INDIRECT IMMUNOPEROXIDASE TEST FOR THE DIAGNOSIS OF LEPTOSPIROSIS

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Abstract. Leptospirosis is a widespread zoonotic disease that affects all mammals, including humans, in different parts of the world. Clinical recognition of leptospirosis is challenging, and the definitive serologic diagnosis assay, the microscopic agglutination test (MAT), is time-consuming and difficult to conduct. In this study, an indirect immunoperoxidase (IIP) test to detect *Leptospira*-specific antibodies in human serum samples was developed. The efficacy of the IIP was compared with the indirect immunofluorescent assay (IFA) and MAT. A total of 368 human serum samples were analyzed by MAT, IFA, and IIP. Using a MAT titer of ≥1:100 as the gold standard, the sensitivities for the detection of Leptospiral antibodies at a titer of 1:200 were 94.7% by IFA and 93.6% by IIP; specificities were 95.3% by IFA and 94.9% by IIP; and accuracies were 95.1% by IFA and 94.6% by IIP. With a titer of 1:400, the sensitivity, specificity, and accuracy were 86.2%, 98.9%, and 95.7% by IFA, respectively; whereas, for the IIP, the sensitivity was 85.1%, specificity 98.5%, and accuracy 95.1%. A further evaluation of this test with 80 unknown-febrile-disease sera was also included. We found that the sensitivity and specificity of this test were 100% and 76.8%, respectively. Therefore, the IIP test is a potentially valuable tool for the diagnosis of leptospirosis.

INTRODUCTION

Leptospirosis is an acute febrile disease caused by infection with pathogenic spirochetes of the genus *Leptospira*. The disease occurs throughout the world; the incidence is significantly higher in warm-climate countries than in temperate regions (Everard and Everard, 1993; WHO, 1999). The spectrum of disease is extremely wide and varies from clinically inapparent to severe multisystemic disease. Therefore, a rapid and simple test, with a high sensitivity and specificity, is essential for accurate diagnosis and prompt treatment. A definitive diagnosis of leptospirosis may be accomplished by isolation of the infecting spirochete from blood, urine, or tissue, but this requires special media and prolonged incubation and is not often performed.

Serodiagnosis by microscopic agglutination test (MAT) is time-consuming, requires a large panel of live-cell suspensions to provide adequate coverage of the antigenic diversity represented in a given testing serum, and requires significant expertise that is available in few laboratories. Several alternatives to the MAT have been developed consisting of direct detection of leptospiral material or indirect detection of anti-leptospiral specific antibodies, including the indirect immunofluorescent method for detecting immunoglobulins G, M and A (Torten *et al*, 1966; Appassakit *et al*, 1995; Naigowit *et al*, 2001). Previous studies showed that the IFA test, for the detection of antibodies against *Leptospira* in clinical samples, was more sensitive and specific than several other tests (Appasakit *et al*, 1995; Naigowit *et al*, 2000; Pradutkanchana *et al*, 2003).

In our study, we modified and developed the indirect immunoperoxidase (IIP) test to detect leptospira-specific antibodies for the diagnosis of leptospirosis. The sensitivity, specificity, and accuracy of the test were compared with the indirect immunofluorescent test (IFA) and the reference standard method, MAT.

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MATERIALS AND METHODS

Specimens

A total of 368 serum samples were included in this study: 94 samples from 80 patients (14 paired sera and 66 single sera) confirmed to have leptospirosis by MAT were obtained from the Department of Microbiology and Immunology, Faculty of Tropical Medicine; 202 samples from patients with various diseases other than leptospirosis, including scrub typhus (n=112), syphilis (n=5), influenza (n=5), dengue fever (n=3), other rickettsial diseases (n=21), malaria (n=26), and typhoid fever (n=30) were obtained from the well established serum bank. In addition, 30 blood donor sera from volunteers in Bangkok, and 42 blood donor sera from the Blood Bank, Maharat Nakhon Ratchasima Hospital, Nakhon Ratchasima Province, were included for negative controls. For further evaluation of this test, 80 unknown febrile sera from Buri Ram General Hospital Buri Ram Province, were also tested for leptospirosis by IIP and compared with the MAT.

Microscopic agglutination test (MAT)

The MAT was performed as described by Faine (1982). A battery of 12 serovars of *L. interrogans* was used as live antigens. The panel consisted of the following: Ballico, Bratislava, Autumnalis, Bataviae, Canicola, Djasiman, Grippotyphosa, Hyos (Tarassovi), Icterohaemorrhagiae, Pyrogenes, Hardjo and Wolffi. Briefly, the serum was diluted 1:50 in sterile 0.85% normal saline. An equal volume of live organisms from seven-day-old pure cultures of each of the 12 serovars was added to each well of a plastic plate (Tomy, China). The plates were gently shaken to mix the contents, covered with plastic wrap and incubated at room temperature for 2 hours. Next, the degree of agglutination and the end-point titer were determined under dark-field microscope (Carl Zeiss JENA, NrM268259). A sample was considered positive at titer of ≥1:100 when approximately 50% or more of the leptospires were agglutinated.

