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Development of enzyme-linked immunosorbent assay and dot-enzyme-linked immunosorbent assay for detection of *Leptospira* specific IgM antibodies

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Problem/ background : *Since the number of patients with leptospira in Thailand has increased and the standard test for laboratory diagnosis is time-consuming and requires a well-train operator, we have developed two rapid methods two methods for using as rapid diagnostic tests for this infectious disease.*

Objective : *Develop an enzyme-linked immunosorbent assay (ELISA) and a dot-enzyme-linked immunosorbent assay (dot-ELISA) for detection of leptospira specific IgM antibodies. These assays can be used as rapid diagnostic tools for leptospirosis.*

Design : *Descriptive study*

Setting : *Department of Microbiology, Faculty of Medicine, Chulalongkorn University*

Materials and Methods : *ELISA and dot-ELISA were developed and tested with sera from healthy individuals, sera positive for antibodies specific to Treponema pallidum and sera positive by macroscopic agglutination test (MAT), a reference method. Sensitivity and specificity was used to compare both ELISA and dot-ELISA with MAT.*

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Results : *The sensitivity of ELISA and dot-ELISA compared with MAT is 75 and 100 %, respectively. ELISA showed cross-reactivity with sera positive for antibodies to Trepomema pallidum whereas none of the samples in this group gave positive results by dot-ELISA.*

Conclusions : *This study demonstrated that dot-ELISA, an easily performed assay, has high sensitivity and specificity compared with MAT, the reference method, and should be used as a rapid test for detection of leptospira specific IgM antibodies.*

Keywords : *Leptospira, Leptospirosis, ELISA, dot-ELISA.*

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เหตุผลของการทำวิจัย : *เนื่องจากปริมาณผู้ป่วยติดเชื้อเล็ปโตสไปราในประเทศไทยเพิ่มขึ้นอย่างรวดเร็ว แต่วิธีมาตรฐานที่ใช้ในการช่วยวินิจฉัยโรคนี้ (MAT) ทำได้ยาก และการอ่านผลต้องอาศัยผู้ที่มีประสบการณ์ ผู้วิจัยจึงได้พัฒนาวิธีการตรวจที่ทำได้ง่ายและรวดเร็ว*

วัตถุประสงค์ : *พัฒนาการทดสอบวิธี enzyme-linked immunosorbent assay และวิธี dot-enzyme-linked immunosorbent assay สำหรับตรวจหาแอนติบอดีชนิด IgM ต่อเชื้อเล็ปโตสไปรา เพื่อใช้เป็นวิธีทดสอบรวดเร็วสำหรับการช่วยวินิจฉัยโรคติดเชื้อเล็ปโตสไปรา*

รูปแบบการวิจัย : *การศึกษาเชิงพรรณา*

สถานที่ทำการศึกษา : *ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย*

ตัวอย่างและวิธีการศึกษา : *พัฒนาวิธี enzyme-linked immunosorbent assay (ELISA) และวิธี dot-enzyme-linked immunosorbent assay (dot-ELISA) เพื่อใช้ตรวจหาแอนติบอดีชนิด IgM ต่อเชื้อเล็ปโตสไปรา และนำมาทดสอบกับน้ำเหลืองจากกลุ่มคนปกติ น้ำเหลืองผู้ป่วยที่ให้ผลบวกแอนติบอดีต่อเชื้อ Treponema pallidum และน้ำเหลืองผู้ป่วยที่ให้ผลบวกโดยวิธี macroscopic agglutination test (MAT) ซึ่งเป็นวิธีมาตรฐาน และคำนวณค่า sensitivity และ specificity เพื่อเปรียบเทียบทั้ง 2 วิธีกับวิธีมาตรฐาน*

