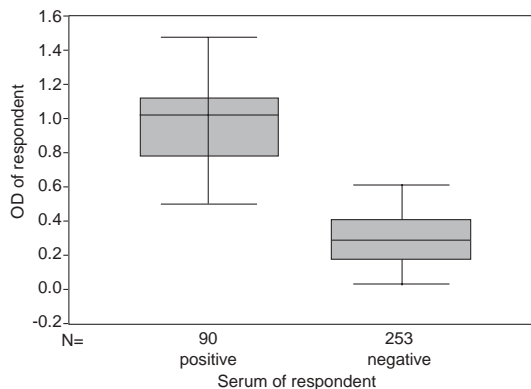


Fig 2—Box plot of the optical densities (OD) between the positive group sera and the negative group sera from 343 serum samples as determined by the IgM-ELISA using the cutoff OD of 0.60.

1:7,500 dilution and an incubation time of 30 minutes with substrate before stopping the enzymatic reaction gave the best result for use in IgM ELISA testing with all 343 sera.

The IgM titers in the positive-group sera (n=96) ranged from 40 to $\geq 10,240$, and in the negative normal healthy control (n=192) from < 10 to 160, and other diseases sera (n=55) from < 10 to 80. The cutoff titer was determined among the negative-control group with nondisease which showed a GM titer + 2SD of 72. So, the cutoff reciprocal titer of 80 was chosen. At this cutoff titer, 91 of 96 (95%) of positive cases showed true-positive, with 5% false-negative; while the negative healthy control group showed 93% true-negative and 7% false-positive; and the negative other disease group showed 95% true-negative and 5% false positive. At this 1:80 serum dilution, the mean ODs from the sera of the healthy individuals were calculated; and the mean plus two standard deviations was 0.60. This cutoff OD of 0.60 was chosen to clarify the lowest level at which specific IgM was considered to be present.

Of the 96 positive MAT samples, 84 (87.50%) samples had OD values higher than the cutoff point, mean \pm SD = 0.93 ± 0.25 . The maximum OD was 1.406 and the minimum OD was 0.389. Of the 192 negative-group sera from healthy individuals, 5 (2.60%) had OD values higher than the cutoff point, mean \pm SD = 0.30 ± 0.15 . The maximum OD was 0.63 and the minimum OD was 0.047. Of the 55 negative-group sera from patients with other diseases, one se-

rum sample (1.82%) had OD values higher than the cutoff point, mean \pm SD = 0.25 \pm 0.10. The maximum OD was 0.590 and the minimum 0.111. For all 343 samples tested by the IgM ELISA using the cutoff OD of 0.60 (Fig 2), 90 samples (84 MAT-positive and 6 MAT-negative samples) had OD values higher than the cutoff point and were considered to be positive by IgM ELISA, whereas 253 samples (12 MAT-positive and 241 MAT-negative) had OD values lower than the cutoff point and were considered negative by IgM ELISA.

Standardization of IgM dot-ELISA

Of various antigen concentrations tested against one known positive- and one negative-standard serum, serially diluted 1:20 to 1:640 (Fig 3), the low concentration (0.25 μ g/ml) could not discriminate between the two sera, while at concentrations of 0.5-1.0 μ g/ml, the reaction clearly discriminated, but the reaction of the positive serum was not strong, whereas using higher concentrations of 2-8 μ g/ml, the reactions more clearly discriminated between the positive and negative sera, especially at 8 μ g/ml. However, for economic reasons, only antigen concentrations of 2-4 μ g/ml were retested and the results showed both 3 and 4 μ g/ml gave a clear cut between the two sera. In this study, 3 μ g/ml antigen concentration was selected as the best result for all subsequent optimization of conjugate dilutions. The result showed a dilution of conjugate at 1:2,500 gave a strong reaction and discriminated clearly between the positive and negative standard sera tested (Fig 4).