Antigen slide preparation

The antigen was prepared from a well-grown culture, non-pathogenic strain Patoc I (*Leptospira biflexa* serovar Patoc) in neopeptone liquid medium (DIFCO, USA) containing 8% rabbit serum (Biologicals, USA) for 2-4 days in the dark at 28º-30ºC. Then the culture was washed three times by centrifugation at 10,000 rpm at 4ºC for 15 minutes. The pellet was resuspended with PBS pH 7.2 to make the concentration approximately 1×10^7 leptospires/ml. Two microliters of leptospiral suspension were placed onto a 12-well clean telflon-coated slide (BDH, England) and air-dried at room temperature. Each slide was then fixed in cold acetone for 30 minutes, thoroughly air-dried and stored at -20ºC until used.

Indirect immunofluorescent assay (IFA)

Appassakij *et al* (1995) selected a titer of 1:100 as the cut-off titer. Later, Pradutkanchana and colleagues (2003), selected a cut-off titer of 1:400 for the IFA test. Therefore, each serum sample in this study was tested by IFA as described by Appassakij *et al* (1995) and three different titers, 1:100, 1:200, and 1:400, were selected. Briefly, 10 μl of three titers of each sample were dropped onto the well of each slide and incubated for 30 minutes at 37ºC. The slide was washed three times in 0.01% Tween-PBS pH7.2 and air-dried. Then, the slide was incubated with fluorescence 1:40 FITC-conjugated anti-human immunoglobulins (IgM+IgG+IgA) (DAKO, Denmark) for 30 minutes at 37ºC, and then washed as above. The slide was mounted and observed under a fluorescence microscope (Olympus, BH_2RLL). The result of the titer was taken as the highest dilution to show the apple green fluorescence of leptospires. Positive and negative controls were included in each batch tested.

Indirect immunoperoxidase (IIP) test

The IIP test was modified from the technique of Kelly *et al* (1990), which was used for the diagnosis of scrub typhus. Briefly, three titers (1:100, 1:200, 1:400) of each serum were diluted and 10 μl of each were dropped onto the well of each slide and incubated at 37ºC for 30 minutes. Positive and negative controls were included in each batch tested. After incubation, the slide was washed in 0.01% Tween-PBS pH7.2 (5 minutes, 3 times). The slide was air-dried and incubated with 1:100 peroxidase conjugated rabbit anti-human immunoglobulin antibodies (IgM+IgG+ IgA) (DAKO, Denmark) for 30 minutes, at 37ºC, and washed as previously

described. Then, the slide was reacted with 3-amino-9-ethyl carbazole (AEC) (Sigma, USA), which was used as a substrate solution to give a specific color, and incubated for 15 minutes at room temperature in the dark, then counter-stained with diluted hematoxylin for 5 minutes. The slide was mounted and examined under a light microscope. The reaction was considered positive when the leptospires were visible as light brown spiral organisms.

Statistical analysis

The statistical analysis for diagnostic sensitivity, specificity, and accuracy of the IFA and IIP for the diagnosis of leptospirosis were calculated by the method of Griner et al (1981), and compared with the results of the MAT test. The kappa coefficient (κ) was calculated to determine a degree of agreement between the two methods.

RESULTS

Since this study was intended to develop a simple and rapid diagnosis of leptospirosis by indirect immunoperoxidase (IIP) test, only three titers (1:100, 1:200, 1:400) were selected for determining the cut-off titer. Ninety-four leptospirosis sera were tested with *Leptospira biflexa* serovar Patoc strain Patoc I antigen in both the IFA and IIP tests. The positive results obtained at different titers were different. As shown in Table 1, at a titer of 1:100, 92 samples (97.9%) were positive and 2 were negative by

IFA. The number of positive sera reduced to 89 (94.7%) and 81 (86.2%) when tested at dilutions of 1:200 and 1:400, respectively. For the IIP, at a titer of 1:100, 91 (96.8%) samples gave positive results. At a titer of 1:200, positive results reduced from 91 to 88 (93.6%), whereas at a titer of 1:400, 80 (85.1%) samples were positive. False-positive results were found in other diseases commonly confused with leptospirosis, and healthy blood donors, as shown in Table 2. At a titer of 1:100, 33 samples gave positive results by IFA (12.0%) and 34 samples were positive by IIP (12.4%). At a titer of 1:200, 13 samples were positive by IFA (4.7%) and 14 were positive by IIP (5.1%), whereas at a titer of 1:400, the positive results were reduced to 3 (1.1%) and 4 (1.5%) by IFA and IIP, respectively.