ผลการศึกษา : *ความไวของวิธี ELISA และ dot-ELISA เทียบกับวิธี MAT เป็น 75 และ 100 % ตามลำดับ วิธี ELISA ให้ผลบวกกับน้ำเหลืองจากผู้ป่วยบางรายที่ให้ผลบวกแอนติบอดีต่อเชื้อ Treponema pallidum ในขณะที่วิธี dot-ELISA ให้ผลลบเมื่อทดสอบกับน้ำเหลืองในกลุ่มดังกล่าวทุกรายซึ่งสอดคล้องกับวิธี MAT*

สรุป : *จากการศึกษาพบว่าวิธี dot-ELISA ซึ่งเป็นวิธีที่ทำได้ง่าย ไม่ต้องใช้เครื่องมือพิเศษ มีความไวและความจำเพาะสูงเทียบกับวิธี MAT ซึ่งเป็นวิธีมาตรฐาน น่าจะใช้เป็นวิธีทดสอบรวดเร็วสำหรับการช่วยวินิจฉัยโรคติดเชื้อเล็ปโตสไปรา*

คำสำคัญ : *เล็ปโตสไปรา, เล็ปโตสไปโรซิส, ELISA, dot-ELISA*

Leptospirosis is a worldwide zoonosis caused by bacteria belonging to the genus *Leptospira*. Symptoms can be high fever, chills, muscle aches, conjunctivitis and vomiting. It can be fatal in some cases which results from injury to kidney and liver especially with virulent strains and in elderly people.

The number of patients diagnosed with leptospirosis in Thailand reported by the Public Health Ministry (1999) has increased about 10 times within 5 years. It is difficult to diagnose this infectious disease because of the wide spectrum of clinical manifestations. Patients can show mild to rapidly fatal symptoms. The mortality rate is high in patients with severe symptoms and the clinical manifestations may be confused with other common febrile illnesses such as dengue fever, typhoid, viral hepatitis and malaria. [1-3] Therefore, laboratory diagnosis is essential for effective therapy.

The culture of leptospirosis from body fluids is the most accurate test. However, the isolation may take up to 2 months and it is not always successful. For that reason, the definitive diagnosis of leptospirosis relies mainly on serological tests. Antibodies to leptospira can be detected 6 -10 days after infection. The microscopic agglutination test (MAT) is the reference standard serodiagnostic test. However, MAT is a time-consuming test and requires a well trained-operator. The maintenance of live organisms is necessary for the test, which increases the risk of laboratory-acquired infection. For this reason, MAT is done only in a few laboratories. In addition, the antibody levels determined by MAT can be detected for several years after infection. The use of paired sera is always required to demonstrate a four-fold rise in antibody titers. Several serological tests such as macroscopic slide agglutination, hemagglutination and latex agglutination have been developed.[4-11] It is easier to performed those tests than MAT. However, paired sera are still required to demonstrate a four-fold rise in antibody titers. A polymerase chain reaction (PCR) assay for detection of DNA of leptospira in body fluid has also been developed [12-13] but is not widely used because it requires special equipment and a well-trained operator In addition, the cost of PCR assay is still too high to be used as a differential diagnostic tool.

A serological test that can demonstrate whether antibodies detected in serum samples are IgM or not, will be very useful as a rapid and effective diagnostic tools for leptospirosis and will be an excellent alternative to MAT. We have developed an enzyme-linked immunosorbent assay (ELISA) and a dot-enzyme-linked immunosorbent assay (dot-ELISA) for the detection of IgM antibodies specific to leptospires. Our results demonstrate that the sensitivity of ELISA is low (75 %) compared with MAT. It also showed cross-reactivity with serum samples from patients with syphilis. In contrast, the sensitivity of our dot-ELISA is 100 % compared with MAT which suggests that dot-ELISA may be used as a screening test for diagnosis of leptospirosis.

