13. Suputtamongkol Y, Sarawish S, Silpasakorn S, *et al*: Microcapsule agglutination test for the diagnosis of leptospirosis in Thailand. Am. Trop. Med & Parasitol. 1998 ; 92 : 797-801.

14. Taylor CR : Immunoperoxidase Technique. Arch. Path. Lab. Med. 1978 ; 102 : 113-121.

15. Ellis TM, Robertson GM, Hustas L, *et al*: Detection of leptospirosis in tissue using an immunoperoxidase staining procedure. Aust. Vet. J. 1983 ; 60 : 364-367.

16. Ferreira Alves VA, Vianna MR, Yasuda PH, *et al*: Detection of leptospiral antigen in the human liver and kidney using an immunoperoxidase staining procedure. J. Patho. 1987 ; 151 : 125-131.

17. Lim TS : A sensitive malaria immunoperoxidase assay for the detection of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 1988 ; 38 : 255-257.

18. Yamamoto S, and Minamishima Y : Serodiagnosis of Tsutsugamushi fever (scrub typhus) by the indirect immunoperoxidase technique. J. Clin. Microb. 1982 ; 15 : 1128-1132.

19. Altman DG : Practical statistics for medical research. Chapman & Hall, London, 1991.

20. Mayer D : Essential evidence-based medicine. Cambridge University Press, Cambridge, UK, 2004.

21. Smiths HL, *et al* : Internataional multicenter evaluation of the clinical utility of a dipstick assay for detection of Leptospira-specific immunoglobulin M antibodies in human serum samples. J. Clin. Microb. 1999; 37: 2904-2909.

22. Levett PN, and Branch SL. : Evaluation of two enzyme-linked immunosorbent assay methods for detection of immunoglobulin M antibodies in acute leptospirosis. Am. J. Med. Hyg. 2002; 66: 745-748.

23. Yitzhaki S, *et al* : New approach for serological testing for leptospirosis by using detection of leptospira agglutination by flow cytometry light scatter analysis. J. Clin. Microb. 2004; 42 : 1680-1685.

24. Jeandel P, *et al* : Late positive blood cultures in leptospirosis (report of four cases). Trans. R. Soc. Trop. Med. Hyg. 1984: 78: 143-145.

25. Bunnag D, Jaroonyesama N, Harinsuta C : A clinical study of leptospirosis. J. Med. Assoc. Thai 1965; 48:231-245.

26. Heisey GB, *et al* : Epidemiology and characterization of leptospirosis at an urban and provincial site in Thailand. Southeast Asian J. Trop. Med. Health 1988; 19:317-322.

27. Sundharagiati B, *et al* : Leptospirosis as a cause of pyrexia of unknown origin in Thailand. Ann. Trop. Med. Parasitol. 1996; 60:247-250.

28. Nimmanitya S, Thisyakorn U, Ningsanonda C: Leptospirosis in children. Bull. Dept. Med. Sci. 1984; 9:69-78.

29. Ratanasang P, *et al* : Prevalence of leptospiral antibody in agricultural group, Kosumpisai district, Mahasarakam province, 1998. Health Sci. 2001; 10:502-507.

30. Phulsukuksombati D, *et al* : Prevalence of leptospirosis antibody in military recruits, Khon Kaen, 1999. RTA Med. J. 2001; 54:209-214.

31. Tangkanakul W, *et al* : Prevalence of asymptomatic leptospirosis infection among high risk group 1998. Health Sci. 2000; 9:56-62.

32. Adler B, *et al* : Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. J. Clin. Microb. 1980; 11: 452-457.

33. Pappas MG, *et al* : Rapid serodiagnosis using the IgM-specific dot-ELISA: Comparison with the microscopic agglutination test. Am. J.Trop.Med 1985; 34: 346-354.

34. Silva MV, *et al* : Behavior of specific IgM, IgG and IgA class antibodies in human leptospirosis during the acute phase of the disease and during convalescence. J. Trop. Med. Hyg. 1995; 98: 268-272.

35. Guerreiro H, *et al* : Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infection and Immunity 2001; 69: 4958-4968.

36. Silva MV, *et al* : Immunodiagnosis of human leptospirosis by dot-ELISA for detection of IgM, IgG, and IgA antibodies. Am. J. Trop. Med. Hyg. 1997; 56: 650-655.

ภาคผนวก

1. บทความเผยแพร่ คาดว่าจะพิมพ์บทความในวารสารนานาชาติจำนวน 3 เรื่อง ดังนี้ :

1.1 Leptospiral IgM, IgG, IgA, or IgS isotype antibody determinations. Which one to choose for the diagnoss of human leptospirosis?

1.2 Serodiagnosis of human leptospirosis by immunoperoxidase test

1.3 A comparative evaluation of the indirect immunoperoxidase and indirect immunofluorescent antibody tests for diagnosis of human leptospirosis.

2. กิจกรรมที่เกี่ยวข้องกับการนำผลจากโครงการไปใช้ประโยชน์

- **ชุดตรวจวินิจฉัยแยกโรคไข้ฉับพลันไม่ทราบสาเหตุ (อยู่ในระหว่างการขอจดสิทธิบัตร)**

Leptospiral IgG, IgM, IgA, or IgS isotype antibody determinations. Which one to choose for the diagnosis of human leptospirosis?

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ABSTRACT

A study was done to compare the IgG, IgM, IgA and the total immunoglobulin (IgS) isotype responses in leptospiral-infected patients. Using 57 paired sera from proven leptospirosis patients, the respective sensitivities of acute and convalescent patient's sera tests were as follows: IgG, 63.2% and 100.0%; IgM, 57.9% and 91.2%; IgA, 3.5% and 56.1%; and IgS, 64.9% and 100.0%. The geometric mean titers for each leptospiral antibody in sera from both acute and convalescent patients were as follows: IgG, 55.0 and 650.6; IgM, 51.8 and 362.9; IgA, 19.2 and 52.5; and IgS, 76.0 and 995.8; respectively. Additionally, leptospiral IgS antibodies showed an earlier detection and a faster declination when compared to the standard MA antibodies. The persistence of leptospiral IgS antibodies was up to five and a half months. A single or simultaneous assay for IgG, IgM or IgA leptospiral antibodies had no advantage over IgS leptospiral assay.

INTRODUCTION

The diagnosis of human leptospirosis usually relies on serological-antibody based assays (1). Early and/or sensitive serological tests of human leptospirosis are essential because early treatment using doxycycline or other tetracycline antibiotics usually results in a rapid and complete recovery.

Human leptospiral antibodies are characterised by IgG, IgM, and IgA isotypes (2-3). Leptospiral IgM antibodies, in particular, have been suggested as a marker of the early phase of the disease, but this type of antibody was present in less than half of the cases (4-6). Assays that use the crude leptospiral antigen to detect leptospiral IgM antibodies appear to be more sensitive assays but subject to variation in specificity (7-11). Additionally, the diagnostic utility of leptospiral IgM antibody detection has been questioned by the occasional persistence of leptospiral IgM antibodies for several years (9, 12-13). On the other extreme there have been no leptospiral IgM response in some patients with proven leptospirosis, especially in some endemic area (4, 8, 3, 14), and false positive results were often detected by genus-specific leptospiral IgM antibodies (7, 10-11). As a consequence, paired sera were required to confirm the rising of leptospiral IgM antibody titers for a definite diagnosis.

As an alternative, it has been suggested that simultaneous detection of leptospiral IgG and IgM antibodies increases the sensitivity of early detection when compared to the detection of leptospiral IgG or IgM antibodies alone (2, 6, 8-9, 12-13). Since not all leptospirosis patients

produce leptospiral IgG or IgM antibodies (2, 8, 15), the additional detection of leptospiral IgA antibodies may help to maximize the sensitivity. Silva and his co-workers have successfully improved the sensitivity of the assay by simultaneously assayed the leptospiral IgG, IgM, and IgA (2, 3). In their reports, leptospiral IgM and IgA detected by ELISA were the predominant antibody isotypes found throughout the course of the leptospiral infection (2, 3). This implies that, in order to increase the sensitivity of the test, at least two methods are required. One by using paired sera testing or two, by simultaneous detection of leptospiral IgG and IgM and/or IgA antibodies. However, which method provides the better yield has not yet been documented.

In the present study, we compared the leptospiral IgG, IgM, IgA, and total immunoglobulin (IgS) antibody responses in patients with leptospirosis to determine which isotype is the most appropriate for use in serodiagnosis. Additionally, the IgS leptospiral antibody kinetics throughout the course of leptospiral infection were also investigated and compared with the MA leptospiral antibody.

MATERIALS AND METHODS

Study design

The study was conducted at Songklanagarind Hospital, Hat Yai, Thailand, between 2001 and 2003 with the approval of the Ethics Committee of the Faculty of Medicine, Prince of Songkhla University. Paired sera obtained from patients, regardless of sex and age, who presented with acute (2-14 days) fever without signs and symptoms suggestive of obvious foci of infection, were included in this study. Acute sera were collected within a week of illness onset with subsequent sera samples taken 1-2 weeks later.

The definitive diagnosis of leptospirosis was defined as a four-fold or greater increase in the leptospirosis microagglutinin (MA) titers (1). There were two groups of leptospirosis populations studied. The first group consisted of 57 patients with proven leptospirosis who were tested for the presence of various leptospiral antibody isotypes. The second group of leptospirosis patients consisted of 37 patients who were investigated for the kinetic and the persistence of leptospiral antibodies. In total, 88 serum samples were obtained from the patients in this group. All sera were collected over a period of one to 216 days after the onset of illness. All the serum samples used in this study were kept at -70°C before being assayed.