The sensitivities, specificities, and accuracies of the IFA and IIP in detecting anti-Leptospiral antibodies at the three different titers were

Table 1
Antibodies detected by IFA and IIP tests in sera collected from leptospirosis patients.

Cut-off titers	Leptospirosis sera (n=94)	
	Positive-IFA	Positive-IIP
1:100	92 (97.9%)	91 (96.8%)
1:200	89 (94.7%)	88 (93.6%)
1:400	81 (86.2%)	80 (85.1%)

Table 2
Cross-reaction assessment to determine the background antibody titer of the IFA and IIP for the detection of Leptospiral antibody.

Other disease and negative control group	IFA			IIP		
	1:100	1:200	1:400	1:100	1:200	1:400
Syphilis (n=5)	2	1	0	2	1	0
Influenza (n=5)	1	0	0	1	0	0
Dengue fever (n=3)	0	0	0	0	0	0
Other rickettsial diseases (n=21)	1	1	0	1	1	0
Malaria (n=26)	2	0	0	2	0	0
Typhoid fever (n=30)	6	4	0	6	4	1
Scrub typhus (n=112)	9	4	3	10	5	3
Healthy blood donor (n=72)	12	3	0	12	3	0
Total (n=274)	33 (12.0%)	13 (4.7%)	3 (1.1%)	34 (12.4%)	14 (5.1%)	4 (1.5%)

Table 3
Evaluation of three cut-off titers for the diagnosis of leptospirosis by IFA and IIP.

Statistical values	IFA			IIP		
	1:100	1:200	1:400	1:100	1:200	1:400
Sensitivity (%)	97.9	94.7	86.2	96.8	93.6	85.1
Specificity (%)	88.0	95.3	98.9	87.6	94.9	98.5
Accuracy (%)	90.5	95.1	95.7	90.0	94.6	95.1
Kappa coefficient (κ)	0.774	0.860	0.882	0.762	0.854	0.860

Table 4
Comparison of IIP and MAT at a titer of 1:400 for detecting anti-Leptospiral antibodies in 80 unknown febrile samples from Buri Ram General Hospital.

IIP	MAT		Total
	Positive	Negative	
Positive	11	16	27
Negative	0	53	53
Total	11	69	80

calculated and are shown in Table 3. A total of 368 blood samples were investigated for Leptospiral antibodies by MAT, IFA and IIP tests. The statistical values of the IFA and IIP were compared with the MAT test, which was used as the reference standard. The sensitivity of the IFA test was different among the three selected titers. It was found that a titer of 1:100 gave the highest sensitivity (97.9%) and reduced to 94.7% and 86.2% at titers of 1:200 and 1:400, respectively. The sensitivity of the IIP test gave similar results, as follows: 96.8% at a titer of 1:100, 93.6% at 1:200, and 85.1% at 1:400. Regarding the specificity of both tests, the IFA showed a high specificity (98.9%) at a titer of 1:400, whereas it reduced to 95.3% and 88.0% at titers of 1:200 and 1:100, respectively. The IIP specificities were similar to the IFA. A titer of 1:100 gave a low specificity (87.6%), while titers of 1:200 and 1:400 showed higher specificities, at 94.9 and 98.5%, respectively. The accuracy of the IFA and IIP tests, was not much different between the three titers. The kappa coefficient (κ) of the IFA was 0.882 and of the IIP 0.860, when compared with the MAT test, indicating very good agreement between the two methods. The kappa coefficient of the IIP showed a good correlation with the IFA test (κ = 0.947), indicating almost perfect agreement between the IFA and IIP methods. These results show that the IIP test can replace the IFA for detecting antibodies against leptospires in clinical samples.

In this study, the decision for the cut-off titer at 1:400 for the IIP test was based on the optimum sensitivity and specificity with the highest percentage of accuracy. When the 14 paired sera were evaluated, the IIP test showed the ability for early detection of antibodies for leptospirosis, better than MAT. It could detect 4 (28.6%) of acute phase sera from 14 paired sera, while the MAT detected only 1 (7.1%) serum. In convalescent sera, all 14 (100%) paired sera were positive by both MAT and IIP tests.

For further evaluation, 80 unknown febrile illness serum samples were blindly examined in this study by both MAT and IIP. These 80 samples were diagnosed by MAT as 11 positive leptospirosis cases infected with different serovars (8 Bratislava, 1 Icterohaemorrhagiae, and 2 samples reacted with both Bratislava and Sejroe). The results from this 1:400 titer are shown in Table 4. Eleven samples were positive by both MAT and IIP. However, 16 samples gave positive results with IIP but showed no agglutination by MAT. From this evaluation, the sensitivity and specificity for applying the IIP test to clinical samples were 100 and 76.8%, respectively.

