UNIVERSITI PUTRA MALAYSIA

EVALUATION OF TWO COMMERCIAL IMMUNOCHROMATOGRAPHIC RAPID TESTS FOR DETECTION OF IgM ANTIBODIES AGAINST LEPTOSPIRES IN HUMAN SERUM

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Leptospirosis is a zoonotic disease which shows the increasing cases over the recent years. In Malaysia, leptospirosis became a notifiable disease in 2011. The treatment of leptospirosis is not difficult, however, the diagnosis is a big challenge. Leptospirosis is a biphasic infection, where the first phase (acute or septic phase) is from 3 to 7 days and followed by the second phase that happens from 7 to 14 days. The patients are mostly asymptomatic in the first few days. During infection, the bacteria can be found in the blood. Then, it moves to kidney and starts shedding in the urine. Antibodies start to develop into detectable amount during the second phase. Diagnosis of leptospirosis involves testing for the presence of bacteria or their DNA in the first phase and antibodies in the second phase. Since the actual onset of illness is not clear, laboratory diagnosis should include both microbiological and serological tests. Clinical diagnosis is another big challenge as the symptoms are similar to several febrile illnesses in the tropics. However, clinical suspicion and positive laboratory investigation are very important to start the therapy. The microbiological examination involves culturing the bacteria and PCR based detection which needs DNA from the clinical samples. However, microbiology is not useful for rapid and early diagnosis as *Leptospira* needs minimum 2 to 4 weeks to grow, while PCR needs DNA extraction, technical ies, and expensive PCR machines and reagents. In the context of serology, microscopic agglutination test, which is the gold standard, is technically tedious as well as the interpretations are very subjective. Most importantly, it also requires a large of live cultures. From the hospital point of view, a rapid test, which at least be able to give a laboratory indication, will be sufficient to direct the treatment options. Many hospitals do not have the facilities to perform MAT or PCR and the assays ; moreover, will take few days. To date, there are several commercial rapid tests which detect IgM specific to *Leptospira*

antigens. The sensitivity and specificity of those tests vary geographical regions. The present study was aimed at evaluating the diagnostic accuracy of two rapid tests namely Leptocheck-WB (Zephyr Biomedicals, India) and ImmuneMed IgM Duo Rapid (IMMUNEMED, Inc Korea) for detection of acute leptospirosis in human.The study used two approaches namely prospective and retrospective evaluation to determine the diagnostic accuracy in the two rapid tests. In the prospective approach, the evaluation was performed on clinically suspected leptospirosis patients from a single hospital and used MAT and qPCR as the reference test. The accuracy of Serion ELISA *classic* also had been evaluated and results of diagnostic accuracy were compared to Leptocheck-WB and ImmuneMed IgM Duo Rapid. In the retrospective approach, the confirmed leptospirosis positive samples were obtained from the Public Health Laboratory Kota Bharu. The samples were evaluated and used only MAT results as the reference test. In the prospective evaluation, among the 50 blood samples tested from clinically suspected leptospirosis cases, six were positive MAT and 13 were positive qPCR. The Leptocheck-WB showed sensitivity and specificity of 47.3% and 80.65% respectively. ImmuneMed IgM Duo Rapid showed sensitivity and specificity of 15.79% and 90.32% respectively. The results of sensitivity and specificity of Leptocheck-WB and ImmuneMed IgM Duo in prospective evaluation samples were compared to the result from Serion ELISA *classic*. Serion ELISA *classic* showed 26.32% sensitivity and 83.87% specificity. For retrospective samples, Leptocheck-WB showed sensitivity and specificity of 90.72% and 76.32% respectively. ImmuneMed IgM Duo Rapid showed sensitivity and specificity of 40.21% and 89.47 respectively. In conclusion, from the present study for both prospective and retrospective samples, Leptocheck-WB was found to be more sensitive in detecting acute leptospirosis in human while ImmuneMed IgM Duo Rapid was found to be more specific in detecting acute leptospirosis.

