Detection of IgM Specific Antibody Using Indirect Immunofluorescent Assay for Diagnosis of Acute Leptospirosis

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Abstract

Leptospirosis is a worldwide zoonosis, caused by *Leptospira interrogans.* At the earlier stage of the disease, the IgM immunoassays are expected to have more sensitivity than other immunoassays. Previous reports showed that the indirect immunofluorescent assay for detection of immunoglobulins (IFA-Igs) against *Leptospira* spp showed higher sensitive and specific than some genus specific tests. The authors determined an efficacy of the indirect immunofluorescent assay for detection of IgM specific leptospiral antibody (IFA-IgM). One hundred and eighty patients with acute febrile illness without localizing signs admitted to Hat Yai Hospital were studied. Using the cut-off value of ≥1 : 400, the overall sensitivity, and overall specificity of the IFA-IgM were 89.2 per cent and 95.1 per cent, respectively. They were slightly greater than those of the conventional IFA-Igs (86.5% and 91.6%). The first sera obtained from patients including acute sera and single sera showed a low sensitivity (32.4%) but still higher than the IFA-Igs (29.7%). None of the patients with various diseases commonly confused with leptospirosis or healthy blood donors gave a titer greater than 1 : 200. In conclusion, the IFA-IgM has the same protocol as the IFA-Igs but the sensitivity and specificity is slightly greater than the IFA-Igs. This is another alternative test for the diagnosis of acute leptospirosis.

Key word : Leptospirosis, Indirect Immunofluorescent Assay, IgM, Immunoglobulins

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Current diagnostic methods for acute leptospirosis are usually based on the demonstration of serum antibodies using serological tests[1]. Since isolation of causative organisms from body fluids is difficult and less sensitive[2,3], the microscopic agglutination test (MAT) is a reference method for diagnosis and detection of antibodies at serovar levels. However, this assay is time-consuming and requires expertise to perform and interpret as well as to maintain a battery of live leptospires[4]. Other serologic approaches have been developed, including the indirect immunofluorescent technique for detection of immunoglobulin IgG, IgM and IgA[5].

Previous studies showed that indirect immunofluorescent assay (IFA) for detection of immunoglobulins (IFA-Igs) against *Leptospira* spp were more sensitive and specific than several serologic tests[6,7]. Since the immunoglobulin M is the first immunoglobulin class appearing in antibody response and most commonly occurs within a few weeks, the IFA for detection of IgM specific leptospiral antibody (IFA-IgM) should be more sensitive than the total immunoglobulins for diagnosis of acute leptospirosis. The authors compared the efficacy of the IFA-IgM and the IFA-Igs with the MAT, a reference method.

MATERIAL AND METHOD

Specimens

Two sets of specimens were used in the present study. The first set was sera which was prospectively collected from 180 patients (95 pairs and 85 singles) who presented at Hat Yai Hospital, Songkhla province, Thailand which is located 930 kilometers to the south of Bangkok, from 1 to 31 December 2000. The patients had acute fever > 38°C for more than 1 day but not exceeding 3 weeks. Exclusion criteria were the presence of profuse rhinorrhea, exudative pharyngitis, pneumonia, urethritis and diarrhea. The second set of specimens was determined for the background of leptospiral antibody using 101 healthy blood donors, 20 patients with syphilis (fluorescent treponema antibody-absorbed test positive 3+ to 4+), 20 patients with anti nuclear antibody (ANA) IFA positive titer ≥ 1 : 1,280 and 62 patients with diseases commonly confused with leptospirosis. These patients consisted of 20 with scrub typhus (IFA titer against *Orientia tsutsugamushi* ≥ 1 : 400)[8], 22 with murine typhus (IFA titer against *Rickettsia typhi* ≥ 1 : 400)[9], and 20 with dengue fever (hemagglutination inhibition antibody titer against dengue virus ≥ 1 : 2,560)[10]. All sera were stored at -80°C until tested.

Microscopic agglutination test (MAT)

The MAT followed the method described by Galton et al[11]. All 23 serovars of *Leptospira* spp were used as antigens: Australis, Ballico, Bratislava, Akayami, Rachamati, Bataviae, Canicola, Cellidoni, Djasiman, Grippotyphosa, Hebdomadis, Hyos, Tarasovi, Icterohemorrhagiae, Copenhageni, Javanica, Saigon, Pomona, Pyrogenes, Sejroe, Hardjo, Wolffi and Andamana. Sera were screened at 1 : 100 by adding 25 µl of serum diluted to 1 : 50 with phosphate buffer saline (PBS) into 23 wells of microtiter plate. Twenty-five µl of each live leptospires serovars was added to each well and then mixed gently. After leaving at room temperature for 2-3 hours, 3 µl of the suspension was dropped on a slide. The agglutination was observed under a dark field microscope (OLYMPUS model BH-2) at a final magnification of 100X. Any serum specimen with a positive reaction was then retested against the respective serovars to determine the endpoint titer which gave the highest dilution. This would be more than 50 per cent agglutination of leptospires. A titer of ≥ 1 : 400 in single serum or a four-fold rising in antibody titer in paired serum was considered as that patient having leptospirosis[6].