Materials and Methods

Serum samples

The following groups of serum samples were used : 20 samples obtained from patients admitted at King Chulalongkorn Memorial Hospital and leptospirosis was suspected. Sera were confirmed positive by a macroscopic agglutination test (MAT) at the National Institute of Health (NIH), Thailand ;

20 samples which were positive for antibodies specific to *Treponema pallidum* (were collected and tested by indirect immunofluorescence assay at Department of Microbiology, Faculty of Medicine Chulalongkorn University) ; and 20 sera samples from healthy individuals.

Microscopic agglutination test (MAT)

MAT was performed at NIH, Thailand. Briefly, diluted serum samples were incubated with different serovars of leptospira and agglutination was observed under the microscope.

Antigen preparation for enzyme-linked immunosorbent assay (ELISA) and dot-ELISA

Leptospira biflexa was kindly provided by Dr. Wipe J. Terpstra (Royal Tropical Institute, Amsterdam, Netherlands). Heat-stable antigen was prepared from a culture of *Leptospira biflexa* as previous described.[9] Briefly, leptospires were grown in leptospira medium base EMJH (Difco, USA) containing leptospira supplement (Difco) for 5 days. Bacteria were heated for 30 minutes and centrifuged. The culture supernatant was collected to be used as the antigen. Protein concentration was determined by Lowry's method and antigen is kept frozen at –70°C .

ELISA

The appropriate antigen concentration used in the assay was determined using negative control samples (from healthy individual with MAT negative) and positive control samples (from patient where leptospirosis was diagnosed and MAT was positive). The antigen concentration used in this assay was 3 μg/ml. ELISA for detection of leptospira specific IgM antibodies was performed as follows: Flat bottom microtiter plates were coated overnight at 4 °C with 50 μl of antigen (diluted in 0.1 M bicarbonate buffer, pH 9.6) per well. The wells were washed three times with PBS containing 0.1 % Tween 20 (PBST) and blocked with blocking solution (2 % bovine serum albumin in PBST) for two hours at room temperature. After washing with PBST, 50 μl of sera diluted in blocking solution (the serum dilution 1:640 was used in the assay since with this dilution, negative control serum gave an absorbance reading of less than 0.2) were added and incubated for 1 hour at room temperature. The wells were washed with PBST and 50 μl of anti-human IgM conjugated to horse-radish peroxidase (DAKO, Denmark) diluted in blocking solution was added to each well and incubated for 60 minutes at room temperature. The appropriate dilution of conjugate was determined and the dilution 1:1000 was used. After the wells were washed with PBST, 50 μl of substrate solution (0.4 mg/ml o-phenylene-diamine hydrochloride with 0.009 % H_2O_2) was added to each well. After 5 minutes, optical density at 492 nm was measured with ELISA reader. All samples were tested in duplicate.

Dot-ELISA

The appropriate antigen concentration used in the assay was determined using the same control sera as in the ELISA. The concentration of antigen used was 60 ng/ml. The assay was performed as previously described.[9] Briefly, 1 μl of antigen diluted in 0.05 M bicarbonate, pH 9.6, was dotted on 5-mm nitrocellulose membrane. Antigen disks were placed in a 96-well plate and incubated at room temperature for 30 minutes. Disks were then blocked with

triethanolamine-buffer saline (TBS) containing 5 % nonfat milk for 1 hour at room temperature with shaking. Serum samples were serially diluted starting at 1:20 in TBS containing 1% nonfat milk. Fifty microliters of diluted sera were added onto antigen disks and incubated for 90 minutes at room temperature with shaking. After washing with washing buffer [TBS containing 0.05 % IGEPAL (Sigma, USA)], 50 µl of anti-human IgM conjugated to horse-radish peroxidase, diluted in TBS containing 1% nonfat milk, was added to each well. Disks were incubated by shaking for 60 minutes at room temperature, washed and incubated with 50 µl of substrate solution (0.5 µg/ml 4-chloro-1-napthol with 0.01% H_2O_2) for 30 minutes at room temperature. The results were observed after washing with TBS. Serum samples that give blue-purple dots on antigen disks are considered positive.

