Evaluation of Enzyme-Linked Immunosorbent Assay and Indirect Hemagglutination Assay for Detection of Leptospiral Antibody by Using Three Different Antigens

Janya Nakarin PhD*,
Sukone Pradutkanchana MSc**

** Department of Microbiology, Faculty of Science, Prince of Songkla University*
*** Department of Pathology, Faculty of Science, Prince of Songkla University*

Evaluation of enzyme linked immunosorbent assay (ELISA) and indirect hemagglutination assay (IHA) for detection of leptospiral antibody were performed using three different soluble antigens extracted from Leptospira interrogans serovar Bataviae including heat extracted antigen (HEA), sonicated antigen (SA) and deoxycholate extracted antigen (DEA). One hundred seventy-eight sera from 85 confirmed leptospirosis cases and 202 non-leptospirosis cases were examined. SA-IHA showed the highest sensitivity of 98.8% followed by SA-ELISA-IgM and DEA-ELISA-IgM which showed an equal sensitivity of 97.6%. SA-ELISA-IgM was the most specific (96.5%) test followed by DEA-ELISA-IgM and SA-IHA which gave an equal specificity of 94.1%. The IgG-ELISA tests were less sensitive and specific than the others. These data indicated that the IgG-ELISA tests were not suitable as diagnostic tests for acute leptospirosis in humans. Using acute phase sera, the sensitivity of DEA-ELISA-IgM, SA-ELISA-IgM and HEA-ELISA-IgM were 22.4%, 20.0% and 20.0% respectively, while those of the IgG ELISA tests were 32.9%, 17.6% and 10.6% respectively, the sensitivity of SA-IHA and DEA-IHA were 22.4% and 10.6% respectively. In conclusion, both the IgM-ELISA tests using SA and DEA and the IHA using SA are not different in diagnosis of human leptospirosis. SA is the most suitable antigen for detection of leptospiral antibody by IgM ELISA and IHA.

Keywords : *Leptospirosis, Evaluation, ELISA, IHA, Antigens*

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Serodiagnosis of human leptospirosis by enzyme-linked immunosorbent assay (ELISA) and indirect hemagglutination assay (IHA) have been recognized as sensitive and specific methods[1-4]. The soluble antigens used in serodiagnostic assays for detection of acute human leptospirosis are various types of antigen preparation including heat extracted antigen[5], sonicated antigen[1], deoxycholate extracted antigen[2], cell surface antigen[6], and ethanol extracted antigen[7]. These antigens are genus-specific which showed broadly reactive between serovars. However, it is not certain which antigen is the most suitable antigen for a screening test by ELISA and IHA. Previous studies showed that the sensitivity and specificity of the assays were varied depending on the antigen used[2], diagnostic criterion of the disease[8,9] and the reference method to compare it with[3,10]. The objective of the present work was to evaluate the efficacy of ELISA and IHA for detection of leptospiral antibody using three different antigens. These are heat extracted, sonicated and deoxycholate extracted antigens.

Correspondence to : Nakarin J, Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkla 90112, Thailand. Phone: 0-7428-8311-3, Fax: 0-7444-6661, E-mail: janya.n@psu.ac.th

Material and Method

Study population

Serum samples were obtained from patients admitted to Songklanagarind and Hat Yai Hospitals, which are two major tertiary hospitals serving 200,000 inhabitants of Hat Yai city which is 930 kilometers from Bangkok, in the south of Thailand. One hundred seventy-eight sera from 85 patients who had acute fever > 38°C for more than 1 day but not exceeding 3 weeks were collected. Exclusion criteria were the

presence of profuse rhinorrhea, exudative pharyngitis, pneumonia, urethritis and diarrhea. All sera were assays for leptospiral antibody by microscopic agglutination test (MAT). Only those who had at least two separate serum samples which showed four-fold or greater increase in MAT titer were included in the study. In addition, the background of leptospiral antibody was determined from 100 healthy blood donors, 20 patients with syphilis (fluorescent treponema antibody-absorbed test positive 3+ to 4+), 20 patients with anti-nuclear antibody IFA positive titer $\geq$ 1:1,280 and 62 patients with diseases commonly confused with leptospirosis. These patients consisted of 20 with scrub typhus (IFA titer against *Orientia tsutsugamuchi* $\geq$ 1:400)[11], 22 with murine typhus (IFA titer against *Rickettsia typhi* $\geq$ 1:400)[12] and 20 with dengue fever (hemagglutination inhibition antibody titer against dengue virus $\geq$ 1:2,560)[13]. All sera were kept at -80°C until used.

