A Test Strip IgM Dot-ELISA Assay Using Leptospiral Antigen of Endemic Strains for Serodiagnosis of Acute Leptospirosis

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A test strip IgM dot-ELISA assay for the detection of leptospire-specific IgM antibodies in human sera was developed. Antigen dotted on a nitrocellulose paper strip was the pool sonicated antigen prepared from three predominant reactive Leptospira serovars currently in endemic area, i.e., Bratislava, Sejroe and Pyrogenes. The ability of the test to diagnose acute leptospiral infection was assessed by testing 343 single serum samples from 96 laboratory-confirmed leptospirosis case patients with positive result in the standard microscopic agglutination test (MAT), 55 serum samples from patients with various diseases other than leptospirosis, and 192 serum samples from healthy individuals. Using the results of the MAT as a gold standard, the sensitivity and specificity of the test strip IgM dot-ELISA assay were 98.96 and 93.93 per cent, respectively. The assay offered relatively high negative predictive values (99.57%) thus making the assay ideally suited for rapid screening. The stability of the test strip was assessed with a panel of five positive and five negative control sera after storage at 4°C and -20°C at different times. The results showed a good performance of the test strip at both storage temperatures for up to one year. In conclusion, the test strip IgM dot-ELISA assay was sufficiently sensitive for use as a screening test for serodiagnosis of acute leptospirosis. The assay was simple, inexpensive, and easy to perform for both a single test format and a large number of specimens. However, further studies are still needed to improve the stability of the test strip and assay reagents at ambient temperature, and to make the assay more rapidly and more user friendly.

Keywords: *Leptospirosis, Serodiagnosis, Test strip IgM dot-ELISA assay, Endemic leptospiral antigens*

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Leptospirosis is a major public health problem worldwide, particularly in the tropics[1,2]. The clinical presentation of leptospirosis in humans is variable, and can range from a mild flu-like illness to a severe disease with pulmonary hemorrhage, renal failure, and occasionally death[2]. Consequently, leptospirosis is easily mistaken for other febrile illnesses including influenza, dengue fever, meningitis, or hepatitis. Therefore, rapid and appropriate laboratory diagnostic tests are needed to aid clinical case identification and to facilitate the implementation of rapid outbreak investigations for optimal treatment and patient management.

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Laboratory confirmation of human leptospirosis relies mainly on serological assays aimed at the detection of specific antibodies in serum samples. The microscopic agglutination test (MAT) is considered the standard serologic test that is specific and provides useful epidemiologic data in the form of presumptive serogroup[3]. However, this assay is not suitable for routine laboratories since it is technically demanding, costly, and requires the maintenance of live, hazardous stock serovar cultures and also requires analyses of paired sera to verify the seroconversion which delays the diagnosis[1].

Ideally, a diagnostic test should be easy to perform, rapid and using only a single specimen[4]. Some potentially useful screening tests for use in all routine laboratories have been proposed. Among these

serologic approaches, enzyme-linked immunosorbent assay (ELISA) for both IgG- and IgM-leptospiral antibodies have been developed[5,6]; and several commercial test kits are available[3,7-9] and mostly using broadly reactive *Leptospira* antigen obtained from nonpathogenic *L. biflexa* serovar Patoc. However, the use of this serovar may affect the sensitivity of testing in some regions where different leptospiral serovars predominate that do not induce antibodies that crossreact with serovar Patoc[10]. Most of the testing aimed for the detection of leptospiral IgM which is detectable from about the 2nd- 5th day of symptoms[11,12] that can help in the rapid diagnosis of the disease by using a single serum sample.

Recently, the authors developed a microplate IgM-ELISA and an IgM dot-ELISA using broadly reactive *Leptospiral* antigens prepared from locally prevalent serovars including Bratislava, Sejroe and Pyrogenes[13]. Compared with the MAT, both assays had high sensitivity, specificity, and efficiency. The IgM dot-ELISA results correlated well with those of microplate IgM-ELISA. As the results of the high sensitivity and specificity of the IgM dot-ELISA and the test is easy, needs no special equipment, thus makes it suitable for further development of a test kit which could be produced at a lower cost and easily standardized for use in field settings.

In the present study the authors prepared a test strip with the utility of antigen from locally prevalent serovars in a form suitable for diagnostic format. The test strip was evaluated and compared with the standard MAT using single serum samples from patients with known MAT titers. In addition, the stability of antigens dotted on membrane and storage at different temperatures and times was also studied.