The cutoff serum dilution was determined with 12 positive- and 12 negative-standard sera, serially diluted at 1:10-1:10,240 (data not shown). The reactions at 1:10-1:40 were little different between the two sera groups, while those higher than 1:640 could discriminate more clearly; however, those dilutions were not suitable for sera with weakly reactive or low titers. At 1:320 serum dilution, 8 of 12 positive sera gave strong color reactions, while 4 of 12 positive sera gave weak reactions (faint color); all 12 negative sera tested were nonreactive. While dilutions at either 1:80 or 1:160 were not different, all positive sera gave strong color reactions and all negative sera were nonreactive. In this study, the cutoff dilution, of 1:160, was chosen because a small volume of sample could be

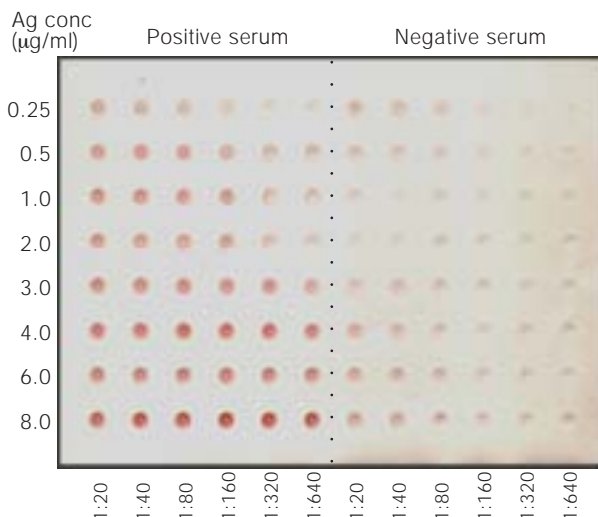


Fig 3—Nitrocellulose membrane showing the result for optimization of antigen concentration for IgM dot-ELISA of leptospirosis at the condition of 0.25 - 8.0 μ g/ml pool antigen concentrations and 1:20 - 1:640 of serum dilution.

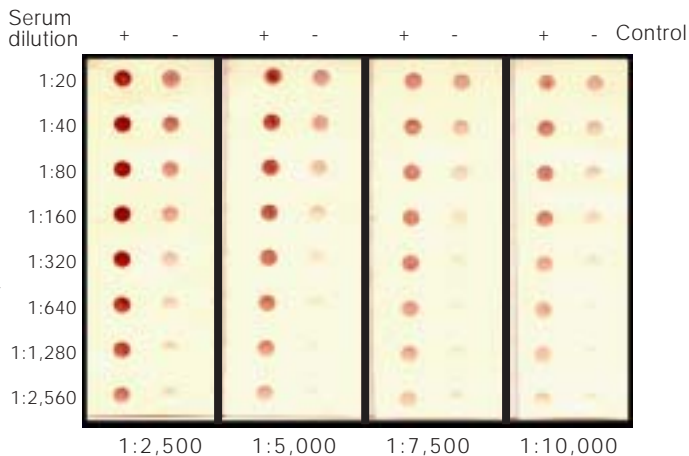
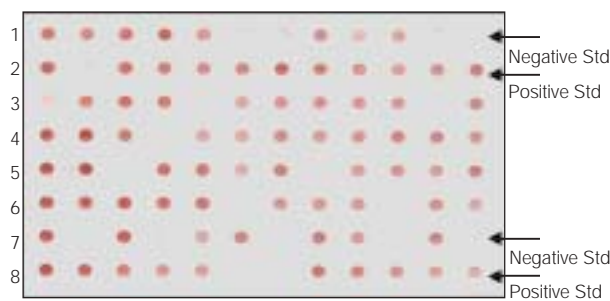


Fig 4—Nitrocellulose membrane showing the result for optimization of conjugate concentration for IgM dot-ELISA of leptospirosis at the condition of 1:20 - 1:2,560 of serum dilution and 1:2,500 - 1:10,000 of conjugate concentrations.

used, and it was enough for several repeated testings.

Fig 5 shows representative results of 92 serum samples tested by the optimized IgM dot-ELISA at 3 μ g/ml of pool antigen concentration (0.3 μ g/dot), at 1:160 serum dilution and 1:2,500 conjugate concentration. Positive reactions showed distinct red-pink spots on the labeled sites, whereas negatives showed colorless or faint-colored spots compared with two positive-



Ag 0.3 µg/dot, serum dilution 1:160, conjugate 1:2,500

Fig 5—Nitrocellulose membrane showing the result of 92 serum samples tested by the IgM dot-ELISA at 3 µg/ml of pool antigen concentration (0.3 µg/dot), 1:160 of serum dilution and 1:2,500 of conjugate concentration. Positive reaction showed the distinct red-pink spot on the labeled sites whereas negative showed colorless or faint color spot compared with two positive and two negative standard sera.

and two negative-standard sera.