Keywords: Leptospirosis, Rapid Diagnostic test, Immunochromatographic Test; Serological Tests; Serum IgM Antibodies, Prospective samples, Retrospective sample.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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PENILAIAN TERHADAP DUA UJIAN IMMUNOKROMATOGRAFIK SEGERA UNTUK MENGENALPASTI ANTIBODI IgM TERHADAP *LEPTOPSIRA* DALAM SERUM MANUSIA

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Leptospirosi adalah wabak zoonosis yang menunjukkan peningkatan kes dalam beberapa tahun lepas. Di Malaysia, Leptospirosis telah menjadi wabak yang perlu diambil perhatian pada tahun 2011. Perawatan leptospirosis tidak susah, walaubagainamapun, diagnosisnya adalah cabaran yang besar. Leptospirosis adalah penyakit dwifasa, yang mana pada fasa pertama (fasa akut atau septik) adalah dari 3 hingga 7 hari dan diikuti dengan fasa kedua yang berlaku dari 7 hingga 14 hari. Pesakit kebanyakkannya bersifat asimptomatik pada beberapa hari pertama. Ketika jangkitan, bakteria boleh dijumpai dalam darah. Kemudian, ia bergerak ke buah pinggang dan mula menggelupas di dalam darah. Antibodi mula terbina dalam bentuk yang mudah dikesan ketika fasa kedua. Diagnosis Leptospirosis melibatkan ujian untuk mengesan bakteria atau DNA pesakit dalam fasa pertama dan antibodi pada fasa kedua. Oleh kerana mula penyakit ini tidak begitu jelas, diagnosis makmal sepatutnya melibatkan kedua dua ujian mikrobiologi dan serologi. Diagnosis klinikal ialah merupakan satu lagi ujian besar kerana simptomnya sama dengan beberapa penyakit febril di Kawasan tropika. '*Clinical Suspision'* dan siasatan *'Positive laboratory'* adalah sangat penting untuk memulakan terapi. Pemeriksaan mikrobilogi adalah melibatkan pengkulturan bakteria dan pengesanan berdasarkan PCR yang memerlukan DNA daripada sampel klinikal. Walaubagaimanapun, mikrobilogi tidak berkesan untuk diagnosis awal dan pantas kerana Leptospira memerlukan 2 hingga 4 minggu minimum untuk pertumbuhan; pada masa yang sama PCR memerlukan ekstrak DNA, ies teknikal serta mesin PCR yang mahal dan bahan uji tak organik.Dalam konteks serologi, ujian aglutinasi mikroskop, yang merupakan standard emas, adalah rumit secara teknikalnya dan interpretasinya sangat subjektif. Yang paling penting, ia juga memerlukan kultur hidup yang banyak. Dalam konteks pandangan hospital, uji cepat, yang sekurang-kurangnya memberikan penanda makmal, sudah cukup untuk menunjukkan pilihan rawatan. Kebanyakan hospital tidak mempunyai kemudahan untuk menjalankan

MAT dan PCR serta menilai keberkesanan; selain itu, ia memakan masa beberap hari. Kini, terdapat beberapa uji cepat komersial yang mengesan spesifik IgM kepada antigen Leptospira. Sensitiviti dan spesifikasi ujian tersebut berlainan mengikut Kawasan geografi. Kajian ini bertujuan untuk menilai ketepatan diagnosis dua uji cepat iaitu Leptocheck-WB (Zephyr Biomedicals, India) and ImmuneMed IgM Duo Rapid (IMMUNEMED, Inc Korea) untuk pengesanan Leptospirosis akut pada manusia. Kajian ini menggunalan dua pendekatan iaitu penilaian prospektif dan retrospektif untuk menegnalpasti ketepatan diagnosis dalam dua uji cepat. Dari segi pendekatan prospektif, penilaian telah dilakukan ke atas pesakit leptospirosis yang disyaki secara klinikal dari sebuah hospital dan telah menggunakan MAT dan qPCR sebagai rujukan ujian. Ketepatan Serion ELISA classic juga telah dinilai dan keputusan ketepatan diagnosis dibandingan dengan Leptocheck-WB dan ImmuneMed IgM Duo Rapid.Dari segi pendekatan retrospektif, sampel positif leptospirosis yang telah dipastikan diambil daripada Makmal Kesihatan Awam Kota Bharu. Sampel tersebut dinilai dan menggunakan hanya hasil keputusan MAT sebagai rujukan ujian. Dalam penilaian prospektif, daripada 50 sampel darah dari kes yang disuspek leptospirosis secara klinikal, enam antaranya positif kepada MAT dan 13 positif kepada qPCR. Leptocheck-WB telah menunjukkan sensistivtii dan spesifikasi masing-masing adalah 47.3% dan 80.65%. ImmuneMed IgM Duo Rapid telah menunjukkan sensitiviti dan spesifikasi masing-masing adalah 15.79% dan 90.32%. Keputusan sensitiviti dan spesifikasi Leptocheck-WB dan ImmuneMed IgM Duo dalam sampel penilaian prospectif telah dibandingkan dengan keputusan daripada Serion ELISA *classic*. Serion ELISA classic menunjukkan keputusan sensitiviti pada 26.32% dan spesifikasi pada 83.87%. Dari sudut lain bagi sampel retrospectif, Leptocheck-WB telah menunjukkan sensitiviti dan spesifkasi masing-masing 90.72% dan 76.32%. ImmuneMed IgM Duo Rapid telah menunjukkan sensitiviti dan spesifkasi masing-masing 40.21% and 89.47. Kesimpulannya, berdasarkan kedua-dua kajian prospektif dan retrospektif ini, Leptocheck-WB dilihat lebih sensitif dalam pengesanan leptospirosis akut pada manusia, manakala ImmuneMed IgM Duo Rapid lebih spesifik dalam mengesan leptospirosis.