DISCUSSION

Many serological tests for the diagnosis of leptospirosis, which are more sensitive and spe-

cific than MAT, have been developed recently (Terpstra *et al*, 1985; Appassakij *et al*, 1995; Bal *et al*, 1994; Gussenhoven *et al*, 1997; Levett *et al*, 2001; Smits *et al*, 2001). Although MAT is the reference standard method, its disadvantages are requiring a battery of different serovars to provide live antigens for the many possible strains present in the region; it is also time-consuming and insensitive. Many studies developed the IFA test for the diagnosis of leptospirosis (Torten *et al*, 1966; Appassakij *et al*, 1995, Naigowit *et al*, 2001; Pradutkanchana *et al*, 2003). In our study, *Leptospira biflexa* serovar Patoc strain Patoc I was selected as an antigen because it detects broadly cross-reactive antibodies. Several studies selected this genus-specific antigen for use in many methods, including indirect hemagglutination (IHA); ELISA (Terpstra *et al*, 1985; Gussenhoven *et al*, 1997; Levett *et al*, 2002); dipstick assay (Sehgal *et al*, 1999; Levett *et al*, 2001) and IFA (Torten *et al*, 1966). However, the different methods appear to have different sensitivities and specificities. Gussenhoven *et al* (1997) used *L. biflexa* as an antigen in ELISA and dipstick tests and found that the sensitivities were 88.5 and 86.8%, while the specificities were 94.2 and 92.7%, respectively. Levett *et al* (2001) concluded that the IgM-dipstick was more sensitive than the IHA. It was found that the sensitivity of the IgM-dipstick (98%) was slightly higher than the IHA (92.2%) and the specificities of both tests were slightly different, 90.6 and 94.4%, respectively. In this study, the IFA method gave a high sensitivity and specificity of 86.2 and 98.9%, respectively, similar to a previous report by Pradutkanchana *et al* (2003), with a sensitivity and specificity being 86.5 and 91.6%. Nevertheless, they selected *L. interrogans* serovar Bataviae as the antigen. From this point, the IFA can detect antibodies to genus-specific antigens; therefore different antigens of leptospires of any serovar can be used for the test. Antigens from leptospires of the serovars generally found in the endemic area probably increase sensitivity and specificity. However, the IFA is suitable only for a well-equipped laboratory having a fluorescence microscope. Thus, it would be a great advantage to have a simple and rapid method for detecting anti-Leptospiral antibodies in clinical samples, which can be used in any non-reference laboratory.

Kelly *et al* (1990) developed the IIP test for the diagnosis of scrub typhus. The IIP test is a modification of the IFA that replaces fluorochrome with peroxidase, allowing the use of an ordinary light microscope, and seems to perform similarly to the IFA. As a consequence, the IIP test may be the test of choice to replace the IFA test, which has not been widely used within endemic regions owing to the scarcity of fluorescence microscopes. Here, the IIP method was modified and developed to detect anti-Leptospiral antibodies in clinical specimens. The results showed that this method gave a high sensitivity (85.1%) and specificity (98.5%) with the highest accuracy (95.1%) at a titer of 1:400. The IIP also showed a good correlation with the IFA test (κ = 0.947), indicating almost perfect agreement between the IFA and IIP tests.

This IIP method detects IgM, IgG or IgA and can be used to determine the state of infection, such as acute or convalescent. Sehgal *et al* (1999) found that the MAT showed a low sensitivity (41.1%) during the first week, increasing to 83.3% during the second to fourth weeks of infection. In this study, 14 pairs of leptospirosis serum samples were examined. The MAT could detect antibody in only 1 (7.1%) acute serum from 14 paired sera. The IIP test had better sensitivity, and could detect antibodies in 4 (28.6%) acute sera from the same paired sera group. This indicates that IIP can give a better chance of detecting early infection.

The application of the IIP test developed in this study was further evaluated using 80 unknown febrile illness single sera. Based on a cut-off titer of 1:400, the IIP test could identify 11 of 11 leptospirosis positive samples correctly (100%). This assay showed 100% sensitivity and 76.8% specificity. The lower specificity is a result of 16 samples giving false-positive results. A possible explanation for these many unexpected false-positives was the small number of serovars (12 serovars) that were used in the MAT test, which did not cover the leptospires present in the region. Another explanation may be the lower sensitivity of the MAT, in detecting acute sera, than the IIP test, as proven in this study

only one acute serum from 14 confirmed letospirosis patients was detected by MAT. This shows the IIP test may have an advantage as a screening test over the MAT at a reference laboratory. However, a larger number of unknown febrile sera should be used for further evaluation of this test compared with other serological tests, preferably in multi-center trials.

In conclusion, the IIP test is not only sensitive and specific but also simple to perform. The result can be obtained within 2 hours with the aid of a light microscope. Therefore, the IIP test is a potentially valuable tool for the diagnosis of leptospirosis.

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