Sensitivity, specificity and efficiency of both IgM-ELISA assays

Table 2 shows all validities for both ELISA assays compared with MAT as the 'gold standard' for diagnosis of human leptospirosis. Of the 96 samples that were judged positive ($\geq 1:400$) by MAT, 84 were positive by microplate IgM-ELISA (sensitivity, 87.50%) and 95 by IgM dot-ELISA (sensitivity, 98.96%). False-negative reactions by IgM-ELISA were found in 12 samples (12.50%) and in IgM dot-ELISA, 1 sample (1.04%).

Of the 247 samples negative by MAT, 241 were negative by IgM-ELISA (specificity, 97.57%)

and 232 by IgM dot-ELISA (specificity, 93.93%). False-positives by IgM-ELISA were found in 6 (2.43%) samples, *ie*, 5 from healthy individuals, and 1 from a dengue fever patient. False-positives by IgM dot-ELISA were found in 15 (6.07%) samples, *ie*, 12 from healthy individuals and 3 from patients with other diseases (*ie* dengue fever, murine and scrub typhus). The efficiency of the two tests was 94.75 and 95.33%, respectively. Both IgM ELISA methods showed results that were statistically significantly different from MAT ($p < 0.05$).

Correlation and agreement rate between the two IgM-ELISA methods

Table 3 shows a *t*-test comparison of the microplate IgM-ELISA and IgM dot-ELISA results for diagnosing human leptospirosis. Of 343 samples, 90 were positive and 232 were negative by both methods. One sample was IgM-ELISA-positive but IgM dot-ELISA-negative, while 20 were IgM dot-ELISA-positive but IgM-ELISA-negative. The agreement rate of IgM dot-ELISA, compared with IgM ELISA, was 0.85 by Kappa analysis. The correlation of the two methods was statistically significantly different ($p < 0.001$).

DISCUSSION

Diagnosis of human leptospirosis continues to be a serious medical and public health problem in Thailand. The need for simple, rapid and appropriate diagnostic tests has become more urgent, to aid clinical case identification and to facilitate rapid outbreak investigations. To detect specific antibody, MAT is still the standard refer-

Table 2

Validities of the microplate IgM-ELISA and IgM dot-ELISA performed on 343 sera from positive-case-group sera ($n=96$) and negative-control-group sera ($n=247$) compared with MAT as the 'gold standard' for diagnosis of human leptospirosis.

MAT	IgM-ELISA (microplate) ^a			IgM dot-ELISA ^b		
	Positive	Negative	Total	Positive	Negative	Total
Positive	84	12	96	95	1	96
Negative	6	241	247	15	232	247
Total	90	253	343	110	233	343

^aUsing cutoff OD of 0.60; sensitivity, 87.50%; specificity, 97.57%; efficiency, 94.75%; positive predictive value, 93.33%; and negative predictive value, 95.26%.

^bSensitivity, 98.96%; specificity, 93.93%; efficiency, 95.33%; positive predictive value, 86.36%; and negative predictive value, 99.57%.

Table 3

The correlation and agreement rate by the microplate IgM-ELISA comparing with IgM dot-ELISA for serodiagnosis of human leptospirosis performed on 343 serum samples.

IgM -ELISA (Microplate)	IgM dot-ELISA		Total
	Positive	Negative	
Positive	90	1	91
Negative	20	232	252
Total	110	233	343

Kappa = 0.85; *t*-test $p < 0.001$

ence test to diagnose leptospirosis. However, MAT is inadequate for rapid case identification, since it is technically demanding, costly, and requires the maintenance of live, hazardous stock serovar cultures (Pappas *et al*, 1985) and also requires analyses of paired sera to verify the seroconversion, which delays diagnosis.