Antigen preparation

Leptospires of serovar Bataviae were grown in neopeptone liquid medium (Difco Laboratories, USA) which contained 10% young rabbit serum (Biochrom AG, Germanys). Cultures were kept at room temperature for 5-7 days. The organisms were observed under a dark field microscope (OLYMPUS model BH-2). The amount of suitable leptospires was approximately 3+ to 4+ with active movement and no clumping was observed[14].

Heat extracted antigen:

HEA was prepared by a modified Smits's method[5]. Leptospires were centrifuged at 10,000 g, 4°C for 10 minutes. The sediment was washed with phosphate buffer saline (PBS) pH 7.4, then resuspended in PBS. The suspension was boiled for 15 minutes. After centrifugation to remove cell debris, the supernatant was aliquoted into small vials and was stored at -80°C.

Sonicated antigen:

SA was prepared by the modified Adler's method[1]. After washing leptospires with PBS twice by centrifugation at 10,000 g, 4°C for 10 minutes each, leptospires were sonicated in an ice bath at a setting of 20 kHz, 30 seconds each, for the total time of 5 minutes. The sonic extract was aliquoted and kept at -80°C.

Deoxycholate extracted antigen:

DEA was prepared by a method described by Petchclai[2]. Leptospires was washed once with normal saline solution by centrifugation at 10,000 g, 4°C for 10 minutes, then resuspended to 1 in 80 of the original volume in deionized distilled water. The suspension was mixed with an equal volume of 2% deoxycholate in 0.1 M tris-HCl buffer pH 7.4 and incubated at 37°C for 3 hours then centrifuged at 10,000 g, 4°C for 25 minutes. The supernatant was dialyzed against deionized distilled water at 4°C for 4 days and centrifuged at 10,000 g, 4°C for 25 minutes. The supernatant was collected, aliquoted into small vials then kept at -80°C.

Microscopic agglutination test (MAT)

MAT was performed as previously described by Galton *et al*[15], with all 23 serovars of *Leptospira spp.* used as antigens: Australis, Ballico, Bratislava, Akayami, Rachamati, Bataviae, Canicola, Cellidoni, Djasiman, Grippotyphosa, Hebdomadis, Hyos, Tarassovi, 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 PBS pH 7.2 into 23 wells of microtiter plate. Twenty-five µl of each live leptospires serovars were added to each well. The specimens were 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 giving more than 50 per cent agglutination of leptospires.

IgM and IgG-ELISA tests

One hundred µl of an optimal dilution of each leptospiral antigens diluted in 0.01 M carbonate buffer pH 9.6 were added to a microtiter plate (Polysorb, Nunc-Immuno Plate, Denmark). After incubating at 4°C overnight, the plate was washed 5 times with 0.05% tween-20 in PBS pH 7.4. Test and control sera were diluted 1:200 in PBS for HEA and those were diluted 1:200 in 0.05% tween-20 in PBS for SA and DEA. One hundred µl of appropriate serum diluted were added to the wells. After incubating at 37°C for 1 hour, the plate was washed 5 times as described above. An optimal dilution of rabbit anti-human IgM (P0215, Dako A/S, Denmark) or IgG (P0214, Dako A/S, Denmark) peroxidase conjugated was added 100 µl per well and incubated at 37°C for 1 hour, then washed 5 times as

above. One hundred μl of freshly prepared 37 mg orthophenylinediamine dihydrochloride substrate (Sigma Chemical, USA) in 10 ml of 0.1 M citric acid phosphate buffer pH 5.0, with 10 μl of 30% H_2O_2 were added to the wells. The plate was incubated at room temperature for 15 minutes. The reaction was then stopped with 100 μl of 4 N sulphuric acid and measured the optical density (OD) was measured at 490 nm by ELISA reader (Bio-Tek Instruments Inc, USA).