Microagglutination (MA) test

The leptospiral agglutinins were tested by the MA method (1) against a battery of the following 23 *Leptospiral interrogans* serovars: *akayami A, ballico, bangkoki, bataviae, bratislava, bullum, canicola, copenhageni,*

cellidoni, djasiman, grippotyphosa, hardjo, hebdomadis, hyos, icterohaemorrhagiae, javanica, pomona, pyrogenes, rachmati, saigon, sejroe, tarassovi, wolffi.

Indirect immunoperoxidase (IIP) test

The IIP test used in this study was modified slightly from the immunofluorescent antibody (IFA) test that previously described (16).

Briefly, the test was as followed: 5-7 day-old cultures containing approximately 10^7 leptospires/ml of *Leptospiral interrogans* serovar *bataviae* were air-dried, fixed with acetone at room temperature for 10 min, and stored at -20°C until used.

Fifty microlitres of the sera diluted to 1:100 were placed on each circle of the dried antigens and incubated in a moist chamber at 37°C for 30 min. The slides were rinsed once with 0.15 M phosphate-buffered saline (PBS), pH 7.2, and then washed with PBS. Fifty microlitres of the optimal dilution of peroxidase enzyme-conjugated rabbit anti-human immunoglobulin IgG, IgM, IgA, or total immunoglobulins (IgS) antiserum was then placed by adding on each antigen circle and incubated in a moist chamber at 37°C for 30 min. The slides were rinsed once and washed with PBS and the peroxidase enzyme substrate was added to the antigen circle for 10 min. After stopping the reaction with distilled water, the antigen circles were air-dried and subsequently examined at x400 magnification using an ordinary microscope. A positive result was identified and read when the leptospires appeared with their characteristic spirochaetal morphology as a brownish colour. All the positive serum samples were tested to their end-point titers. Furthermore,

both positive and negative serum controls were included in each batch of the assays.

RESULTS

Leptospiral isotype antibody responses in patients with leptospirosis

Fifty-seven paired sera obtained from patients with proven leptospirosis were tested using the IIP test for various leptospiral antibody isotypes, as shown in Table 1.

Analysis of the acute and convalescent sera showed for the detection of leptospiral antibodies as followed: IgG, 63.2% and 100.0%; IgM, 57.9% and 91.2%; IgA 3.5% and 56.1%; and IgS, 64.9% and 100.0%, respectively. The magnitude of titers for each of the leptospiral antibodies in the acute and convalescent sera, as expressed by geometric means, were as followed: IgG, 55.0 and 650.6; IgM, 51.8 and 362.9; IgA, 19.2 and 52.5; and IgS, 76.0 and 995.8; respectively. Leptospiral IgS antibodies provided the highest sensitivity and magnitude of any antibody titers for both the early and convalescent phases of diseases (McNemar test, p value <0.01).

The background IIP total IgS antibodies in patients with non-leptospirosis controls was 8.8% and all of them showed IIP total IgS antibody titers not more than 1:200. These results were similar to the background IIP antibodies of IgG and IgM isotypes (data not shown).

The kinetics of leptospiral antibodies responses in patients with leptospirosis

Eighty-eight sera obtained from 37 patients with proven leptospirosis were investigated by the IIP test and by the standard MA test (Figures 1 and 2) to determine the persistence of leptospiral antibodies over a period of time ranging from one day up to 216 days after the onset of illness. In general,

the IIP antibodies showed an earlier detection and a faster declination when compared to the MA antibodies.

On serial testing, the IIP-IgS antibodies appeared during the first week of illness in 12 of the 20 (60.0%) patients with leptospirosis, and plateaued during the second and third week (100.0%), and after that, declined and persisted for five and a half months. In contrast, the MA antibodies appeared during the first and second weeks of illness in only one of 20 (5.0%) patients and 12 of 26 (46.5%) patients, respectively, and peaked by the fourth week (100.0%). The persistence of MA antibodies was longer than that of the IIP-IgS antibodies.

DISCUSSION

The genus-specific leptospiral antibodies demonstrated in the present study were characterised by leptospiral IgG, IgM, and IgA antibodies where IgG and IgM were the predominant isotypes. However, our findings were different from those reported by Silva and his co-workers (2) in that leptospiral IgA antibodies were not as that prevalent in our patients with leptospirosis. Only 3.5% and 56.1% of our patients with leptospirosis showed positive results for leptospiral IgA antibodies in their acute and convalescent sera, respectively. The magnitude of leptospiral IgA antibody titers were also much lower than those of leptospiral IgG and IgM antibodies. Thus, assay for IgA anti-leptospiral antibody is not a sensitive diagnostic test for leptospirosis at all.

As expected, both IgG and IgM were the predominant leptospiral antibodies in both the acute and convalescent patient's sera because the cell surface leptospiral antigens used in this study consisted of protein and lipopolysaccharide antigens (6, 17). These results were in accordance with other studies in endemic area for leptospirosis (13, 15, 17), and other infectious diseases such as scrub typhus (18), and toxoplasmosis (19) in that early and high titers for both IgG and IgM isotypes were demonstrated. Some investigators, however, found that the IgM isotype titers response in leptospirosis were greater than those of specific IgG isotypes (2-3, 6, 8-9). The leptospiral IgG antibodies were recognised in all of our 57 patients with leptospirosis while leptospiral IgM antibodies were absent in five patients. The possibility of delayed-IgM production should be taken into account

because of the host factors or the early initiation of antibiotic therapy (8, 16, 20). For example, Terpstra and his co-workers mentioned of a proven case of leptospirosis who remained seronegative for leptospiral IgM antibodies for up to three months after the onset of the disease (8). Unfortunately, sera of our IgM-seronegative patients were unavailable for serial testings. The other explanation for the absence of leptospiral IgM antibodies may be due to re-infection type of leptospirosis which are not uncommon in hyperendemic area (8).

Taken together, the factor(s) which might explain the apparently different results from various other investigations for leptospiral antibody isotypes in patients with leptospirosis were as follows: Firstly, the individual hosts may response to the leptospiral antigens from the different infected leptospira strains by generating the different leptospiral isotype antibodies (12). Secondly, the type of leptospiral antigens used in the studies may determine the different leptospiral isotype antibodies. For example, the lipopolysaccharide antigens predominantly induce IgM isotype antibodies (6, 12) *versus* the leptospiral protein antigens which evoke more IgG responses (15). Thirdly, the variability of leptospiral antigens expressed during the course of infection (17, 21), and the timing of the collection of serum samples, (2, 6, 8- 9, 12) may lead to determine the different leptospiral isotype antibodies. Fourthly, the type of assay techniques used in the various studies were reported to determine the different leptospiral isotype antibodies. For example, the leptospiral antibodies detected by a microagglutination test were mainly of the IgM isotypes (6, 12) while ELISA antibodies were mainly of the IgM and IgG isotypes (6, 13, 15, 17). Lastly,

the different antibody responses were partly due to the host factors such as the severity of the disease, the abnormal of host responses or the early initiation of antibiotic therapy (8, 9, 20, 21).

Regardless of whether IgG or IgM isotype antibody determinations had any advantage over one and another, an important point of the present study was that singly or assays for leptospiral IgM, IgG, and IgA antibodies had no advantage over a single assay for leptospiral IgS antibodies. Moreover, the IgS leptospiral assay was less-time consuming and more economic. The potential diagnostic value of IgS leptospiral antibodies assay was also supported by the earlier detection and a faster declination when compared to the standard MA antibodies (Figure 1 and 2).

In conclusion, from our study we identified a predominance of both specific IgG and IgM isotype leptospiral antibodies specific to cell surface antigens. A single or simultaneous assay for IgG, IgM or IgA leptospiral antibodies had no advantage over IgS leptospiral assay.

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REFFERENCES

1. Faine, S. 1982. Guidelines for the control of leptospirosis. W.H.O. Offset Publication no.67. World Health Organization, Geneva.

2. Silva, M.V., E.D. Camargo, L. Batista, A. J. Vaz, A.P. Brandao, P.M. Nakamura, *et al.* 1995. Behavior of specific IgM, IgG and IgA class antibodies in human leptospirosis during the acute phase of the disease and during convalescence. J.Trop. Med. Hyg. 98: 268-272.

3. Silva, M.V., P.M. Nakamura, E.D. Camargo, L. Batista, A. J. Vaz, E. Romero, *et al.* 1997. Immunodiagnosis of human leptospirosis by dot-ELISA for the detection of IgM, IgG, and IgA antibodies. Am. J. Trop. Med. Hyg. 56:650-655.

4. Eapen, C.K., S. Sugathan, M. Kuriakose, T. Abdoel, H.L. Smits. 2002. Evaluation of the clinical utility of a rapid blood test for human leptospirosis. Diagn. Microbiol. Infect. Dis. 42:221-225.

5. Levett, P. N. and U. C. Whittington. 1998. Evaluation of the Indirect Hemagglutination Assay for Diagnosis of Acute Leptospirosis. J. Clin. Microbiol. 36:11-14.