Kata kunci: Leptospirosis, Ujian Diagnostik Pantas, Ujian Immunokromatografik, Ujian Serologi; Serum IgM Antibodi, sampel Prospektif, sampel Retrospektif.

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LIST OF ABBREVIATIONS

Buffer AL	DNA Binding Buffer
Buffer AW1	Wash Buffer
Buffer AW2	Desalting Buffer
Buffer AE	Elution Buffer
CFS	Cerebral spinal fluid
DFM	Dark-field Microscope
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacid acid
EMJH	Liquid Ellinghausen, Mccullough, Johnson, Harris
ELISA	Enzyme Linked Immunosorbent Assay
GBD	Global Burden Disease
IU/ µl	International unit per microliter
LPS	Lipopolysaccharide
MAT	Microscopic Agglutination Test
mg	Milligram
mg/ml	Milligram per milliliter
min	Minute
ml	Milliliter
MOH	Ministry of Health
nm	Nanometer
OD	Optical density
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction

pmol	Picomoles
POC	Point of care
pH	Potential of hydrogen
qPCR	Quantitative polymerase chain reaction
RDT's	Rapid diagnostic tests
sec	Second
SPSS	Statistical Package for the Social Sciences
WHO	World Health Organization
µl	Microliter
%	Percent
°C	Degree Celsius
5-FU	5-Fluorouracil

CHAPTER 1

INTRODUCTION

1.1 Study Background

Leptospirosis is one of the emerging zoonotic diseases which are potentially fatal with worldwide distribution (Mathieu Picardeau et al., 2014). It is caused by the pathogenic spirochetes *Leptospira* and affects both human and animals (Haake & Levett, 2015). Classified as a Gram-negative bacterium, *Leptospira* replicates and maintained in nature through chronic renal colonization of rodents and other small mammals. However, leptospirosis in rodents are asymptomatic and the animals appear healthy even when *Leptospira* bacteria are constantly replicating in their kidneys and excreted through (Noguchi, 1917; Stokes et al., 1917). Morphological alteration were reported in a study on bacteria isolated from kidney of rodents (Tucunduva de Faria et al., 2007). The *Leptospira* colonize by forming an amorphous, biofilm-like structure in the renal tubules of the asymptomatic carrier (Thiermann, 1981). Humans acquire the infection through direct or indirect contact with the urine or tissue of an animal infected or colonized with *Leptospira*. The bacteria enter directly into the body through cuts and abrasion on skins, mucous membrane, conjunctivae, or aerosol inhalation of microscopic droplets. However, infection through inhalation of water or aerosol, animal bites or inter-human transmission has been rarely demonstrated (Musso & La Scola, 2013). Thus humans becomes accidental hosts after being infected by *Leptospira* (Sánchez-Montes, Espinosa-Martínez, Ríos-Muñoz, Berzunza-Cruz, & Becker, 2015). Clinical leptospirosis can be divided into "leptospiraemic" phase which happens four to seven days after onset of infection and "leptospiruric" phase that last for 4 to 30 days. Incubation period range from 2 to 20 days while acute symptoms usually develop from 7 to 12 days after infection (Eugene et al., 2015; Levett, 2001). *Leptospira* can be found in the bloodstream during the acute phase and disappear during the convalescent phase. Antibody generally appears during "leptospiruric" phase which begin with the appearance of IgM antibody and usually persist for several months (Chernukha, Shishkina, Baryshev, & Kokovin,1976; Haake & Levett,2015).