Indirect immunofluorescent assay (IFA) for detection of IgM and immunoglobulins (IgG, IgM, IgA)

The IFA was a modified Appassakij's method[5]. Briefly, normal yolk sac was added to the 5-7 days culture of *L. interrogans* serovar Bataviae in neopeptone medium to reach the final concentration of 0.5 per cent. The culture was dotted on the wells of clean taflon-coated slides and air-dried at room temperature. Each slide was then fixed in acetone for 10 minutes, left to air-dry and stored at -70°C until used.

Ten µl of diluted serum (start at 1 : 100) was applied on the slide and incubated at 37°C in a moist chamber for 30 minutes. Each slide was washed three times in phosphate buffer saline (PBS) pH 7.2, then rinsed once with distilled water and air-dried. Ten µl of optimal dilution of fluorescein isothiocyanate (FITC) conjugated rabbit anti-human immunoglobulins M (Dako A/S, Code F0203, Denmark) or immunogloblulins (IgG, IgM, IgA) (Dako A/S, Code F0200, Denmark) was placed on the slide and incubated at 37°C in the moist chamber for 30 minutes. Each slide was washed as done previously. After mounting with glycerol buffer, each slide was examined under a fluorescent microscope (OLYMPUS model BH-2 with FITC filter and exciter filters at a 400X magni-

fication). All positive sera were further diluted and final titers were determined. The endpoint titer was the highest serum dilution giving a visible fluorescence of leptospires. Positive and negative reference sera were included in every batch tested. In the case of detection of immunoglobulins, a patient who had a titer of ≥ 1 : 400 in single serum or a four-fold increase in antibody titer in paired serum was considered as positive[5].

Statistical analysis

The sensitivity, specificity, false positive rate, false negative rate, positive predictive value and negative predictive value of the assays were calculated according to standard method[12]. The kappa statistics for agreement between two independent observers was determined using Statistical Packages for the Social Science release 9.05 (SPSS, Chicago, USA).

RESULTS

A total of 281 serum samples from 180 patients were investigated for leptospiral antibody by the MAT, the IFA-Igs and the IFA-IgM. Thirty-seven patients were found to have leptospirosis and 143 patients were found not to have leptospirosis using the diagnostic criterion by the MAT. The sensitivity and specificity of the indirect immunofluorescent assay for detection of IgM specific leptospiral antibody are summarized in Table 1. The background antibody titer was investigated in healthy blood donors, syphilis, sera with anti nuclear antibody positive and various diseases commonly confused with leptospirosis. The cross reaction results are summarized in Table 2. When using the cut-off value of ≥ 1 : 400, the performance of the IFA-IgM and the IFA-Igs are summarized in Table 3. Although the IFA-IgM was subjective, most of the antibody titers examined by two independent experimenters showed the same titer as the kappa statistic value for agreement of 0.83.

DISCUSSION

An accurate and sensitive method for diagnosis of acute leptospirosis is essential for both clinicians and patients to avoid serious complications

Table 1. The sensitivity and the specificity of the indirect immunofluorescent assay for detection of immunoglobulin M for diagnosis of acute leptospirosis.

Cut-off value	Overall sensitivity*	%	Overall specificity*	%	Sensitivity of the first sera* obtained from patients	%
≥ 1 : 3,200	4/37	10.8	143/143	100	2/37	5.4
≥ 1 : 1,600	18/37	48.6	141/143	98.6	7/37	18.9
≥ 1 : 800	24/37	64.9	139/143	97.2	7/37	18.9
≥ 1 : 400	33/37	89.2	136/143	95.1	12/37	32.4
≥ 1 : 200	36/37	97.3	130/143	90.9	16/37	43.2
≥ 1 : 100	36/37	97.3	127/143	88.8	16/37	43.2

* No of positive sera/No of sera tested

Table 2. Cross reaction assessment to determine the background antibody titer of indirect immunoflorescent assay for detection of IgM specific leptospiral antibody.

IgM titer	Other diseases and negative control group (n)					
	Healthy blood donors	Scrub typhus	Murine typhus	Dengue fever	Syphilis	ANA positive sera
< 1 : 100	100	15	21	20	20	19
1 : 100	1	2	1	0	0	1
1 : 200	0	3	0	0	0	0
1 : 400	0	0	0	0	0	0
Total	101	20	22	20	20	20

Note : ANA = anti nuclear antibody

Table 3. The performances of the IFA-IgM and the IFA-Igs for diagnosis of acute leptospirosis.