6. Gowri Priya, C., K. Bhavani, S. R. Rathinam, and V. R. Muthukkaruppan. 2003. Identification and evaluation of lipopolysaccharide antigen for serodiagnosis of uveitis associated with leptospirosis. J. Med. Microbiol. 52:667-673.

7. Winslow, W. E., D.J. Merry, M.L. Pirc, and P.L. Devine. 1997. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection. J. Clin. Microbiol. 35: 1938-1942.

8. Terpstra, W.J., G. S. Ligthart and G. J. Schoone. 1985. ELISA for the detection of specific IgM and IgG in human leptospirosis. J. Gen. Microbiol. 131: 377-385.

9. Adler, B., A. M. Murphy, S. A. Locarnini, and S. Faine. 1980. Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. J. Clin. Microbiol. 11:452-457.

10. Smits, H.L., C.K. Eapen, S. Sugathan, M. Kuriakose, M.H. Gasem, C. Yersin, D. *et al.* 2001. Lateral-flow assay for rapid serodiagnosis of human leptospirosis. Clin. Diagn. Lab. Immunol. 8:166-169.

11. Smiths, H. L, Y.V. Ananyina, A. Chereshsky, L. Dancel, A.F.R.F. Lai, H.D. Chee, *et al.* 1999. Internataional multicenter evaluation of the clinical utility of a dipstick assay for detection of Leptospira-specific immunoglobulin M antibodies in human serum samples. J. Clin.Microbiol. 37:2904-2909.

12. Cumberland, P., C.O.R. Everard, J.G. Wheeler, and P.N. Levett.

2001. Persistence of anti-leptospiral IgM, IgG and agglutinating in patients presenting with acute febrile illness in Barbados 1979-1989. European J. Epidemiol. 17: 601-608.

13. Finsterer, J., C. Stollberger, E. Schnal, G. Stanek. 2005. Mild leptospirosis with three-year persistence of IgG- and IgM-antibodies, initially manifesting as carpal tunnel syndrome. J. Infect. 51:E67-70.

14. Wuthiekanun, V., P. Amornchai, W. Chierakul, A.C. Cheng, N. J. White, S.J. Peacock, *et al.* 2004. Evaluation of Immunoglobulin M (IgM) and IgG Rapid Cassette Test Kits for Diagnosis of Melioidosis in an Area of Endemicity. J. Clin. Microbiol. 42:3435-3437.

15. Flannery, B., D. Costa, F. P. Carvalho, H., J. Matsunaga, E. D. da Silva, A. G. Pinto Ferreira, *et al.* 2001. Evaluation of Recombinant *Leptospira* Antigen-Based Enzyme-Linked Immunosorbent Assays for the Serodiagnosis of Leptospirosis. J. Clin. Microbiol. 39:3303-3310.

16. Appassakij, H., K. Silpapojakul, R. Wansit, and J. Woodtayakorn. 1995. Evaluation of the immunofluorescent antibody test in the diagnosis of human leptospirosis. Am. J. Trop. Med. Hyg. 52 :340-343.

17. Natarajaseenivasan, P., P. Vijayachari, A.P. Sugunun, S. Sharma, and S.C. Sehgal. 2004. Leptospiral proteins expressed during acute and convalescent phases of human leptospirosis. Indian J. Med. Res. 120:151-159.

18. Coleman, R.E., V. Sangkasuwan, N. Suwanabun, C. Eamsila, S. Mungviriya, P. Devine, *et al.* 2002. Comparative evaluation of selected diagnostic assays for the detection of IgG and IgM antibody to Orientia

tsutsugamushi in Thailand. Am. J. Trop. Med. Hyg. 67: 497-503.

19. Rossi, C.L. 1998. A simple, rapid ELISA foe evaluating antibody immunoglobulin G avidity in toxoplasmosis. Diagn. Microbiol. Infect. Dis. 30:25-30.

20. Jeandel, P., D. Raoult, Y. Rougier, C. Auger, and M. Mailloux. 1984. Late positive blood cultures in leptospirosis (report of four cases). Trans. R. Soc. Trop. Med. Hyg. 78:143-145.

21. Abdulkader, R.C., E.F. Daker, E.D. Camargo, C. Spinosa, M.V. da Silva, 2002. Leptospirosis severity may be associated with the intensity of humoral immune response. Rev. Inst. Med. Trop. Soa Paulo 44:79-83.

Table 1 Leptospiral IgG, IgM, IgA, and IgS antibody determinations in acute and convalescent sera of patients with leptospirosis

Disease phase	No. of patients	IIP antibodies : no of positive, % positive, [95% confidence interval], (geometric mean)			
		IgS	IgG	IgM	IgA
Acute	57	37, 64.9% [52.2-77.6%] (75.6)	36, 63.2% [50.3-75.9%] (55.0)	33, 57.9% [44.7-71.0] (51.8)	2, 3.5% [0.0-8.4%] (19.2)
Convalescence	57	57, 100.0% [100.0-100.0%] (995.8)	57, 100.0% [100.0-100.0%] (650.6)	52, 91.2% [83.7-98.7%] (362.9)	32, 56.1% [42.9-69.3%] (52.5)

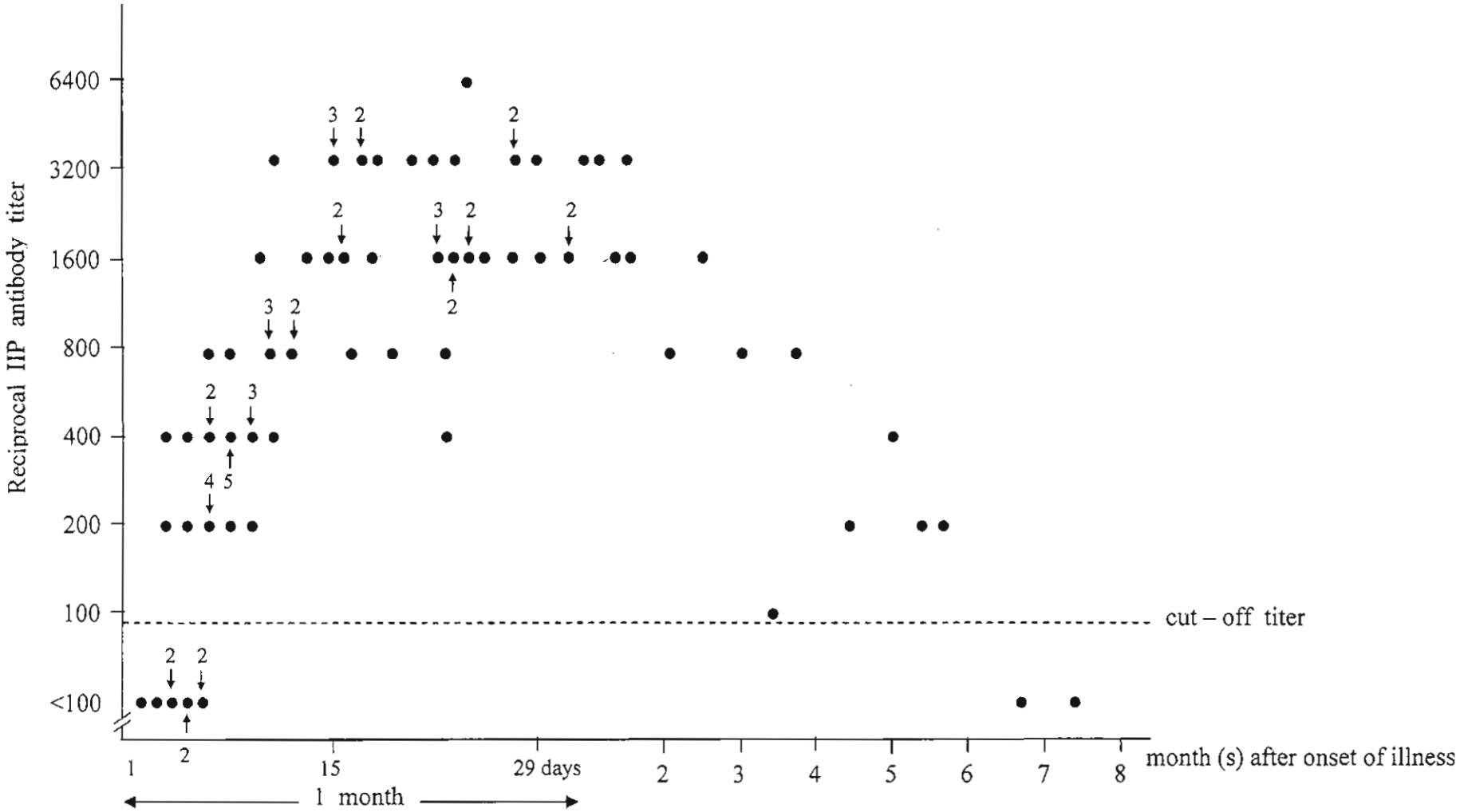

Figure 1 The persistence of leptospiral antibodies in patients with leptospirosis as detected by indirect immunoperoxidase test