Several rapid methods for antibody detection have subsequently been developed to diagnose leptospirosis early. Among these assays, IgM antibody detection by ELISA has been widely used, because IgM antibodies usually become detectable during the first week of illness, allowing the diagnosis to be confirmed and treatment initiated. Several studies have shown that assays which detected IgM were more sensitive than MAT, gave positive results earlier in the acute phase of disease (Cumberland *et al*, 1999), and permitted the use of a single serum sample. da Silva *et al* (1997) also showed that in acute-phase leptospirosis, dot-ELISA with sera from patients collected until the 14th day of illness detected IgM antibodies in 98%, IgG in 70%, and IgA in 76%.

However, the reported sensitivities and specificities of these assays varied according to the *Leptospira* serovars used and the antigen extraction procedures. Several test kits are now available commercially. A comparative evaluation of commercial test kits using different methods, *ie*, IHA, MSAT, Lepto-Dipstick, ELISA, and MCAT for serological diagnosis of leptospirosis, showed sensitivities of 72, 58, 78, 84, and 70%, respectively and specificities of 98, 82, 90, 89, and 94%, respectively (Nigowit *et al*, 2000). However, all of these test kits are expensive and some are not appropriate for single-sample testing, for use

in the field or in less-equipped laboratories.

So, in this study, an effort to develop a simple diagnostic test to achieve high sensitivity in the acute phase focused on detecting IgM binding to whole-cell antigen prepared from three dominant serovars of *Leptospira* associated with disease in Thailand. The approaches take advantage of cross-reactive antigens in crude extracts which are shared among diverse leptospiral serovars. These broadly reactive antigens include proteins and components of leptospiral lipopolysaccharide (LPS). ELISA methods were applied with modifications. An IgM-specific dot-ELISA was developed in this study, since it used smaller volumes of reagents and visual readings were possible; no special equipment was required, so the method can be used to diagnose patients with leptospirosis in peripheral laboratories with relatively little expertise.

The result of the present study showed that three pathogenic serovars of leptospira-Bratislava, Sejroe, and Pyrogenes - reacted most commonly serum samples collected from groups of patients with leptospirosis during the years 1998-1999. Therefore, these three serovars were chosen to prepare broadly reactive *Leptospira* antigens and used as the antigenic-pool. However, not only the *Leptospira* serovars used, but also the procedures for antigen extraction, may affect the sensitivity and specificity of an ELISA assay. Several previous studies used whole-cell antigen or partially purified extract from surface antigenic fractions from saprophytic and pathogenic leptospira of different serovars, rather than purified antigen (Terpstra *et al*, 1985; Gussenhoven *et al*, 1997). The present study also used whole-cell antigen, because it was simple and inexpensive to prepare. However, ELISA and other rapid serologic tests based on whole-cell leptospiral antigen preparations are suitable for use as alternative methods to screen for leptospiral infection, although MAT is still required for confirmation.

The optimal antigenic-pool concentration that discriminated best between ELISA reactions of sera from positive and negative control groups, for a microplate format, was 2 µg/ml. At this concentration, the results showed high reciprocal titers of 160 to 5,120 in the positive-standard-sera group, and low reciprocal titers of 20-40 in the negative-standard-sera group.

The cutoff point, the threshold of positivity, was 1:80. In the IgM dot-ELISA, the optimal concentration of the antigenic pool was 3 µg/ml, when 100 µl (or 0.3 µg) of this antigenic pool concentration were spotted onto a membrane. da Silva *et al* (1997) also showed the optimal protein concentration of the antigenic-pool for use in IgM, IgG, and IgA dot-ELISA, was 0.2 µg/ml, when 1 µl of the antigenic pool was dotted onto a membrane. The conjugate concentration, 1:7,500, was chosen for the microplate IgM-ELISA, and 1:2,500 for IgM dot-ELISA because they best discriminated between reactions of sera from positive- and negative-control groups, and the amount of conjugate could be minimized. However, the conjugate concentrations used in ELISA varied depending on the batches and types of antigen and conjugate.