Indirect hemagglutination assay

The IHA was performed according to the method described by Petchclai[16]. Briefly, sheep red blood cells (SRBC) were washed 3 times at 3,000 rpm for 5 minutes in normal saline solution, then adjusted to 10% SRBC with PBS pH 7.2. 2.5% glutaraldehyde was added to 1 in 4 of the volume of suspension. The mixture was rotated for 2 hours at room temperature. After washing 3 times with normal saline solution, the cell suspension was adjusted to 10% with PBS pH 7.2 containing 0.1% sodium azide. The preserved SRBC can be stored at 4°C for a year. Three hundred μl of the preserved SRBC were washed with PBS pH 7.2 then an equal volume of 1:20,000 tannic acid was added. The mixture was incubated at 37°C for 30 minutes. After washing twice with PBS pH 6.4, the cells were adjusted to 10% suspension. Optimal dilution of each antigen was added. The mixture was incubated at 37°C for 30 minutes, then washed twice with PBS pH 7.2 and adjusted to 0.5% SRBC in PBS pH 7.2 containing 0.5% bovine serum albumin and 0.1% sodium azide.

Twenty-five μl of twofold dilution of tested sera (starting at 1:25) were added to 7 wells of U-bottom microtiter plate. Twenty-five μl of sensitized SRBC were then added to each well. A serum control which consisted of 1:25 serum dilution and uncoated SRBC were included for each patient's sera. The suspension was mixed and incubated at room temperature for 2 hours. Hemagglutination was read on a scale of 0 to 4+. The endpoint titer was the highest serum dilution giving ≥ 2+ hemagglutination. Positive and negative reference sera were included in every batch tested.

Statistical analysis

Diagnosis statistic:

The sensitivity, specificity, false positive rate and false negative rate of the assays were calculated according to the method described by Griner[17]. Areas under the receiver operating characteristic (ROC) curves and 95% confidence level were determined using Statistical Packages for the Social Science release 9.05 (SPSS, Chicago, USA). A p-value ≤ 0.05 was considered significant.

Results

ROC curves of the IgM-ELISA tests using three different antigens including HEA, SA and DEA in 85 confirmed leptospirosis cases and 202 non-leptospirosis cases are shown in Fig. 1(a). The area under the curve values was obtained from the ROC curve of HEA-ELISA-IgM, SA-ELISA-IgM and DEA-ELISA-IgM were 0.90, 0.99 and 0.99 respectively (99% confidence level, $P < 0.001$). Even though SA-ELISA-IgM showed an equal value of area under curve to DEA-ELISA-IgM, the sensitivity and specificity of the SA-ELISA-IgM were 97.6% and 96.5%, respectively, at cut-off point of 0.9. DEA-ELISA-IgM had the sensitivity of 97.6% and specificity of 94.1%, when cutting at 0.7. HEA-ELISA-IgM with a cut-off value of 0.9, had the sensitivity of 83.5% and specificity of 80.2%. The analysis of the IgG-ELISA tests using HEA, SA and DEA are shown in Fig. 1(b). The area under the ROC curve values were 0.59, 0.86 and 0.87, respectively (95% confidence level, $P < 0.05$). DEA-ELISA-IgG showed the most accurate results, at a cut-off point of 0.4, with the sensitivity of 84.7% and specificity of 79.2%. SA-ELISA-IgG had the sensitivity of 78.8% and specificity of 71.3%, at a cut-off value of 0.7. HEA-ELISA-IgG gave the lowest accurate results, at a cut-off point of 0.6, the sensitivity and specificity were only 54.1% and 63.4%, respectively. HEA could not be evaluated for the IHA because hemagglutination had not occurred although undiluted HEA was used to coat on SRBC. The ROC curve of the SA-IHA and DEA-IHA is shown in Fig. 1(c). The area under the curve of SA-IHA was the highest (0.99) followed by DEA-IHA (0.90) (99% confidence level, $p < 0.001$). The sensitivity and specificity of SA-IHA were 98.8 and 94.1, respectively at a cut-off value of 1:200, while DEA-IHA had a sensitivity of 81.2% and specificity of 95.0% when cutting at 1:50.