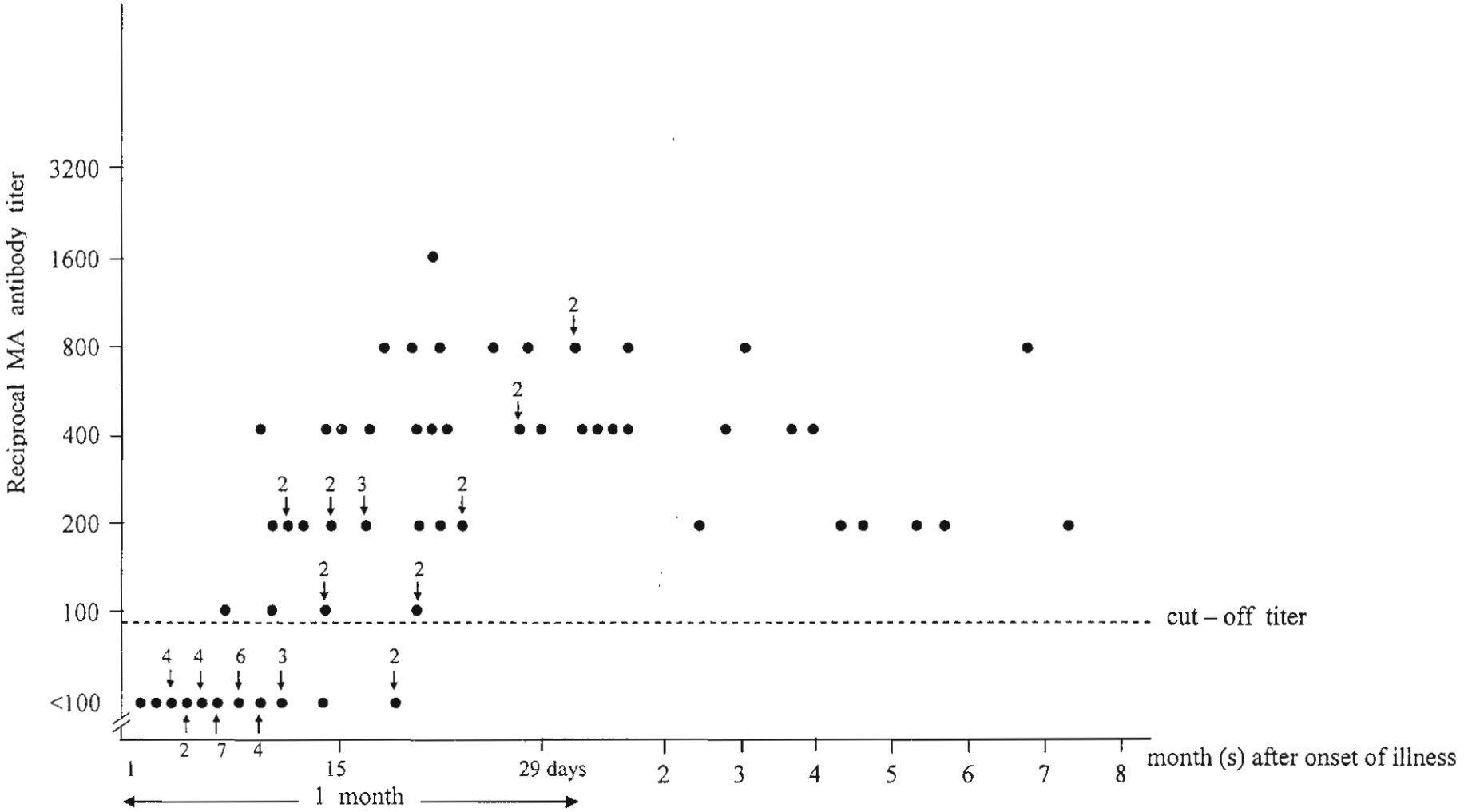

Figure 2 The persistence of leptospiral antibodies in patients with leptospirosis as detected by microscopic agglutination test

SERODIAGNOSIS OF HUMAN LEPTOSPIROSIS BY IMMUNOPEROXIDASE TEST

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ABSTRACT

An indirect immunoperoxidase (IIP) test was developed for the detection of genus-specific anti-leptospiral antibodies. Paired sera from 320 patients with clinically suspected pyrexia of unknown origin were tested with both the IIP test and the microagglutination (MA) test, and the results compared. Overall, the IIP test recognized all 78 proven MA-positive patients with leptospirosis. The performance of the IIP test as a screening test (at the titer ≥ 1:100) was 74.5% sensitive, 91.3% specific, and the likelihood ratios of positive and negative results were 8.6 and 0.3, respectively. While the performance of the IIP test as a confirmatory test at the titer ≥ 1:400 was 65.8% sensitive, 99.4% specific, and the likelihood ratios of positive and negative results were 106.2 and 0.3, respectively. The IIP test in the early phase of disease was more sensitive than the MA test (sensitivity = 47.4% versus 19.4%, respectively). However, all patients demonstrated seroconversion in the paired sera. Regarding the clinical utility of the IIP test in terms of calculations by post-test probabilities of disease, the greatest incremental gain occurred when the pre-test probability was in an intermediate range, 10-50% respectively.

The IIP test can offer an earlier diagnosis than the standard MA test and could be used as a confirmatory test if the paired sera showed seroconversion, or an IIP titer of 1:400 or greater was considered as the cut-off point in single serum. Additionally, the IIP test is practical for use as a routine service in hospitals with limited facilities.

INTRODUCTION

Human leptospirosis is an acute febrile zoonotic disease resulting from infection with *Leptospira interrogans*. The clinical manifestations of leptospirosis are diverse and are often indistinguishable from those of patients with various other diseases including dengue infection, scrub typhus, murine typhus, influenza infection, and typhoid (1). Early treatment with doxycycline or tetracycline antibiotics usually results in a rapid and complete recovery. Thus, laboratory testing to confirm the clinical diagnosis is essential for optimal treatment and patient management.

The microagglutination (MA) test is the current reference method for the serodiagnosis of leptospirosis (2). This test, however, is relatively insensitive in the acute phase of the illness (3). In addition, because the test is relatively serovar specific, it usually requires a battery of more than 20 live leptospira serovars to provide the antigens for many possible strains present in the region. This is time consuming and increases the risk of laboratory-acquired infection.

Several serological tests have been developed with variable results (3-10). In an earlier article the current authors reported on the immunofluorescent antibody (IFA) test for the early diagnosis of human leptospirosis (3). The IFA test has been considered the routine standard serological assay but it is not widely used because of a lack of fluorescence microscopes in leptospirosis endemic areas. To help overcome this limitation, the authors are proposing an alternative assay for the serodiagnosis of human leptospirosis by using an indirect

immunoperoxidase (IIP) test. The IIP test is a modification of the IFA test by replacing the fluorochrome with peroxidase thus allowing the use of an ordinary light microscope instead of a fluorescent microscope to read the test results (11). As the result, the IIP test would be practical for routine use in hospitals with limited facilities.

The main aims of the present study were to develop, standardized, and evaluate the performance and the technical characteristics of the IIP test for the serodiagnosis of human leptospirosis.

MATERIALS AND METHODS

Study design

The study was conducted at Songklanagarind Hospital, Hat Yai, Thailand between 2001 and 2003 with the approval of the Ethics Committee of the Faculty of Medicine, Prince of Songkhla University. Only adults (>15 year old) patients who presented with acute (2-14 days) fever without signs and symptoms suggestive of obvious foci of infection and had paired sera were included in this study. Acute sera were collected from the patients within a week of onset of illness and the subsequent sera were taken 1-2 weeks later. These serum samples were kept at -70°C before being assayed.

The definitive diagnosis of leptospirosis was defined as a patient who had a four-fold or greater increase in the microagglutination (MA) titers (2). Non-leptospirosis patients, who had negative MA tests in their paired sera, were further differentially diagnosed based on serological assays for scrub and murine typhuses and dengue infection. Patients with scrub typhus was defined as having a four-fold or greater immunofluorescent titers for *O. tsutsugamushi* ≥ 1:400. Murine typhus was defined as having a four-fold or greater immunofluorescent titers for *R. typhi* ≥ 1:400. Dengue infection was diagnosed in patients who had four fold or greater dengue hemagglutination titer ≥ 1:1280 and/or positive dengue specific IgM ≥ 40 units/ml) (3). Those who remained nonreactive to the above tests were labeled as having unknown acute undifferentiated fevers.

Cross-reactivity with other spirochetal disease was examined using sera from 35 patients with syphilis (Venereal Disease Research Laboratory titer ≥ 1:8 and positive fluorescent treponemal absorption test)

Autoimmune serum samples obtained from patients with autoimmune diseases, who had positive rheumatoid factors ≥ 20 IU/ml and positive anti-nuclear antibodies, were also tested to determine whether the leptospiral indirect immunoperoxidase test might be affected by non-specific anti-immunoglobulins.

Microagglutination (MA) test

The leptospiral agglutinins were tested using the MA method (2, 3) against a battery of the following 23 *Leptospiral interrogans* serovars: *akayami A, ballico, bangkoki, bataviae, bratislava, bullum, canicola, copenhageni, cellidoni, djasiman, grippotyphosa, hardjo, hebdomadis, hyos, icterohaemorrhagiae, javanica, pomona, pyrogenes, rachmati, saigon, sejroe, tarassovi, wolffi.*

Indirect immunoperoxidase (IIP) test

The IIP test used in this study was modified slightly from that which we had previously described for the leptospirosis IFA assay (3).

Briefly, the test was done as followed: Five to seven day-old cultures containing approximately 10^7 leptospires/ml of *Leptospiral interrogans serovar bataviae* were air-dried, fixed with acetone at room temperature for 10 min, and stored at -20°C until used.