Material and Method

Serum specimens

Single serum sample from the acute phase of leptospirosis was selected from a bank of samples from cases identified at Maharat Nakhon Ratchasima Hospital during active hospital-based surveillance from 1998-1999[14,15]. There were 96 patients (50 males and 46 females) aged 12-50 years (mean age 30 years) during hospital admission. A median period of serum collection was 7 days (range, 3 to 14 days) after the onset of symptoms. It was judged to be positive by the MAT at serum titer $\geq$ 1:400. Control serum samples were obtained from 192 apparently healthy individuals with no clinical or epidemiological history of leptospirosis who lived in Nakhon Ratchasima province, and from 55 patients with various diseases other than leptospirosis including syphilis (n = 8), haemorrhagic fever (n = 8), hepatitis B and C (n = 20), scrub typhus (n = 10), and murine typhus (n = 9). None of these control cases reacted in the MAT at serum titer $\geq$ 1:400.

Microscopic agglutination test (MAT)

MAT was performed as described[16], with the following 16 serovars of *L. interrogans* used as antigen: Sejroe, Wolffi, Bratislava, Bangkoki, Ballico, Pyrogenes, Icterohaemorrhagiae, Bataviae, Hebdomadis, Javanica, Poi, Pomona, Canicola, Akiyami, Hyos and Autumnalis. The diagnosis of leptospirosis was confirmed by a MAT titer of $\geq$ 1:400 in one serum specimen.

Preparation of a test strip

Sonicated antigen was prepared from cultures of three serovars, i.e., Bratislava, Sejroe and Pyrogenes. The antigen was used as a pool and prepared as described previously [13]. Briefly, the organism was cultivated in Ellinghausen, McCullough, Johnson and Harris (or EMJH) medium (Difco), and incubated at 30°C with shaking for 7 days to yield a cell density of about 10^8 cells/ml. The organisms were killed with 0.5 mg/L sodium azide, and disrupted by sonication at 20 kHz for 3 periods each of 3 min. The three sonicated leptospiral suspensions were mixed in equal proportions of protein contents (w/v; pool antigen), determined according to Bradford[17].

Pooled sonicated antigen (Ag) was diluted in 0.05 M carbonate buffer (pH 9.6) and 2 µl (protein concentration, 0.3 µg/2 µl) was dotted in duplicate onto a strip (0.8 cm X 2.5 cm) of nitrocellulose (NC) membrane (Hybond-C extra, Amersham). Beside the Ag dot, a control (human IgM prepared from healthy individuals, diluted 1:2) was also dotted and served as a reagent control. After being air-dried, the paper strip was enclosed with blocking buffer (phosphate buffered saline, pH 7.2 containing 0.1% Tween 20 [PBS-T] and 5% bovine serum albumin [BSA]) for 30 min at room temperature. The paper strip was air-dried and was then stored in a small sealed plastic bag at 4°C until used for testing.

Test procedure

The test strip was numbered with the corresponding serum numbers. The test was performed in a 2 ml-microtube by adding 5 µl of serum to 800 µl of PBS-T buffer (PBS, pH 7.2, containing 0.1% Tween 20) to make a dilution of 1:160. Once the serum was mixed

by gentle shaking the microtube, a test strip was added. The microtube was placed down horizontally, and left at room temperature for 30 min with gentle shaking. Each test strip was taken out and washed with PBS-T in the same container for 15 min, 2 times. After blocking in blocking buffer (PBS-T with 5% skim milk) for 15 min, the strips were incubated with horseradish peroxidase (HRP)-conjugated anti-human IgM (Sigma) (1:2500 in PBS-T containing 1% BSA) for 15 min, then washed twice with PBS-T for 15 min, and developed with 3-amino-9-ethyl carbazole (Sigma) for 15 min. The reaction was stopped by rinsing the strips with PBS. Positive and negative control sera were tested simultaneously with all unknown samples. Positive results were indicated visually by the development of the distinct red-pink on spotted sites, whereas negative reactions showed no color on spotted sites.

Stability testing

In order to assess the stability of the antigen test strip, a number of test strips were made and stored at 4 °C and -20 °C. The test strips at each storage temperature were divided into 6 sets (10 strips/set) and were tested at different times, i.e., 0, 1, 2, 4, 6 and 12 months with a panel of five positive and five negative control sera. The control sera (dilution of 1:160) were aliquoted (0.8 ml amount) and stored at -20°C until used for testing. The test results obtained at different times were compared with those results of the first time.