When the appropriate cut-off value of each test was considered, SA-IHA showed the highest sensitivity of 98.8% followed by SA-ELISA-IgM and DEA-ELISA-IgM which showed an equal sensitivity of 97.6%. SA-ELISA-IgM was the most specific (96.5%) test followed by DEA-ELISA-IgM and SA-IHA which gave an equal specificity of 94.1%. The evaluated statistical data is summarized in Table 1.

In the acute phase of leptospirosis, an evaluation of the ELISA and the IHA were analyzed with 85 acute sera. The sensitivity of DEA-ELISA-

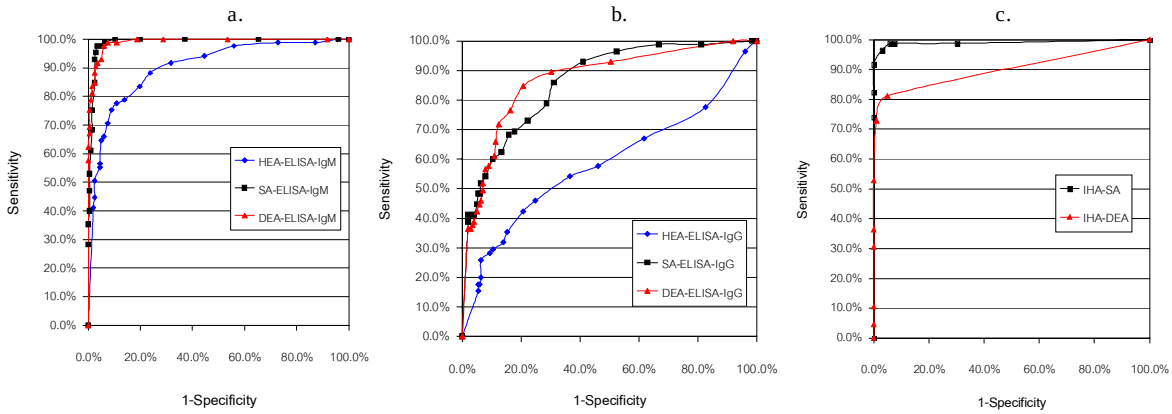

Fig. 1 ROC curve obtained from analysis of the ELISA tests and the IHA using three different antigens. The true-positive rate (sensitivity) is plotted against the false-positive rate (1-specificity)
a. ROC curved of the IgM-ELISA test, b. ROC curved of the IgG-ELISA test, c. ROC curved of the IHA test

IgM, SA-ELISA-IgM and HEA-ELISA-IgM were 22.4%, 20.0% and 20.0%, respectively, while those of IgG-ELISA tests were 32.9%, 17.6% and 10.6%, respectively, and those of the IHA were 22.4% for SA and 10.6% for DEA.

The reproducibility of the ELISA assays were assessed by assaying one reactive serum and one non-reactive serum repeatedly for 20 consecutive days. The result is shown in Table 2. In addition, the precision of the IHA was determined. The titer of positive and negative reference sera did not change within 20 consecutive days. Similar results were observed for the within-run precision. The titers of both reference sera for the SA-IHA did not change until 4 weeks and those of DEA-IHA were longer than 6 weeks.

Discussion

The three antigens used in the present study were prepared from *L. interrogans* serovar Bataviae while most of the antigens used in commercial immunoassays for detection of anti-leptospiral were *L. biflexa* serovar Patoc which was nonpathogenic serovar and broadly reactive. However, previous reports showed that sera from patients with a high titer of MAT reacted strongly with the antigen from Bataviae serovar more than the antigen from Patoc serovar[2,18]. This is not surprising because *L. interrogans* serovar Bataviae is the most common serovar in Thailand, especially in the southern region of Thailand where the study was located[19]. In addition, an antigen from serovar Bataviae also cross-reacted with other serovars in genus specific immunoassay like ELISA or IFA[2,18].