Fifty microlitres of the sera diluted to 1:100 were placed on each circle of the dried antigens and incubated in a moist chamber at 37°C for 30 min. The slides were rinsed once with 0.15 M phosphate-buffered saline (PBS), pH 7.2, and then washed with PBS. Fifty microlitres of the optimal dilution of peroxidase enzyme-conjugated rabbit anti-human total immunoglobulins (IgS) antiserum was then placed by adding on each antigen circle and incubated in a moist chamber at 37°C for 30 min. The slides were rinsed once and washed with PBS and then peroxidase enzyme substrate was added to the antigen circle for 10 min. After stopping the reaction with distilled water, the antigen circles were air-dried and subsequently examined at x400 magnification using an ordinary microscope. A positive result was identified and read when the leptospires appeared with their characteristic spirochaetal morphology as a brownish colour. All the positive serum samples were tested to their end-point titers. Furthermore, both positive and negative serum controls were included in each batch of the assays.

Reproducibility of the IIP test

To demonstrate the reproducibility of the IIP test, regarding the intra-observer variation, a subset of 107 serum samples were tested by the same medical technologists twice. Each time, the serum samples were randomized and the performers were blinded as to the number of the specimens. For the inter-observer study, a subset of 36 serum samples with IIP antibody titers ranging from 1:100 to 1:1600 were similarly and independently tested by two medical technologists.

Statistical methods

Statistical parameters in terms of sensitivity, specificity, likelihood ratios, post-test probabilities and predictive values and their corresponding 95% confidence intervals were calculated in accordance with Bayes Theorem (12). For the evaluation of intra- and inter-observers variation, the degree of agreement between any two tests in classifying an individual sample correctly was calculated using the *kappa* statistic (14). A *kappa* value of more than 0.8 indicates excellent agreement between tests.

RESULTS

Patients

A total of 320 paired sera obtained from 320 patients with acute undifferentiated fevers were independently tested using both the MA and IIP tests. Seventy-eights patients were proven to have leptospirosis on the basis of a four-fold or greater increase in MA antibody titers. The presumptive infective serovars were *bataviae* (69), *hebdomadis* (1), *canicola* (2), *grippotyphosa* (1), and *copenhageni* (5). For non-leptospirosis (MA negative) group, 73 patients were identified as patients with scrub typhus, 10 patients had murine typhus, 45 patients were infected with dengue viruses, and 114 patients remain as unknown acute undifferentiated fever.

Assessment of the performance characteristics of the IIP test

The validities of the IIP test were evaluated in patients with leptospirosis (78) and a non-leptospirosis control group (242), by using the MA test as a gold standard. To determine the optimal cut-off values for the IIP, the performance characteristics were calculated at cut-off titers 1:100, 1:200, 1:400, and 1:800.

Specificity, Sensitivity and the Likelihood Ratios

IIP seropositivity were observed in 1.1% of the patients with dengue infection, 22.6% of the patients with scrub typhus, and 3.3% of the patients with unknown acute undifferentiated fevers. Their sera, which consisted of

21 acute and 18 convalescent samples, had IIP antibody titers ≤ 1:200 with the exception of three patients with unidentified pyrexia of unknown origin who had their IIP antibodies titers of <1:100 and 1:400 in their acute and convalescent sera, respectively. At the titer ≥ 1:400, the performance characteristics of the IIP test as a confirmatory test were 99.4% specific and 65.8% sensitive, and the likelihood ratios of positive and negative results were 106.2 and 0.3 respectively (Table 1).

All the patients with leptospirosis had at least one serum sample reactive at an IIP titer ≥ 1:100. Thus, a cut-off titer of 1:100 can be considered as a positive screening test in a single serum sample (12). At this titer, the IIP test were 74.5% sensitive, 91.3% specific, and the likelihood ratios of positive and negative test results were 8.6 and 0.3, respectively (Table 1). On acute sera testing, the IIP test was 47.4% sensitive at this titer (Table 2). False negative results were found in 41 of 78 (52.6%) patients with leptospirosis upon acute sera testing. However, all 78 patients demonstrated IIP seroconversion in the subsequent tests. The performance of the MA test on acute sera testing, at a cut-off titer of 1:100, was 19.4% sensitive, 98.3% specific, and the likelihood ratios of positive and negative results were 11.8 and 0.8, respectively (data not shown).

Post-test probabilities

Based on the clinician's perspective the post-test probabilities and/or predictive values of disease were evaluated in order to assess the clinical utility of the IIP test for the diagnosis of leptospirosis.

Table 3 shows the outcome of customising the pre-test probabilities and the calculated post-test probabilities of the disease. The greatest incremental gain occurred when the pre-test probability was in the intermediate range, 10-50% but was of little benefit when the pre-test probability was very high (*e.g.* 70% pre-test probability) or very low (*e.g.* 1% pre-test probability). In a situation where the prevalence of the disease is known a clinician's perspectives can be evaluated in terms of predictive values. The predictive values of the IIP test were dependent on the prevalence disease (Table 4). Similar to the resulting post-test probabilities, the greatest incremental gain occurred when the prevalence of disease was in an intermediate range between 10 and 40%, but was of little benefit when the prevalence of disease was very high (*e.g.* 70%) or very low (*e.g.* 1%).

Evaluation of technical characteristics of the IIP test

Cross-reactivity

All these 118 patients with either dengue or scrub typhus infection had IIP titers ≤ 1:200. Only 3 patients with unidentified acute pyrexia of origin had IIP >1:200. (Their IIP antibodies in acute and convalescent patient's sera were <:100 and 1:400, respectively.) In addition, IIP seropositivity up to a titer of 1:1600 were observed in 23% (8/35) of patients with syphilis.

Effect of interference substances in the IIP test

14.0% (7/50) of the sera from patients with autoimmune diseases were reactive in the IIP test (range of titers, 1:100 to 1:400).

Reproducibility of the IIP test

In the IIP reproducibility study, 107 blind serum samples were in 100% agreement by intra-observation testing when performed as qualitative assays (*kappa* value = 1.00). A series of 36 blind serum samples were in 91.7% agreement by inter-observation testing when performed as semi-quantitative assays (*kappa* value = 0.9). Thus, degree of the agreement between two tests was excellent. In no case did the repeated titer differed by more than one dilution.

DISCUSSION

Detection of genus-specific leptospiral antibodies has been suggested as useful for the early diagnosis of human leptospirosis when compared with the MA test (3, 8). Several serological tests have been developed to detect leptospiral antibodies with reports of variable results (3-10). The main explanations given by those reports were due to differences in case definition (14), the spectrum of disease controls used in the study (3, 5), timing of serum sample collection (3, 13), or a combination of any or all of these factors (3-10, 13-14). Additionally, technical problems and the perspective of test result information could limit the clinical utility of those published papers.

In the present study, the genus-specific antibodies detected by IIP test recognised all the 78 MA-positive proven patients. An important point was that the sensitivity of the IIP test depended upon the phase of the diseases and it was found to be relatively insensitive in the early phase of the diseases. False negative results were found in 47.4% (41/78) of the initial sera which were collected within less than seven days of the onset of illness. These negative results were partially caused by collecting serum samples too early. The reasons were supported by our earlier report that the genus-specific leptospiral antibodies appear late during the first week of illness (approximately 52.0%), and peak by the second week of illness (3). Therefore, the significant leptospiral antibodies determined in any single serum sample should be interpreted with relation to the course of illness. Additionally, it is advisable that a second blood sample should be tested if a

negative result is obtained in an initial testing. In the present study, all 78 proven leptospirosis patients seroconverted by the follow up IIP tests.

Generally, the IIP test was highly specific but the important issue was that some patients with clinically suspected leptospirosis (non-leptospirosis controls) had the IIP titers up to 1:200 and based on the cut-off titer of ≥ 1:400, false positive results were found in sera of three patients with unknown acute undifferentiated fevers. These three patients might have been leptospirosis patients but could not be diagnosed by means of the MA test used in this study. Similar results were previously reported by the current authors (3), and by other investigators (14). For example, Jeandel and others have described three culture-proven cases of leptospirosis that remained seronegative upon serial testing up to 35-150 days after the onset of the diseases (14). The reasons for these findings might be the presence of new Leptospira serovar not included in the battery of serovars used in the MA testing, the severity of the disease, the abnormal host response, or the early initiation of antibiotic therapy, which can suppress or delay MA antibody production (3, 14). The other possible explanation for the false positivity includes the cross-reactivity which was also observed in patients with clinically unrelated diseases such as syphilis and autoimmune diseases as demonstrated in this study.

The principal goal of diagnostic tests is to help clinicians increase the probability of making a correct diagnosis. Likelihood ratios are useful in this regard. Thus, one can use the likelihood ratios to link the pre-test to the post-test probabilities by calculation (12) or alternatively use a nomogram for converting pre-test probabilities to post-test probabilities of diseases (15).

Our results have shown that the IIP test was most useful for diagnosis of human leptospirosis in patients with an intermediate range of pre-test probabilities or the prevalence of the disease *e.g.* 10-50% and so was of little benefit when the pre-test probability was very high or very low. These findings support the aim of the diagnostic test in that the IIP test can reduce the level of uncertainty to a level that allows optimal therapeutic decisions (12-13).

The value of a diagnostic test often depends on its reproducibility when applied to patients. The IIP test was reproducible and applicable.

In conclusion, the IIP can offer an earlier diagnosis than that of the standard MA test and was found to be most useful in patients with intermediate pre-test probabilities or prevalence of the disease. It was practical for use as a routine service in hospitals with limited facilities.