Statistical analysis

Indices of sensitivity and specificity of the test strip IgM dot-ELISA assay were calculated as described[18] by using the results of MAT as the "gold standard". Statistical comparison was performed by using t-test analysis; p of < 0.05 was considered significant.

Results

Among the 343 specimens examined, 96 fulfilled the standard criteria for diagnosis (a titer of $\geq$ 1:400 in MAT for single samples) and were considered as cases of leptospirosis, while 247 control samples including samples from patients with diseases other than leptospirosis (n = 55), and normal healthy controls (n = 192) were negative for leptospirosis and were considered as non-cases. Diagnoses in the 55 patients without leptospirosis included hepatitis B and C (n = 20), syphilis (n = 8), scrub typhus (n = 10), murine typhus (n = 9), and dengue fever (n = 8). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the test strip IgM dot-ELISA assay are summarized in Table 1.

The test strip IgM dot-ELISA assay detected 95 cases of leptospirosis; there were 15 (6.07%) false-positive results; 12 from healthy individuals and 3 from patients with other diseases (i.e., dengue fever, murine typhus, and scrub typhus), and one false-negative result (1.04%). The sensitivity of the test strip IgM dot-ELISA assay for the detection of acute leptospirosis cases was 98.96 per cent, its specificity was 93.93 per cent, its PPV was 86.36 per cent, and its NPV was 99.57 per cent. The test strip IgM dot-ELISA assay showed the results with statistically significant differences from MAT ($p < 0.05$).

For stability study of the test strips which had been stored at 4°C and -20°C, and tested at 0, 1, 2, 4, 6 and 12 months with a panel of 5 positive sera and 5 negative sera, the test results obtained at different times were compared with those results at the beginning of storage. The same results as at the beginning were obtained at different times of storage and at both storage temperatures (4°C and -20°C); however, the color reaction of the test strips with storage at 4°C seem to be more distinct than storage at -20°C. The color reaction of the 5 positive sera could be clearly discriminated from the 5 negative sera, by the positive reactions showed the distinct red-pink spot on the antigenic label sites, whereas the negative reactions were colorless or fading color (data not shown).

Fig. 1 shows some representative results of the test strip IgM-dot ELISA-based detection of

Table 1. Sensitivity and specificity of the test strip IgM dot-ELISA assay performed on 343 serum samples from positive case group sera (n=96) and negative control group sera (n = 247) for serodiagnosis of acute leptospirosis using MAT as a "gold standard"

IgM dot-ELISA	MAT		Total
	Positive	Negative	
Positive	95	15	110
Negative	1	232	233
Total	96	247	343
Sensitivity	98.96%	95%CI = 96.90%-100.00%	
Specificity	93.93%	95%CI = 91.00%-96.80%	
Efficiency	95.33%	95%CI = 93.10%-97.50%	
Predictive value of positive	86.36%	95%CI = 86.15%-86.57%	
Predictive value of negative	99.57%	95%CI = 99.49%-99.65%	

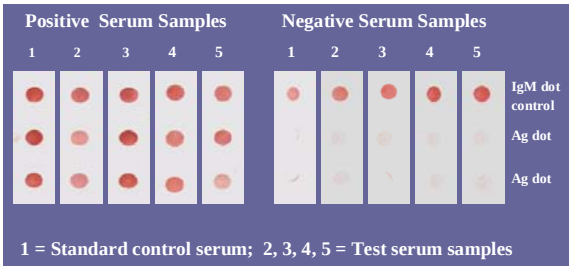

Fig. 1 Representative results of the test strip IgM-dot ELISA using endemic leptospiral antigens for serodiagnosis of acute leptospirosis. The test strip IgM-dot ELISA was tested with 8 test serum samples (4 positive and 4 negative serum samples), and standard control sera (one positive and one negative control sera). Red-pink dots denote positive results while colorless means negative. A positive reaction was defined as a visible test and control dot (IgM), a negative reaction was defined as a positive control dot only

leptospire-specific IgM antibodies. The test strip IgM dot-ELISA assay was tested with 8 test serum samples (4 positive and 4 negative serum samples), and one positive and one negative standard control sera were tested simultaneously with all unknown samples. A positive reaction was defined as a visible test and control dot (IgM), and a negative reaction was defined as a positive control dot only.