The cutoff value was determined separately among the groups of healthy individuals who resided in the endemic or nonendemic areas. The mean was 0.62 among the endemic-area sera and 0.36 in the nonendemic control sera, or 1.7 times higher than the nonendemic control sera ($p < 0.01$). If the cutoff value of 0.36 was chosen, the sensitivity was 100%, and the specificity 71.7%. This gave a high number of false-positive results (28.3%). Although it did not create a problem for diagnosis, because the result of the laboratory test showed positive in normal healthy person, it was not a suitable screening test because the specificity was low.

If the cutoff value was determined for both endemic- and nonendemic-area sera, the median absorbance value would be 0.60, which was not different from the endemic-area sera. In this study, a cutoff value of 0.60 was chosen because some people at risk of leptospirosis were normal. A natural history of leptospires was often found and it was endemic disease where carrier states and asymptomatic infections were often reported (Tangkanakul *et al*, 2000). At this cutoff value, the assay demonstrated rather high sensitivity (87.50%) and specificity (97.57%). The false-positives (2.43%) were mostly found in healthy individuals (5 of 6 false-positive samples). As leptospirosis is widespread in Thailand, it is possible that some false-positive reactions were due to inapparent past infections with leptospirosis or other spirochetes. Cumberland *et al* (2001) found patients who had severe leptospirosis

commonly remained seropositive, with IgM, IgG and agglutinating antibodies detectable for several years after infection. This can create problems in interpreting serological results. Thus, in endemic areas where seroprevalence is high, the use of a single elevated titer is not reliable for defining a current infection.

Cross-reactions occurred in several other diseases. Both ELISA assays revealed cross-reactivity with sera from one patient with dengue fever. IgM dot-ELISA revealed cross-reactivity with sera from patients with scrub and murine typhus, due to the similarity of clinical features and endemicity of these diseases in Thailand. However, in most cases the OD values for IgM ELISA ranged from 0.50-0.6, which were near borderline cutoff values (0.60). For the IgM dot-ELISA, the staining intensity showed distinct red-pink spots on the label site, while the MAT result was negative, indicating that these serum samples contained IgM antibodies to leptospira, but were misdiagnosed as hemorrhagic fever, scrub typhus, murine typhus, and hepatitis.

In general, the microplate ELISA values were slightly lower than those of the IgM dot-ELISA (sensitivity 87.50 vs 98.96%; efficiency 94.75 vs 95.33%), except for specificity (97.57 vs 93.93%). The sensitivity and specificity of both tests appeared to agree well ($k=0.85$). The IgM dot-ELISA compared very favorably with the MAT with a sensitivity of 98.96%. Only one (1.04%) sample failed to react in the IgM dot-ELISA, but was reactive by MAT. These data suggested that at the cutoff point selected in this study, the IgM dot-ELISA may be more sensitive in detecting low levels of anti-leptospiral antibodies than the MAT. Both assays offered relatively high negative predictive values (95.26-99.57%), thus making the assays ideally suited for rapid screening. This finding agrees with the study by da Silva *et al* (1997), who evaluated a dot-ELISA using antigen from 5 serovars for detecting human IgM, IgG, and IgA in the acute phase; and they reported a sensitivity of 98% for IgM, but only 70% for IgG, and 76% for IgA. Other studies have also found IgM enzyme immunoassays based upon crude antigen to be more sensitive for serodiagnosis than MAT (Thiermann and Garrett, 1983) but may be subject to variations in specificity. However, MAT is an appropriate test for epidemiological serosurveys, since it can be applied to sera from

any animal species. The MAT data can give a general impression about which serogroups are present within a population.

In summary, the IgM dot-ELISA using endemic leptospiral antigens offered good sensitivity, specificity, and reproducibility, yielding accurate results comparable to the reference MAT and thus could be used as a rapid screening test for acute leptospirosis. Sonicated antigen was used, thus avoiding the risk of infection to those who perform the test, and no maintenance of live, hazardous stock serovar cultures. The assay was simple, inexpensive, and easy to perform, with visual reading of the results that did not require special equipment, *ie* a microscope or spectrophotometer. The assay was completed in approximately 2 hours. Future applications aim to further develop a lower-cost test kit as a test strip using antigen from locally prevalent serovars in a form suitable for diagnosis, for use in peripheral laboratories with little specialized equipment.

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