ACKNOWLEDGMENTS

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REFFERENCES

1. Levett, P. N. 2001. Leptospirosis. Clin. Micro. Rev. 14:296-326.
2. Faine, S. 1982. Guidelines for the control of leptospirosis. W.H.O. Offset Publication no.67. World Health Organization, Geneva.
3. Appassakij, H., K. Silpapojakul, R. Wansit, and J. Woodtayakorn. 1995. Evaluation of the immunofluorescent antibody test in the diagnosis of human leptospirosis. Am. J. Trop. Med. Hyg. 52:340-343.
4. Sulzer, C. R., and W. I. Jones. 1973. Evaluation of a hemagglutination test for human leptospirosis. Appl. Environ. Microbiol. 26:655-657.
5. Smiths, H. L, Y.V. Ananyina, A. Chereshsky, L. Dancel, A.F.R.F. Lai, H.D. Chee, *et al.* 1999. Internataional multicenter evaluation of the clinical utility of a dipstick assay for detection of Leptospira-specific immunoglobulin M antibodies in human serum samples. J. Clin. Microbiol. 37:2904-2909.
6. Levett, P.N., and S.L. Branch. 2002. Evaluation of two enzyme-linked immunosorbent assay methods for detection of immunoglobulin M antibodies in acute leptospirosis.

Am. J. Med. Hyg. 66:745-748.

7. Adler, B., A.M. Murphy, S.A. Locarnini, and S. Faine. 1980. Detection of specific anti-leptospiral immunoglobulins M and G in human serum by solid-phase enzyme-linked immunosorbent assay. J. Clin. Microb. 11:452-457.
8. Pappas, M.G. 1985. Rapid serodiagnosis using the IgM-specific dot-ELISA: Comparison with the microscopic agglutination test. Am. J.Trop. Med 34:346-354.
9. Silva, M.V., E.D. Camargo, L. Batista, L. A. J. Vaz, A.P. Brandao, P.M. Nakamura, *et al.* 1995. Behavior of specific IgM, IgG and IgA class antibodies in human leptospirosis during the acute phase of the disease and during convalescence. J.Trop. Med. Hyg. 98:268-272.
10. Guerreiro, H., J. Croda, B. Flannery, M. Mazel, J. Matsunaga, M. Galvao Reis, *et al.* 2001. Leptospiral proteins recognized during The humoral immune response to leptospirosis in humans. Infection and Immunity. 69:4958-4968.
11. Taylor, C.R. 1978. Immunoperoxidase Technique. Arch. Path. Lab. Med. 102:113-121.
12. Mayer, D. 2004. Essential evidence-based medicine. Cambridge University Press, Cambridge, UK.
13. Suputtamongkol, Y., S. Sarawish, S. Silpasakorn, U. Potha, K. Silpapojakul, P. Naigowit. 1998. Microcapsule agglutination test for the diagnosis of leptospirosis in Thailand. Am. Trop. Med & Parasitol. 92:797-801.

14. Jeandel, P., D. Raoult, Y. Rougier, C. Auger, and M. Mailloux. 1984. Late positive blood cultures in leptospirosis (report of four cases). Trans. R. Soc. Trop. Med. Hyg. 78: 43-145.

15. Fagan, T.J. 1975. Nomogram for Bayes's theorem. N.Engl.J.Med 293:257.

Table 1 The performance characteristics of the IIP test on overall sera (acute and convalescent sera) testing

Titers	IIP test results				Sensitivity% (CI)	Specificity% (CI)	Likelihood ratios (CI)	
	TP	FP	TN	FN			Positive LR	Negative LR
1:100	120	42	442	41	74.5 (64.7-84.4)	91.3 (87.7-94.9)	8.6 ()	0.3 ()
1:200	113	16	463	48	70.2 (59.8-80.6)	96.7 (95.5-97.8)	21.2 ()	0.3 ()
1:400	106	3*	481	55	65.8 (55.1-76.6)	99.4 (98.4-100.0)	106.2 ()	0.3 ()
1:800	71	0	484	90	44.1 (32.8-55.4)	100.0 (100.0-100.0)	∞ ()	0.6 ()

Note : TP = true positive, FP = false positive, TN = true negative, FN = false negative, CI = 95% confidence interval

* patients with seronegative by the standard MA test but seropositive for at least 4 fold rising titers in paired sera by the IIP test

Table 2 The performance characteristics of the IIP test on acute sera testing

Titers	IIP test results				Sensitivity% (CI)	Specificity% (CI)	Likelihood ratios (CI)	
	TP	FP	TN	FN			Positive LR	Negative LR
1:100	37	21	221	41	47.4 (36.1-58.8)	91.3 (87.7-94.9)	5.5 ()	0.6 ()
1:200	26	13	229	46	33.3 (22.6-44.0)	94.6 (91.7-97.5)	6.2 ()	0.7 ()
1:400	23	0	242	55	29.5 (19.2-39.8)	100.0 (100.0-100.0)	∞ ()	0.7 ()
1:800	1	0	242	77	1.3 (0.0-3.8)	100.0 (100.0-100.0)	∞ ()	1.0 ()

Note : TP = true positive, FP = false positive, TN = true negative, FN = false negative, CI = 95% confidence interval

Table 3 The association between pre-test probabilities and post-test probabilities of the IIP test for the diagnosis of human leptospirosis

Disease phase	Post-test probability of disease for different pre-test probabilities (%)							
	pre-test 1.0%	pre-test 10.0%	pre-test 20.0%	pre-test 30.0%	pre-test 40.0%	pre-test 50.0%	pre-test 60.0%	pre-test 70.0%
Acute phase:								
- screening	5.2 (4.2)#	37.8 (27.8)	57.8 (37.8)	70.1 (40.1)	78.5 (38.5)	84.5 (44.5)	89.1 (29.1)	92.8 (22.8)
convalescent phase :								
-screening*	10.3 (9.3)	55.9 (45.9)	74.2 (54.2)	83.2 (53.2)	88.5 (48.5)	92 (42.0)	94.5 (34.5)	96.4 (26.4)
-confirmatory**	44.6 (43.6)	89.9 (79.9)	95.3 (75.3)	97.2 (67.2)	98.2 (58.2)	98.8 (48.8)	99.2 (39.2)	99.5 (29.5)
Overall phase :								
- screening	7.8 (6.8)	48.5 (38.5)	68.2 (48.2)	78.6 (48.6)	85.1 (45.1)	89.6 (39.6)	92.8 (32.8)	95.2 (25.2)
- confirmatory	45.2 (44.2)	92.1 (82.1)	96.4 (76.4)	97.9 (67.9)	98.6 (58.6)	99.1 (49.1)	99.4 (39.4)	99.6 (29.6)

Note : * calculated values at likelihood ratios for IIP titer of 1: 100

** calculated values at likelihood ratios for IIP titer of 1: 400

The incremental gain of the probabilities of a correct diagnosis (%)

Table 4 The positive and negative predictive values of the IIP test for the diagnosis of human leptospirosis, at various prevalence of disease

Disease phase	Prevalence of disease (%)													
	Prevalence 1.0%		Prevalence 10.0%		Prevalence 20.0%		Prevalence 30.0%		Prevalence 40.0%		Prevalence 50.0%		Prevalence 70.0%	
	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV
Acute phase: -Screening-	0.5 (0.5)#	99.4 (0.4)	37.8 (27.8)	94.0 (4.0)	57.7 (37.7)	87.4 (7.4)	70.1 (40.1)	80.2 (10.2)	78.5 (38.5)	65.1 (12.3)	84.5 (34.5)	63.5 (13.5)	92.7 (22.7)	42.7 (12.7)
Convalescent phase : - confirmatory**	44.5 (54.5)	100.0 (1.0)	89.9 (79.9)	100.0 (10.0)	95.3 (75.3)	100.0 (20.0)	97.2 (67.2)	100.0 (30.0)	98.2 (58.2)	100.0 (40.0)	98.8 (48.8)	100.0 (50.0)	99.5 (29.5)	100.0 (70.0)
Overall phase : - confirmatory	51.5 (50.5)	99.8 (0.9)	92.2 (82.2)	96.3 (6.32)	96.4 (76.4)	92.1 (12.1)	97.9 (67.9)	87.2 (17.2)	98.6 (58.6)	81.4 (21.4)	99.1 (49.1)	74.4 (24.4)	99.6 (29.6)	25.5 (55.1)

Note : PPV = positive predictive value; NPV = negative predictive value

* calculated values at the IIP titer of 1:100 ; ** calculated values at the IIP titer of 1:400

The incremental gain of the probabilities of a correct diagnosis (%)

A Comparative Evaluation of the Indirect Immunoperoxidase and Immunofluorescent Antibody Tests for Diagnosis of Human Leptospirosis

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Abstract

The indirect immunoperoxidase (IIP) test was compared to the immunofluorescent antibody (IFA) test, using the microagglutination (MA) test as a "gold standard". With a study group that included 78 patients with leptospirosis and 242 non-leptosprosis controls, the sensitivities of the two tests in the acute phase were 47.4% and 44.9%, respectively, and the specificities were 91.3% and 90.9%, respectively. In the convalescent phase, the sensitivities and the specificities of the IIP and IFA tests were both 100.0% and 98.8%, respectively. The two tests were highly correlated (coefficiency correlation > 0.8 for all isotypes). There were no significant differences in the measurement of anti-leptospiral antibodies for IgG, IgM, IgA, and total immunoglobulins (IgS) isotypes nor the clinical utility. Both tests are most useful for the diagnosis of human leptospirosis. However, the IIP test is practical for use as a routine service in hospitals that have limited facilities.