Discussion

The diagnosis of human leptospirosis continues to be a serious medical and public health problem in Thailand. It is frequently underdiagnosed, because of several factors including the nonspecific symptoms early in the disease, the inappropriate sample collection, the unavailability of testing facilities, and the difficulty of performing both culture and the reference serological test - MAT[19]. Serodiagnosis of human leptospirosis by an IgM-specific ELISA assay is often used as an alternative to MAT in routine diagnostic laboratories[20]. The MAT detects both IgG and IgM antibodies[21] but the MAT titers are usually low during the acute stage of the disease and, hence, diagnosis based on a single serum sample is difficult[22]. Detection of IgM antibodies by ELISA is more sensitive than the MAT[3] and gives a positive result earlier in the acute phase of the disease. It is easier to perform and can easily accommodate a large number of samples and gives a less subjective result than MAT[12]. However, the limited shelf-life of reagents and the requirement of an ELISA reader to read the results and a continuous electrical supply limit its usefulness in less equipped laboratories. To overcome these problems, ELISA derivatives such as immuno-blot, i.e., dot-ELISA have been developed in many laboratories[4,6] due to the use of smaller volumes of reagents and the possibility of visual readings, no requirement of special equipment, thus the method can be used to diagnose the patients with leptospirosis in peripheral laboratories with relatively little expertise.

Though several commercial kits based on IgM-ELISA in a number of modifications are now available for screening of acutely ill patients, the reported sensitivities and specificities of these assays were varied according to the *Leptospira* serovars used and the procedures for antigen extraction. Comparative evaluation of several commercial test kits for use as rapid screening methods for serodiagnosis of acute leptospirosis in different countries[23-25] showed the variability in screening test sensitivities and specificities. The reported sensitivities may be affected by several factors, including the differences in the case definitions, and the prevalence of the various different infecting serovars and serogroups in different areas. The screening test's sensitivity in any given setting is dependent on the ability of test antigens to detect antibodies produced against the site-specific leptospiral serovars. Hence, laboratories need to validate the performance of these screening tests for use in the setting[25].

Although several commercial test kits are available and some offer good sensitivity for testing in Thailand, they are still expensive for use in developing countries and some are not appropriate for single-sample testing or for use in the field and in less equipped laboratories. So, the authors have recently developed an IgM-ELISA in both conventional microplate and dot format for the detection of leptospire-specific IgM antibodies[13]. In order to achieve high sensitivity and specificity in the acute phase of illness, thus, the authors focused on detecting IgM binding to whole-cell antigen prepared from three dominant serovars of *Leptospira* including Bratislava, Sejroe and Pyrogenes which were found to be associated with disease in Thailand.

As the results of the high sensitivity and good specificity of the IgM dot-ELISA assay obtained with the utility of pool sonicated antigen prepared from locally prevalent serovars of *Leptospira* made it suitable for further development of a test kit which could be produced at lower cost and easily standardized for use in field settings. In the present study the authors prepared a test strip dot-ELISA with the utility of endemic leptospiral antigens in a form

suitable for diagnostic format in either a single assay or a large number of samples and aimed to be used in peripheral laboratories to screen for human leptospiral infection. Based on the clinical presentation and occupational activities of the patients will have made the potential usefulness of the test strip to rapid detection of specific IgM antibodies in human leptospirosis.

From the results presented in Table 1, it can be seen that the test strip IgM dot-ELISA assay compared very favorably with the MAT test. Only one serum sample failed to react in the IgM-dot ELISA assay, but was reactive in the MAT (sensitivity, 98.96%; 95%CI, 97%-100%). The possibility of getting high sensitivity in the present study was strongly associated with the MAT titers. Since the criteria for confirmation of current leptospiral infection for a single serum available in the present study was a MAT titer of 1:400 or more. All 96 cases which were considered as cases of leptospirosis in the present study had MAT titer > 1:400. In comparison of eight rapid screening tests by Effler et al[25], also reported specimens with higher MAT titers were significantly more likely to test positive in each of the screening tests; and in recent evaluation of a new assay kit, Lepto lateral flow, Sehgal et al[26] also found the positivity rate of the test increased from 21 per cent in MAT-negative samples to 54.5 per cent in samples with a MAT titer of 1:100 and 81.8 per cent in samples with a titer of 1:400.