Leptospirosis cases were highly reported in tropical and subtropical areas due to the suitable condition for the *Leptospira* to spread in the environment (Musso & La Scola, 2013). For example, the Asia Pacific region (Seychelles, India, Sri Lanka, and Thailand) as well as in Latin America and the Caribbean were reported as the highest endemic countries with the incidence rate of over 10 per 1000,000 people (Pappas et al., 2008). From 873,000 annual cases, 48,000 (5.5%) were reported dead by WHO Leptospirosis Burden Epidemiology Reference Group (LERG) (Pappas et al., 2008).

Occupational and outdoor recreational activities that are related to exposure to animals contaminated water and soil emerged as strong risk factors. For example, people who are involved in the occupation such as agriculture, military troops and recreational activities such as fishing and swimming are at high possibility of acquiring leptospirosis (Adler & de la Peña Moctezuma, 2010; Chiat Han Chang, Riazi, Yunus, Osman, & Noordin, 2014). In Malaysia, there is an increasing trend of leptospirosis cases whereby 8291 cases were reported in year 2015 which higher than cases in 2014 (7806) (Wahab, 2015).

1.2 Problem Statement

Prevention, control and early detection of the causative agent are key factors to reduce mortality and morbidity. Several factors are important to reduce the severity of the leptospirosis infection such as rapid diagnosis, effective treatment, the awareness and knowledge among the population on diseases prevention. Diagnosis is always a challenge at the early stage of leptospirosis since shows a very common febrile illness symptoms, thus most of the physicians tend to misdiagnose it. In Malaysia, leptospirosis also commonly misdiagnosed with other febrile illness especially dengue. Microscopic agglutination test (MAT) has been used as the gold standard for confirmation of leptospirosis. However, MAT is less reliable as the early detection method since the body does not produce specific antibodies against *Leptospira* as soon as the infection occurs. Polymerase chain reaction (PCR) is a rapid, specific and sensitive method to detect the bacteria in the blood sample at the very early stage. However, PCR is not suitable after the leptospiraemic phase when the body produced antibodies that cleared the bacteria. Even though the PCR method is very sensitive, but not all laboratory can perform this method because of the high cost and technical challenge.

1.3 Research Justification

Detailed study on leptospirosis detection methods is still needed, especially rapid diagnosis at early stages of illness to help the physician in starting the right treatment regimen. The method should be easy to handle and affordable. Nowadays there are many rapid test kits available in the market, but not all these rapid test kits are suitable for every country due to different circulating serovars, antibody cut-titer values for MAT confirmation and coinfection with other local febrile illneses. The present study was aimed at evaluating the diagnostic efficacies of two commercially available immunochromatographic based rapid diagnostic test (RDTs) namely Leptocheck-WB and ImmuneMed IgM Duo Rapid and Serion ELISA *classic* for early detection of leptospirosis. Leptocheck-WB was used in this study because reports from several other countries supported this kit for early detection and evaluation in their hospitals, while ImmuneMed IgM Duo Rapid was recently approved to be used as rapid test in hospitals in Malaysia. Serion ELISA *classic* was selected as it is being used in the common reference laboratory in Malaysia. Evaluation of the diagnostics sensitivity and

specificity of these tests would provide important data to guide Malaysians hospitals to choose the rapid test for the early screening of leptospirosis.

1.4 Objectives

1.4.1 General Objective

The study attempted to evaluate the accuracy of three diagnostic tests on samples for the early screening of leptospirosis.

1.4.2 Specific objectives

1. To determine the diagnostic accuracy of two rapid diagnostic tests for the early detection of leptospirosis through prospective evaluation approach.
2. To evaluate the specificity and the sensitivity of the tests through a retrospective evaluation approach.
3. To compare the diagnostic accuracy of two RDTs with ELISA for early diagnosis of leptospirosis

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