Introduction

The detection of genus-specific leptospiral antibodies has been suggested as useful test for the early diagnosis of human leptospirosis when compared with the standard microagglutination (MA) test (1, 16). Several serological tests have been developed to detect leptospiral antibodies with reports of variable results (1-8). The main explanations given by those reports were due to the differences in case definition (8), the spectrum of disease controls used in the study (1, 4), timing of serum sample collection (1, 9), a variety of leptospiral antibodies detection to different leptospiral antigens (1-8) or a combination of any or all of these factors (1-9). Additionally, technical problems and the perspective of the test result information might have limited the clinical utility of those published papers.

The various assays for the detection of leptospiral antibodies specific to cell surface antigens detection by immunofluorescent antibody (IFA) and indirect immunoperoxidase (IIP) showed an earlier detection and a faster declination when compared to the MA antibodies (1, 10). The IFA test has been considered the routine standard serological assay but it is not widely used because of the lack of fluorescence microscopes in leptospirosis endemic areas (1, 8). Recently, the current authors have developed and evaluated the genus-specific leptospiral antibodies detected by the IIP test to investigate antibody isotypes and antibody levels independently (10, 11). It has been suggested that the IIP test is most useful for the diagnosis of human leptospirosis in patients with an intermediate range of prevalence of a disease, *e.g.* 10-40% (10). Of importance is the IIP test can provide a

permanent stained slide for re-examination, which is in contrast to the IFA test that only remains for a few hours (12). This has lead to the facilitation of the development of a quality assurance programme of the IIP test in hospitals with limited facilities located in leptospirosis endemic areas. Ideally, a quality control programme must be performed at each level of the laboratories operations. However, most laboratories in leptospirosis endemic area lack reports on quality assurance results. Relating to the diagnosis of human leptospirosis by the IIP test, large laboratories or central government-supported laboratories with more resources may offer references or inter-laboratory comparisons for small laboratories with greater ease when compared to the other tests.

In general, the IFA and the related IIP are comparable assays and accepted as the "gold standard" test for diagnosis of scrub typhus, murine typhus, other rickettsial diseases (13-14), and leishmaniasis (15). However, a comparison evaluation of the IFA and IIP tests for the clinical utility of human leptospirosis is limited. Thus, a comparative evaluation is further required to support and expand the use of the IIP test as a routine test in those hospitals with limited facilities in the endemic leptospirosis area. The present study describes a comparison of IIP and IFA, using the MA test as the "gold standard", in the evaluation of performance characteristics, test results perspectives and technical characteristics of the tests for the diagnosis of human leptospirosis.

Materials and Methods

Study Design

The study was conducted at Songklanagarind Hospital, Hat Yai, Thailand between 2001 and 2003 with the approval of the Ethics Committee of the Faculty of Medicine, Prince of Songkhla University. Only adults (>15 year old) patients who presented with acute (2-14 days) fever without signs and symptoms suggestive of obvious foci of infection and had paired sera were included in this study. Acute sera were collected from the patients within a week of onset of illness and the subsequent sera were taken 1-2 weeks later. These serum samples were kept at -70°C before being assayed.

The definitive diagnosis of leptospirosis was defined as a patient who had a four-fold or greater increase in the microagglutination (MA) titers (16). Non-leptospirosis patients, who had negative MA tests in their paired sera, were further differentially diagnosed based on serological assays for scrub and murine typhuses and dengue infection. Patients with scrub typhus was defined as having a four-fold or greater immunofluorescent titers for *O. tsutsugamushi* ≥ 1:400. Murine typhus was defined as having a four-fold or greater immunofluorescent titers for *R. typhi* ≥ 1:400. Dengue infection was diagnosed in patients who had four fold or greater dengue hemagglutination titer ≥ 1:1280 and/or positive dengue specific IgM ≥ 40 units/ml) (1). Those who remained non-reactive to the above tests were labeled as having unknown acute undifferentiated fevers.

Cross-reactivity with other spirochetal diseases was exammined using sera from 35 patients with syphilis (Venereal Disease Research Laboratory titer ≥ 1:8 and positive fluorescent treponemal absorption test)

Autoimmune serum samples obtained from 50 patients with autoimmune diseases, who had positive rheumatoid factors ≥ 20 IU/ml and positive anti-nuclear antibodies, were also tested to determine whether the leptospiral indirect immunoperoxidase test might be affected by non-specific anti-immunoglobulins.

Microagglutination (MA) Test

The leptospiral agglutinins were tested using the MA method (16) against a battery of the following 23 *Leptospiral interrogans* serovars: *akayami A, ballico, bangkoki, bataviae, bratislava, bullum, canicola, copenhageni, cellidoni, djasiman, grippotyphosa, hardjo, hebdomadis, hyos, icterohaemorrhagiae, javanica, pomona, pyrogenes, rachmati, saigon, sejroe, tarassovi, wolffi.*

Immunofluorescent Antibody (IFA) Test

The IFA test used in this study was modified slightly from that which we had previously described (1).

Indirect Immunoperoxidase (IIP) Test

The IIP test used in this study was previously described by the current authors (10-11). The various conditions used for the assay were optimised

before being assayed in the serum samples. A stained slide was mounted with permount for keeping as a permanent slide.

Reproducibility of the IFA and IIP Tests

To demonstrate the reproducibility of the IFA and IIP tests, the same medical technologists performed a repeat examination of 60 of the blined serum samples two times for intra-observation testing. Two medical technologists independently examined the same serum samples on two occasions for inter-observation.

Statistical Methods

Statistical parameters in terms of sensitivity, specificity, likelihood ratios, and predictive values were calculated in accordance with Bayes Theorem (17). For the degree of agreement between any two tests in classifying an individual sample correctly was calculated using the *kappa* statistic (17). A *kappa* value of more than 0.8 indicates excellent agreement between tests.

Results

1. Leptospiral Genus-specific Antibodies Detected by the IIP and IFA Tests

One hundred and fourteen serum samples obtained from patients with proven leptospirosis were tested for leptospiral antibodies by the IIP and IFA.

Both tests demonstrated a high correlation (correlation coefficient, r) for the detection of leptospiral total immunoglobulins (IgS) (r=0.9), IgG (r=0.8), IgA (r=0.9), and IgM (r=0.9) (data not shown). The agreement between both tests for the detection of any leptospiral antibody isotypes was excellent (17). There were no significant differences in the measurement of anti-leptospiral antibodies for IgG, IgM, IgA, and total immunoglobulins (IgS) isotypes (Table 1).

2. Assessment of the Performance Characteristics of the IFA and IIP Tests

The validities of the IFA and IIP tests were evaluated in patients with leptospirosis (78) and non-leptospirosis controls (242), and the results compared with that of the MA test. The optimal cut-off values of the IIP and IFA have been characterised previously by the current authors at 1:100 for a screening test and 1:400 for a confirmatory test in a single serum sample (10).

As the results show in Table 2; that generally, both the IFA and IIP showed comparable results for the performance characteristics of the

diagnostic assays. The respective sensitivities of the IFA and IIP tests in the acute phase were 44.9% and 47.4%, respectively, and the respective specificities were 90.9% and 91.3%, respectively. The likelihood ratios of positive results of the IFA and IIP were 4.9 and 5.5, respectively while the negative results were 0.6 and 0.6, respectively.

In the convalescent phase, the sensitivities and the specificities of the IFA and IIP tests were both 100.0% and 98.8%, respectively. The likelihood ratios of positive and negative results of the IFA and IIP were both 80.6 and ∞, respectively. Both assays demonstrated seroconversion in all the patients with leptospirosis.

When both acute and convalescent patient's sera were analysed as a confirmatory test in a single serum sample, the sensitivities and the specificities of the IFA and IIP tests were both 65.8% and 99.4%, respectively. The likelihood ratios of positive and negative results of the IFA and IIP were both 106.2 and 0.3, respectively.

The magnitude of the leptospiral antibody titers of IIP was slightly higher than those of IFA (data not shown).

3. Evaluation of the IFA and IIP as Test Results Perspectives

As the results show in Table 3, both the IFA and IIP tests showed similar results from the test results perspectives in so far as the respective positive predictive values as a screening test in the acute phase were 61.4% and 63.8%, respectively, and the respective negative predictive values were 83.7% and 84.3%, respectively.

In the convalescent phase, the positive predictive values and the negative predictive values as a confirmatory test of the IFA and IIP were both 96.3% and 100.0%, respectively.

When both acute and convalescent patient's sera were analysed as a confirmatory test in a single serum sample, the positive predictive values and the negative predictive values of the IFA and IIP tests were both 97.2% and 90.0%, respectively.

4. Evaluation of the IFA and IIP as Technical Perspectives

4.1 Cross-reactivity

Cross-reactivities at a serum dilution of 1:100 were obtained from patients with dengue infection (1.1%); patients with scrub typhus (22.6%); and patients with unidentified acute pyrexia of origin (3.3%) in both assays. In addition, cross-reactivity in patients with syphilis was observed in 22.9% of the cases by both assays.