In the present study the assay was sensitive for infections with strains of several serogroups as described previously when using these MAT-positive serum samples (serum titers $\geq$ 1:400)[13]. These serogroups included Sejroe, Pyrogenes, Australis (serovar bratislava, balico, bangkoki), Hebdomadis, Javanica and Pomona. For epidemiological interest, further studies may be needed to determine whether the assay is sensitive for strains of other serogroups. However, knowledge of the serogroup has no clinical implications.

The reported sensitivities may be affected also by the time of specimen collection. All tests evaluated by Effler et al[25], were insensitive for testing of samples collected within the first week following illness onset, but test sensitivities improved during the second week post-illness onset. In the present study, serum specimens were collected from patients during hospital admission, a median of 7 days (range, 3-14 days) after the onset of symptoms. Thus, the test which is sensitive early in the acute phase of illness would be of great benefit to both the clinician and the patient. Further study may be needed to determine the sensitivity of the assay for samples collected at different times.

Specificity was 93.93 per cent (95% CI, 91-97%) in the test strip IgM-dot ELISA compared to the MAT test. Fifteen dot ELISA-positive/MAT-negative were obtained from 12 healthy individuals and 3 patients with other diseases. The false positive reactions were mostly found in healthy individuals. The authors could not determine whether the IgM antibody detected in these healthy individuals and in patients with nonleptospiral infections was persisting antibody from a previous leptospiral infection or cross-reacting antibody. In this study, the test strip IgM dot-ELISA assay performed with sera at 1:160 dilution as previously optimized[13] to eliminate the low antibody background. However, antigens dotted on the test strip were a crude sonicated preparation of 3 pathogenic leptospiral serovars, they are broadly reactive antigens which include proteins and lipopolysaccharides shared among diverse leptospiral serovars both pathogenic and saprophytic leptospires and other spirochetes. Currently Cumberland et al[27] found patients who had severe leptospirosis commonly remained seropositive, with IgM, IgG and agglutinating antibodies detectable for several years after infection. This can create problems in the interpretation of serological results. Thus, in endemic areas where seroprevalence is high, use of a single elevated titer is not reliable to define a current infection. However, ELISA and other rapid serologic tests based on whole-cell leptospiral antigen preparations are suitable for use as alternative methods to screen for leptospiral infection, although MAT is still required for confirmation.

This assay offered relatively high negative predictive values (99.57%), thus making the assays ideally suited for rapid screening. The positive predictive value (PPV) of the dot ELISA was 86.36 per cent. However, the predictive values will vary with the prevalence of patients with leptospirosis among those suspected on clinical grounds of having the disease. The prevalence will depend on the endemicity of disease and on clinical practice. When the prevalence is low, the PPV is relatively low and a positive result should be confirmed by the MAT.

Although the tests described can be improved and still have to be evaluated further by analysing more samples both of positive and control serum samples from healthy individuals and from

other febrile illnesses, i.e, influenza, dengue fever, meningitis, salmonellosis, hepatitis, etc. The preliminary results are very promising and give a lead to further studies in order to improve the stability of the test strip and assay reagents at ambient temperature, to make the assay more rapidly and more user friendly. Furthermore, the authors aim to develop another test strip dot ELISA assay with the utility of purified antigen or partially purified antigens extracted from outer membrane. Recently, a recombinant technology was found the utility of recombinant *Leptospira* antigen-based ELISA assays for the serodiagnosis of human leptosprosis[28]. Of several outer membrane proteins, LipL32 is a major leptospiral lipoprotein whose expression is restricted to pathogenic *Leptospira* species and highly conserved across leptospiral species[29]; and rLipL32 has been reported to be an useful antigen for the serodiagnosis of leptospirosis[28]. However, the preparation of purified antigens by using recombinant technology is costly and highly sophisticated and complicated method. An alternative approach will be the use of partially purified antigens which can be isolated from the outer membrane of the organism by using a simple and inexpensive method, i.e. Triton X114-extraction method[29,30].

In conclusion, the test strip IgM dot-ELISA assay using endemic leptospiral antigens offered good sensitivity, specificity and reproducibility yielding accurate results comparable to the reference MAT. The assay was simple, inexpensive, and easy to perform, with visual reading of the results that do not require special equipment. The test strip was stable at either 4°C or -20°C for up to one year. Performance of the assay was also useful for both a single assay format and a large number of specimens which could be completed in approximately 2 hours. Thus, it could be used as an initial screen for leptospiral infection, with subsequent confirmation of positive test results by MAT.