With ROC curve analysis, the area under curve values obtained from the three IgM-ELISA tests were all relatively high when compared to those of IgG-ELISA tests. This indicated that the IgM-ELISA test is more accurate and more suitable use for the diagnosis of acute human leptospirosis than the IgG-

Table 1. Summary of an evaluation of the IgM and IgG-ELISA tests and the IHA for detection of leptospiral antibody using three different antigens with 85 confirmed leptospirosis cases and 202 non-leptospirosis cases

Types of the tests	Sensitivity (%)	Specificity (%)	False positive rate (%)	False negative rate (%)	Cut-off value
HEA-ELISA-IgM	83.5	80.2	19.8	16.5	0.9
SA-ELISA-IgM	97.6	96.5	3.5	2.4	0.9
DEA-ELISA-IgM	97.6	94.1	5.9	2.4	0.7
HEA-ELISA-IgG	54.1	63.4	36.6	45.9	0.6
SA-ELISA-IgG	78.8	71.3	28.7	21.2	0.7
DEA-ELISA-IgG	84.7	79.2	20.8	15.3	0.4
HEA-IHA	NA	NA	NA	NA	NA
SA-IHA	98.8	94.1	5.9	1.2	1:200
DEA-IHA	81.2	95.0	5.0	18.8	1:50

Remark: NA = not available

Table 2. The reproducibility of the ELISA tests for detection of leptospiral IgM and IgG using three different antigens with a single reactive serum and a single non-reactive serum for 20 consecutive days

Types of the tests	Within run (% CV)		Between run (% CV)	
	Reactive	Non-reactive	Reactive	Non-reactive
HEA-ELISA-IgM	7.4	10.3	13.4	12.0
SA-ELISA-IgM	2.5	6.9	8.1	9.8
DEA-ELISA-IgM	4.8	4.6	5.6	13.3
HEA-ELISA-IgG	9.8	11.7	10.7	15.9
SA-ELISA-IgG	7.3	8.8	9.1	11.4
DEA-ELISA-IgG	7.6	11.0	15.1	14.7

CV = coefficient of variance

ELISA test. A previous study showed that the ELISA antibody produced are mainly IgM more than IgG during the early stage of the disease[1]. However, both sonicated antigen and deoxycholate extracted antigen are suitable for the detection of IgM by the ELISA method. SA-ELISA-IgM showed a similar high sensitivity of 97.6% but had a slightly higher specificity (96.5%) than DEA-ELISA-IgM (Fig. 1). These were higher than previous studies that reported a sensitivity of 85-93% and specificity of 89-94%[4,20,21]. Although IgG ELISA tests showed a high value of area under the ROC curve, the sensitivity and specificity of DEA-ELISA-IgG were 84.7% and 79.2%, respectively. These data suggested that the IgG ELISA tests are not suitable for the diagnosis of acute human leptospirosis.

Hemagglutination of the HEA for evaluation of the IHA did not occur. This may be because of the low protein contents of HEA (0.117 μg/μl) or a weak antigenic determinant of the antigen. The ROC curve of IHA indicated that SA is a more suitable antigen for coating on SRBC than DEA. However, when the appropriate cut-off value was considered, the sensitivity and specificity of the SA-IHA were 98.8% and 94.1%, respectively, while those of the DEA-IHA were 81.2% and 95.0%, respectively. Compared to the previous reports, the sensitivity of the IHA was 83-100% and the specificity was 94-99%[22-24].

The sensitivity of all IgM-ELISA tests and IgG-ELISA tests in the acute sera in the present study were very low (less than 33%), compared to a previous report[4]. This is because of the difference in case definition and the early duration of onset of fever. In the present study, only patients who had paired serum samples which showed a four-fold or greater increase in MAT titer were selected. Serum samples with high MAT antibody titer which persisted in acute serum with less than a 4 fold rise in titer were lost. In addition, the average duration of onset of fever in the patients was only 4 days which is too early for antibodies to appear in acute sera.

The reproducibility of the ELISA tests was assessed by assaying one single reactive serum and one single non-reactive serum repeatedly for 20 consecutive days. All sera showed identical results. Most of the tests produced less than 10% of the coefficient of variance (CV). This indicated that the ELISA tests are reliable and have high repeatability. Similar to the IHA, the titers of a single reactive serum and a single non-reactive serum were not changed within 20 consecutive days. This confirmed that the IHA has high precision and reproducibility. The stability of the SA-IHA was 4 weeks while that of DEA-IHA was at least 6 weeks. Imamura et al[25] showed that the IHA using SA was stable for a short period. This may be due to the difference in methods of red blood cell preservation. Imamura used formalin to fix the SRBC before using tannic acid as a coating diluent, while the present study used glutaraldehyde to fix the SRBC then followed by tannic acid. The difference in stability of the test may be due to the difference in methods of RBC preservation. However, Imamura reported that the IHA using DEA had stability for more than 1 year. The stability of the IHA-DEA in the present study was tested for only 6 weeks. But the stability of the IHA-DEA was tended to be longer without changing the antibody titer of the reference sera.