Results

Comparison of MAT and ELISA

The results of ELISA from samples from healthy individuals (A), samples from patients positive for antibodies to *Treponema pallidum* (B), and samples positive for MAT (C) were shown in the ELISA ratio in Table 1. The ELISA ratio is the ratio between the average absorbance of tested samples and absorbance of negative controls. If a ratio greater or equal to 2 is considered positive, there are 15 out of 20 samples positive by MAT that are also IgM ELISA positive. The sensitivity of IgM ELISA is 75 % compared with MAT. Since *Treponema pallidum* are bacteria in the same family as leptospira, serum samples positive for antibodies specific to these bacteria are also tested. Seven out of twenty samples (35 %) that were positive for antibodies to *Treponema pallidum* by IgM ELISA were negative by MAT, suggesting that IgM-ELISA gave cross-reacting results with samples from patients with syphilis. Two out of 20 samples from healthy individuals were also positive by IgM ELISA.

Table 1. ELISA ratios from serum samples from healthy individuals (A), patients positive for anti-*treponema pallidum* antibodies (B), patients with MAT positive (C).

A (n = 20)		B (n = 20)		C (n = 20)	
sample #	ratio	sample #	ratio	sample #	ratio
1	1.54	1	2.21	1	1.52
2	0.77	2	1.93	2	1.94
3	1.08	3	2.15	3	0.84
4	1.41	4	2.58	4	5.54
5	1.59	5	1.74	5	4.99
6	2.10	6	3.53	6	5.23
7	1.00	7	1.83	7	1.81
8	0.76	8	6.67	8	1.93
9	1.30	9	5.81	9	3.21
10	1.48	10	1.13	10	4.46
11	1.37	11	1.72	11	2.18
12	0.68	12	1.26	12	3.18
13	1.00	13	1.94	13	2.35
14	1.50	14	1.23	14	2.55
15	2.24	15	2.11	15	3.42
16	1.54	16	0.96	16	2.67
17	1.66	17	0.74	17	2.68
18	1.37	18	0.89	18	3.72
19	1.02	19	0.12	19	2.48
20	1.58	20	1.13	20	2.17

Note : Underlined numbers are the ratios that are over or equal to 2.0

Table 2. Comparison of the results from ELISA and dot-ELISA for the detection of leptopsira specific IgM antibodies in samples positive for MAT, samples positive for antibodies to *Treponema pallidum* and samples from healthy individuals.

	MAT positive samples (n = 20)	samples positive for antibodies to *T. pallidum* (n = 20)	samples from healthy individuals (n = 20)
ELISA			
positive	15	7	2
negative	5	13	18
dot-ELISA			
positive	20	0	0
negative	0	20	20

Comparison of MAT and dot-ELISA

All samples positive by MAT are also positive for IgM by dot-ELISA. None of samples from healthy individuals or samples positive for antibodies specific to *Treponema pallidum* gave positive results by dot-ELISA. It can be concluded from these data that the sensitivity of dot-ELISA is 100 % compared with MAT. From the samples we studied, the specificity of dot-ELISA is also 100 %.

The results of ELISA and dot-ELISA for detection of IgM specific to leptospira are summarized in Table 2

Discussion

In this study, the diagnostic potential of ELISA and dot-ELISA for detection of leptospira IgM antibodies was compared. Both ELISA and dot-ELISA are easier to perform than MAT. However, dot-ELISA can be done in small laboratories that do not have ELISA readers. The interpretation of results of dot-ELISA is based on the appearance of blue-purple dots, which are easy to observe. Dot-ELISA requires less amount of antigen than ELISA. In addition, our data demonstrate that dot-ELISA has higher sensitivity and specificity than ELISA compared with MAT. The dot-ELISA can lead to the development of a dipstick assay, a more simple screening method for field use. However, a larger number of samples and samples from patients with other febrile illnesses should be further tested to evaluate the clinical utility of the assay.

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