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การตรวจวินิจฉัยโรคเลปโตสไปโรซิสระยะเริ่มแรก ด้วยแผ่นทดสอบ IgM dot-ELISA ที่เตรียมจากแอนติเจนของเชื้อเลปโตสไปราที่ก่อการระบาด

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การตรวจหาแอนติบอดีชนิด IgM ที่เฉพาะต่อเชื้อเลปโตสไปราในซีรัมของผู้ป่วยโรคเลปโตสไปโรซิสระยะเริ่มแรกด้วยวิธี IgM dot-ELISA ได้ถูกพัฒนาขึ้นเป็นแผ่นทดสอบชิ้นเล็กๆ โดยแอนติเจนที่หยดเป็นจุดบนแผ่นไนโตรเซลลูโลสเป็นแอนติเจนชนิดผสมที่เตรียมมาจากเชื้อเลปโตสไปรา 3 สายพันธุ์ที่เกี่ยวข้องกับการระบาดมากสุด คือ bratislava, sejroe และ pyrogenes ซึ่งทำการสลายเซลล์ด้วยคลื่นเสียง ในการทดสอบประสิทธิภาพของแผ่นทดสอบที่เตรียมขึ้นได้ทำการประเมินผลเปรียบเทียบกับวิธี microplate IgM-ELISA โดยอาศัยผลของวิธี microscopic agglutination test (MAT) เป็นวิธีมาตรฐานในการยืนยันผลการตรวจกับตัวอย่างซีรัมทั้งหมด จำนวน 343 ตัวอย่าง ซึ่งประกอบด้วยซีรัมเดี่ยวที่ได้จากกลุ่มผู้ป่วยโรคเลปโตสไปโรซิส จำนวน 96 ราย กลุ่มผู้ป่วยที่เป็นโรคอื่นๆ ที่ไม่ใช่เลปโตสไปโรซิส จำนวน 55 ราย และกลุ่มคนปกติที่ไม่เป็นโรค จำนวน 192 ราย

ผลการศึกษาพบว่า การวิเคราะห์ด้วยแผ่นทดสอบ IgM dot-ELISA ให้ค่าความไว และ ความจำเพาะ คิดเป็นร้อยละ 98.96 และ 93.93 ตามลำดับ การวิเคราะห์ด้วยแผ่นทดสอบ IgM dot-ELISA ยังให้ค่าทำนายผลลบที่สูงคือร้อยละ 99.57 จึงเหมาะสำหรับการตรวจคัดกรอง ส่วนการทดสอบความคงตัวของแผ่นทดสอบที่แยกเก็บที่อุณหภูมิ 4 องศาเซลเซียส และ -20 องศาเซลเซียส ในเวลาต่างๆ กัน และนำออกมาทดสอบกับชุดตัวอย่างซีรัมมาตรฐานกลุ่มบวก 5 ตัวอย่าง และกลุ่มลบ 5 ตัวอย่าง พบว่า แผ่นทดสอบของ IgM dot-ELISA นี้ให้ผลการตรวจที่ถูกต้อง และยังคงประสิทธิภาพของปฏิกิริยาได้เป็นอย่างดีเมื่อครบเวลา 1 ปี

โดยสรุป การตรวจวิเคราะห์ด้วยแผ่นทดสอบ IgM dot-ELISA มีความไวสูงเพียงพอที่จะใช้ในการตรวจคัดกรองผู้ป่วยที่สงสัยโรคเลปโตสไปโรซิสในระยะเริ่มแรกของโรคได้ อีกทั้งยังเป็นวิธีการที่ง่าย ราคาถูก และแผ่นทดสอบยังเหมาะกับการตรวจตัวอย่างซีรัมในจำนวนน้อย ๆ ที่มีเพียงหนึ่งตัวอย่าง หรืออาจตรวจกับหลาย ๆ ตัวอย่างได้พร้อมกัน อย่างไรก็ตาม การศึกษานี้เป็นเพียงพื้นฐานเพื่อการศึกษาพัฒนาในขั้นตอนต่อไป โดยเฉพาะในเรื่องความคงตัวของแผ่นทดสอบและของน้ำยาตรวจสอบ ที่ควรคงตัวอยู่ได้ที่อุณหภูมิห้อง รวมทั้งขั้นตอนของการตรวจที่ง่าย สะดวก และให้ผลที่รวดเร็วขึ้น