In summary, the present study showed that both the IgM-ELISA tests using SA and DEA and the IHA using SA are not different in the diagnosis of human leptospirosis. The IgM-ELISA test is more suitable for the diagnosis of acute human leptospirosis than the IgG-ELISA test. SA is the most suitable antigen for the detection of leptospiral antibody by IgM-ELISA and IHA.

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การประเมินวิธีเอนไซม์ ลิงค์ อิมมูโนซอเบนท์ เอสเสย์ และอินไดเร็ก ฮีมแอ๊กกลูติเนชั่น เอสเสย์ สำหรับตรวจหาแอนติบอดีต่อเชื้อเลปโตสไปรา โดยใช้แอนติเจนที่เตรียมต่างกัน 3 ชนิด

จรรยา นครินทร์, สุคนธ์ ประดุจกาญจนา

การประเมินวิธี enzyme linked immunosorbent assay (ELISA) และ indirect hemagglutination assay (IHA) สำหรับตรวจหาแอนติบอดีต่อเชื้อเลปโตสไปรา โดยใช้แอนติเจนชนิดละลายน้ำที่สกัดจากเชื้อเลปโตสไปรา ซีโรวาร์ ปัตตาเวีย ได้แก่ heat extracted antigen (HEA), sonicated antigen (SA) และ deoxycholate extracted antigen (DEA) เมื่อทดสอบกับซีรั่มของผู้ป่วยจำนวน 178 ตัวอย่างจากผู้ป่วยโรคเลปโตสไปโรซิส 85 ราย และผู้ป่วยที่ไม่ได้ป่วยเป็นโรคเลปโตสไปโรซิสอีก 202 ราย พบว่า วิธี SA-IHA ให้ความไวสูงสุด ร้อยละ 98.8 รองลงมาคือวิธี SA-ELISA-IgM และวิธี DEA-ELISA-IgM มีความไวร้อยละ 97.6 เท่ากัน ขณะที่วิธี SA-ELISA-IgM มีความจำเพาะสูงสุดร้อยละ 96.5 รองลงมาคือวิธี DEA-ELISA-IgM และวิธี SA-IHA มีความจำเพาะร้อยละ 94.1 เท่ากัน ส่วนการทดสอบ ELISA-IgG มีความไวและความจำเพาะค่อนข้างต่ำ ไม่เหมาะที่จะนำมาใช้ในการตรวจวินิจฉัยโรคเลปโตสไปโรซิสในมนุษย์ เมื่อพิจารณาเฉพาะกลุ่มตัวอย่างตรวจที่เก็บซีรั่มครั้งแรก พบว่าความไวของวิธี DEA-ELISA-IgM, SA-ELISA-IgM และ HEA-ELISA-IgM มีค่าเท่ากับร้อยละ 22.4, 20.0 และ 20.0 ตามลำดับ ขณะที่ความไวของวิธีทดสอบ IgG-ELISA มีค่าเท่ากับร้อยละ 32.9, 17.6 และ 10.6 ตามลำดับ ความไวของวิธี SA-IHA และ DEA-IHA เท่ากับร้อยละ 22.4 และ 10.6 ตามลำดับ โดยสรุปทั้งวิธี IgM-ELISA ที่ใช้แอนติเจน SA และ DEA และวิธี IHA ที่ใช้แอนติเจน SA สามารถนำไปใช้ในการตรวจวินิจฉัยโรคเลปโตสไปโรซิสในมนุษย์ได้ไม่แตกต่างกันมากนัก และแอนติเจน SA เป็นแอนติเจนที่เหมาะสมที่สุดสำหรับการนำมาใช้ในการพัฒนาการทดสอบเพื่อวินิจฉัยโรคเลปโตสไปโรซิสด้วยวิธี IgM-ELISAและ IHA