4.2 Effect of Serum Interference Substance by the IFA and IIP Tests

14.0% (7/50) of the sera from patients with autoimmune diseases were reactive in both assays.

4.3 Reproducibility of the IFA and IIP Tests

Both of the IFA and IIP tests were reproducible as the intra-observation testing had 100% agreement. The inter-observation of both assays were 98.3% and 95.0%, respectively.

4.4 The Permanence of Test Results as Detected by the IFA and IIP Tests

To re-examine the test results as detected by the IIP test from a permanent stained slide, we tested each sample (n=15) at a period time of 6, 12, 18, 24 months after an initial record. The IIP assay provided the same results every time they were tested. In contrast to the IFA assay, the test results remained for a few hours or a few days if stored in the dark at 4°C.

Discussion

The results from this study have demonstrated that the IFA and IIP tests were comparable for the detection of genus specific leptospiral antibodies to cell surface antigens. Both tests can detect antibody isotypes and antibody titers independently like the common ELISA techniques, but the preparation of an antigen is easier (1, 10, 2, 7). Because the intact leptospiral cells are used in both of the IFA and IIP assays, the result is a clear-cut reading of the end-point of the distinguished spirochaetal morphology (1, 10-11). Thus, there is no need for highly skilled personnel to perform the test. In contrast, the various extracted leptospiral antigens or purified cloned leptospiral antigens used in the ELISA assays initially required to standardise and perform an ELISA need skilled personnel and a well equipped laboratory (2-4, 6-7). Although the ELISA and related ELISA kit are commercially available, they are expensive and consequently they are unaffordable by hospitals with only limited facilities in leptospirosis endemic areas. Moreover, the results of those commercial tests that have been applied in the endemic areas are still questionable (3, 4, 7).

The other advantage of the IFA and IIP assays is that the predominant infective serovars found in an area can be used to increase the maximum sensitivity and it is also easier to standardise the test than ELISA. Alternatively, a broadly reactive genus-specific antigen derived from a saprophytic leptospira biflexa pathoc I can be used without the significant interpretation of the results (data not shown).

Moreover, earlier studies have suggested that at least two ELISA assays were required to increase the maximum sensitive assay by using either paired sera or simultaneous detection of leptospiral IgG and IgM antibodies (2-3, 6). In contrast to the IIP or IFA, a single assay for leptospiral total (IgS) antibodies has been documented as being a diagnostic tool than the simultaneous assays for leptospiral IgG, IgM, and/or IgA antibodies (1, 11) while the same results detected by ELISA have not yet been documented. Thus, both IFA and IIP assays are reproducible, more economic and more applicable than ELISA especially in a routine service in hospitals with limited facilities.

The IFA and the related IIP are generally accepted as the "gold standard" test for some other infectious diseases *e.g.* rickettsiosis (13-14), leishmaniasis (15). However, the results of both assays in those rickettsial diseases may be interpreted inconsistently because of a difference in the antigen preparation that is used in the assays (13-14). This supposition is unlikely to occur for the detection of leptospiral antibodies by the IFA and IIP because of a clear-cut reading for the end-point of the distinguished spirochaetal morphology (1, 10-11). Of importance is the persistence of the IFA and IIP antibodies for leptospirosis which was up to five and a half months (1, 11) while the IFA and IIP antibodies for scrub typhus persisted for many years after recovery (13). In an earlier article the current authors reported the IIP test to be most useful in patients with an intermediate probability or prevalence of the disease, *e.g.* 10-50% (10). Based on the similarity of the test results perspectives of both the IFA and IIP assays, it is implied that the IFA test is also likely to be most useful for patients with an

intermediate probability or prevalence of the disease, *e.g.* 10-50%. These results supported a potential use of the IFA and IIP tests for being used as an alternative standard assay for the diagnosis of human leptospirosis in endemic area (8, 10).

However, the IIP test is considered to have an advantage over the IFA test for routine use in the hospitals with limited facilities. Firstly, the IIP test results can be read with an ordinary light microscope which will allow it to expand and be used as a routine service (12). Thus, the IIP is more applicable in hospital with limited facilities. Secondly, the IIP provide a permanent stained slide for re-examination for at least two years. In our own previous experience, a permanent stained slide for re-examination can last at least 14 years (Appassakij, H., personal communication). As discussed earlier, this advantage will promote the effective use as a routine test for being used for a quality control, as an inter-laboratory comparison or as a development of the network in the external quality assurance programme. Thirdly, the IIP staining has the potential for a higher sensitive assay than the IFA staining (12). This is because the enzyme labelled on antibody conjugates (*e.g.* peroxidase-antiperoxidase conjugate, avidin-biotin conjugate) can amplify a multi-step of a continuous action on the enzyme-substrate which results in a more amplified signal product at the site where leptospiral antibodies bind on leptospiral antigens (12).

This study has demonstrated that both the IFA and IIP tests were comparable. However, the IIP was more practical for use as a routine test in hospitals with limited facilities.

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References

1. Appassakij, H., K. Silpapojakul, R. Wansit, and J. Woodtayakorn. 1995. Evaluation of the immunofluorescent antibody test in the diagnosis of human leptospirosis. Am. J. Trop. Med. Hyg. 52:340-343.
2. Pappas, M.G., 1985. Rapid serodiagnosis using the IgM-specific dot-ELISA: Comparison with the microscopic agglutination test. Am. J.Trop.Med 34:346-354.
3. Winslow, W. E., D.J. Merry, M.L. Pirc, and P.L. Devine. 1997. Evaluation of a commercial enzyme-linked immunosorbent assay for detection of immunoglobulin M antibody in diagnosis of human leptospiral infection. J. Clin. Microbiol. 35:1938-1942.
4. Smits, H.L., C.K. Eapen, S. Sugathan, M. Kuriakose, M.H. Gasem, C. Yersin, *et al.* 2001. Lateral-flow assay for rapid serodiagnosis of human leptospirosis. Clin. Diagn. Lab. Immunol. 8:166-169.
5. Levett, P.N. and U.C. Whittington. 1998. Evaluation of the indirect hemagglutination assay for diagnosis of acute leptospirosis. J. Clin. Microbiol. 36:11-14.
6. Adler, B., A.M. Murphy, S.A. Locarnini, and S. Faine. 1980. Detection of specific anti-leptospiral immunoglobulins M and G in

human serum by solid-phase enzyme-linked immunosorbent assay. J. Clin. Microbiol. 11:452-457.

7. Flannery, B., D. Costa, F.P. Carvalho, H.J. Matsunaga, E. D. Da Silva, A.G. Pinto Ferreira, *et al.* 2001. Evaluation of recombinant *Leptospira* antigen-based enzyme-linked Immunosorbent assays for the serodiagnosis of leptospirosis. J. Clin. Microbiol. 39:3303-3310.
8. Suputtamongkol, Y., S. Sarawish, S. Silpasakorn, U. Potha, K. Silpapojakul, P. Naigowit. 1998. Microcapsule agglutination test for the diagnosis of leptospirosis in Thailand. Am. Trop. Med & Parasitol. 92:797-801.
9. Jeandel, P., D. Raoult, Y. Rougier, C. Auger, and M. Mailloux. 1984. Late positive blood cultures in leptospirosis (report of four cases). Trans. R Soc. Trop. Med. Hyg 78:143-145.
10. Appassakij, H., K. Silpapojakul, S. Sretrirutchai, and P. Khuntigij. Serodiagnosis of human leptospirosis by immunoperoxidase test. (submitted for publication)
11. Appassakij, H., K. Silpapojakul, S. Sretrirutchai, and S. Chookumnerd. Detection of leptospiral IgG, IgM, IgA, and IgS antibodies for the diagnosis of human leptospirosis. (submitted for publication)
12. Taylor, C.R. 1978. Immunoperoxidase Technique. Arch. Path. Lab. Med. 102:113-121.
13. Suto, T. 1985. Laboratory manual of indirect immunoperoxidase antibody titration for rapid serologic diagnosis of rickettsial diseases. World Health Organization. Geneva.
14. McDade, J.E. 1991. Rickettsiae, p1036-1044. *In* A. Balows, W.J.

Hausler, Jr., K.L. Herrmann, H.D. Isenberg, and H.J. Shadony (ed.).Manual of clinical microbiology. American Society for Microbiology, Washington, D.C.

15. Gradoni, L. 2002. The diagnosis of canine leishmaniasis, p.7-14. *In* Canine leismaniasis: moving towards a solution. Proceedings of the 2nd International Canine Leieshmaniasis Forum. Intervet International by Boxmeer, The Netherland.
16. Faine, S. 1982. Guidelines for the control of leptospirosis. W.H.O. Offset Publication no.67. World Health Organization, Geneva.
17. Mayer, D. 2004. Essential evidence-based medicine. Cambridge University Press, Cambridge, UK.

Table3 **Summary of the comparison of the IIP and IFA, using the MA test as a gold standard, in the evaluation of test results perspectives**

Characteristics of the tests	IFA : test (%)	IIP test (%)
Under condition of "test results perspectives" (prevalence = 24.4%)		
: Acute phase (screening)		
- positive predictive value	61.4%	63.8%
- negative predictive value	83.7%	84.3%
: Convalescent phase (confirmatory)		
- positive predictive value	96.3%	96.3%
- negative predictive value	100.0%	100.0 %
: Overall phase (confirmatory)		
- positive predictive value	97.2%	97.2%
- negative predictive value	90.0%	90.0%