Statistic values	IFA-IgM %	IFA-Igs %
Sensitivity	89.2	86.5
Specificity	95.1	91.6
False positive rate	4.9	8.4
False negative rate	10.8	13.5
Positive predictive value	82.5	72.7
Negative predictive value	97.1	96.3
Accuracy	93.9	90.6

and initiate the appropriate treatment[13]. At the earlier stage of the disease, the IgM immunoassays are expected to be more sensitive than other assays. In the present study, using the first sera obtained from the patients at the cut-off titer of ≥ 1 : 400, the authors found that the IFA-IgM tended to be more sensitive (32.4%) than the IFA-Igs (29.7%) (data not shown). The previous report showed that the IFA-Igs had a greater sensitivity when compared to other genus-specific immunoassays such as indirect hemagglutination assay, Dipstick, ELISA and microcapsule agglutination test[7]. This is a great advantage of the IFA over the others. However, the authors found that the overall sensitivity (89.2%) and the overall specificity (95.6%) of the IFA-IgM were slightly higher than those of the IFA-Igs (86.5% and 91.6%, respectively), at cut-off titer of ≥ 1 : 400. It was clear that using the first sera obtained from patients, IFA-IgM showed only one third of the leptospirosis cases. Requirement of convalescent serum is essential to increase the sensitivity of the test.

A low antibody titer could be observed in healthy blood donors or rickettsial diseases. The authors found only one out of 101 blood donors who gave a titer of 1 : 100. Interestingly, five out of 20 (25%) patients with scrub typhus were cross-reacted at titer ≤ 1 : 200. It was known that leptospirosis was a major causes of false positive result in the serodiagnosis of scrub typhus[14]. It is not surprising that scrub typhus can cross reacted to the immunodiagnostic test of leptospirosis and *vice versa*. The reasons for this are unclear, but these two organisms may probably share the common antigen or previous exposure to leptospires in the patients may occur. However, the cross reaction is a minor problem since none of the patients with the diseases commonly confused with leptospirosis and healthy blood donors gave a titer > 1 : 200 and the diagnostic cut-off value used in this study was ≥ 1 : 400.

In conclusion, the IFA-IgM for diagnosis of acute leptospirosis has the same protocol as the IFA-Igs except using FITC conjugated rabbit anti-human immunoglobulins M instead of anti-human immunoglobulins (IgG, IgM, IgA). The time consumption and cost of the assay were not different, but the sensitivity and specificity of the IFA-IgM were slightly higher than the IFA-Igs. This is another alternative test for the diagnosis of acute leptospirosis.

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การตรวจหาแอนติบอดีย์จำเพาะชนิดไอจีเอ็ม ด้วยวิธีอินไดเร็ก อิมมูโนฟลูออร์เรสเซนต์ เอสเสย์ สำหรับวินิจฉัยโรคเลปโตสไปโรซิส

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โรคเลปโตสไปโรซิสเป็นโรคติดต่อจากสัตว์มาสู่คนที่พบได้ทั่วโลก เกิดจากเชื้อ *Leptospira interrogans* ช่วงระยะเริ่มต้นของโรค การตรวจวินิจฉัยที่ตรวจหาแอนติบอดีย์ชนิด IgM มักมีความไวสูงกว่าการตรวจวินิจฉัยอื่น ๆ รายงานก่อนหน้านี้พบว่าการตรวจวินิจฉัยด้วยวิธี อินไดเร็ก อิมมูโนฟลูออร์เรสเซนต์ เอสเสย์ สำหรับตรวจหาภูมิต้านทานรวม (IFA-Igs) ต่อเชื้อ *เลปโตสไปรา* มีความไวและความจำเพาะสูงกว่าวิธีการตรวจชนิดอื่น คณะผู้วิจัยจึงศึกษาประสิทธิภาพของวิธี อินไดเร็ก อิมมูโน-ฟลูออร์เรสเซนต์ เอสเสย์ สำหรับการตรวจหาแอนติบอดีย์จำเพาะชนิด IgM (IFA-IgM) โดยศึกษาในผู้ป่วยที่ป่วยด้วยอาการไข้สูงเฉียบพลันที่ไม่มีแหล่งติดเชื้อเฉพาะที่และเข้ารับการรักษาในโรงพยาบาลหาดใหญ่จำนวน 180 ราย พบว่าเมื่อใช้ระดับนัยสำคัญที่ 1 : 400 ค่าความไวรวมและความจำเพาะรวมของวิธี IFA-IgM มีค่า 89.2% และ 95.1% ตามลำดับ ซึ่งมีค่าสูงกว่าวิธี IFA-Igs เล็กน้อย (86.5% และ 91.6%) การตรวจหาแอนติบอดีย์ในซีรั่มครั้งแรกแม้ว่ามีความไวค่อนข้างต่ำ (32.4%) แต่ก็ยังสูงกว่าวิธี IFA-Igs (29.7%) การศึกษาในกลุ่มผู้ป่วยที่มีอาการใกล้เคียงกับโรคเลปโตสไปโรซิสและในผู้บริจาคโลหิต ไม่พบว่ามีผู้ป่วยหรือผู้บริจาคโลหิตรายใดมีแอนติบอดีย์สูงกว่า 1 : 200 วิธี IFA-IgM มีขั้นตอนการปฏิบัติงานเหมือนกับวิธี IFA-Igs แต่มีความไวและความจำเพาะสูงกว่า ดังนั้นวิธี IFA-IgM จึงเป็นอีกทางเลือกหนึ่งในการตรวจวินิจฉัยโรคเลปโตสไปโรซิส

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