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Evaluation of IgM LAT and IgM ELISA as compared to microscopic agglutination test (MAT) for early diagnosis of *Leptospira* sp.

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ABSTRACT. Leptospirosis is a worldwide zoonotic disease caused by spirochetes of the genus *Leptospira*. The clinical manifestation of leptospirosis is non-specific and frequently misdiagnosed as other illnesses. The aim of this study was to compare the diagnostic accuracies of two commercial tests for early diagnosis of *Leptospira* species: the IgM latex agglutination test (IgM LAT) and the IgM enzyme-linked immunosorbent assay (IgM ELISA). A total of 140 serum samples were obtained from patients suspected of leptospirosis at the Universiti Kebangsaan Malaysia Medical Centre (UKMMC). These serum samples were tested for the presence of *Leptospira* sp. using IgM LAT, IgM ELISA and MAT. From Table 1, IgM LAT showed 21% (n = 29) positive, 18% (n = 25) inconclusive and 61% (n = 86) negative, while IgM ELISA showed 6% (n = 8) positive, 6% (n = 8) inconclusive, 88% (n = 124) negative and MAT showed 11% (n = 16) positive, 47% (n = 65) inconclusive, 42% (n = 59) negative. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of IgM LAT were 68.8%, 57.6%, 30.6% and 87.2% respectively, while for IgM ELISA they were 37.5%, 89.8%, 50% and 84.1%, respectively as compared to MAT (Table 2). The results showed that IgM LAT had higher sensitivity but lower specificity compared to IgM ELISA. In conclusion, IgM LAT can be useful as an early screening test for early diagnosis of *Leptospira* sp., while IgM ELISA is a suitable method for reducing false negative detection of *Leptospira* sp. As both tests show moderate percentages (~65%) in accuracy, an additional test is required for better detection of *Leptospira* sp.

INTRODUCTION

Leptospirosis is a zoonotic disease caused by spirochetes of the genus *Leptospira* which has a worldwide distribution (Panwala *et al.*, 2015). To date, over 260 serovars of both *Leptospira* sp. have been documented, while serovars with related antigens have been grouped into serogroups (Levett, 2001; Mgode *et al.*, 2015). The *Leptospira* sp. has an outer membrane in which lipopolysaccharide (LPS) is embedded in the outer leaflet, an inner membrane and an intervening, peptidoglycan-containing periplasmic space which is structured similarly as other Gram negative bacteria. The cells have pointed ends, one or both of which are usually bent into a distinctive hook (Adler, 2015). Recently, leptospirosis cases became major problem in Malaysia started from 2012 when 12.5/100,000 population were infected. In 2013, the number of cases was increase to 15.0/100,000 population (4,457 cases with 71 deaths) (Data extracted from official report from the Ministry of Health Malaysia). Furthermore, the leptospirosis cases in

Malaysia was increase dramatically from 263 cases (2004) to 7806 cases (2014) (Ministry of Health, 2015). During flood in Kelantan state, 94 human leptospirosis cases reported between 1 until 18 January 2015. (New Straits Times, 2015). To date, 38 different serovars were successfully identify in Malaysia includes serovar Hardjo, serovar Pomona, serovar Icterohaemorrhagiae and serovar Bratislava isolated from various animals such as cattle, buffalo, sheep, goats and rats with prevalence rate from 8.6 until 40.5% (Benacer *et al*., 2013; Mohamed-Hassan *et al*., 2012; Mohamed-Hassan *et al*., 2010; Bahaman 1991; Bahaman and Ibrahim 1988; Blackmore *et al*., 1982).

Leptospira sp. infections in humans occur by direct contact with infected animals or by indirect contact via water or soil contaminated with urine shed from a reservoir host (most commonly from rodents). The *Leptospira* sp. enters the blood stream via cuts, skin abrasions or through mucous membranes (Victoriano *et al*., 2009; Basker *et al*., 2014). Infected patients will show such symptoms such as flu-like illnesses, headaches, and transient skin rashes which will become more serious, leading to organ failure and eventually death if not treated accordingly (Goris *et al*., 2013; Jain *et al*., 2015). Because these clinical symptoms are common, leptospirosis is often misdiagnosed as other illnesses and diseases such as influenza, aseptic meningitis, encephalitis, dengue fever and hepatitis. Thus, it is difficult to recognize leptospirosis based only on clinical symptoms (Sehgal *et al*., 1995; Effler *et al*., 2002; Sehgal, 2006).

Laboratory diagnosis of leptospirosis is based on several methods such as the microscopic agglutination test (MAT), DNA detection by polymerase chain reaction (PCR) (Brown *et al*., 1995), isolation of the organism through culture methods (Musso and La Scola, 2013) and detection of antibodies to the *Leptospira* antigen (Niloofa *et al*., 2015). However, the diagnosis of leptospirosis is often made by serological tests (Panwala *et al*., 2015) because the isolation of *Leptospira* sp. through cultures is insensitive and requires special media, while detection of specific genes using PCR sometimes leads to amplification of wrong amplicons and is less efficient for certain serovars in some geographical areas (Effler *et al*., 2002: Mullan and Panwala, 2016). The MAT is the serological gold standard test used in reference laboratories for the diagnosis of leptospirosis because of its high degree of sensitivity and specificity (Bajani *et al*., 2003). However, MAT is time consuming, requires significant expertise to perform and the maintenance of live *Leptospira* cultures. As such, there is a need to explore other detection methods for the diagnosis of leptospirosis (Levett and Branch, 2002; Effler *et al*., 2002; Panwala *et al*., 2015). Because of that, IgM LAT and IgM ELISA may become the best alternative methods since both are time effective, easy to perform (follow manufacture manual) and inexpensive (Loong *et al*., 2018). Three commercial alternatives to MAT have been developed for screening leptospirosis: latex agglutination test, IgM ELISA, IgM dipstick assay (LDS) and the indirect hemagglutination assay (IHA). However, the effectiveness of these three tests were only based on single test results, with no comparison done with the MAT gold standard. Futhermore, their diagnostic accuracy was not fully established (Niloofa *et al*., 2015; Panwala *et al*., 2015).

The aim of this study was to compare sensitivity, specificity, predictive values and accuracy of a commercial IgM latex agglutination test (IgM LAT) and a commercial IgM enzyme-linked immunosorbent assay (IgM ELISA) as compared to MAT for early diagnosis of *Leptospira* sp. in serum samples collected over a five month period (October 2015 – February 2016), at the Universiti Kebangsaan Malaysia Medical Centre (UKMMC).

MATERIALS AND METHODS

Sample Collection

This study used 140 serum samples in plain tubes collected from suspected leptospirosis patients at the Virology-Serology Unit, Department of Diagnostic Laboratory Services (JPMD), Universiti Kebangsaan Malaysia Medical Centre (UKMMC) from

October 2015 to February 2016. Ethical approval was obtained from the Research Ethics Committee of the Universiti Kebangsaan Malaysia (Ethical no: NN_2016-025). All samples were kept in -20ºC until further analysis.

IgM-Latex Agglutination Test (IgM LAT)

A Leptorapide® kit [Linnodee Ltd. (Northern Ireland)] was used in the IgM latex agglutination test (IgM LAT) for the detection of *Leptospira* IgM antibody in serum patients suspected with leptospirosis. A 5 µl of the suspension bead solution was added on one circle of the agglutination card containing leptospirosis suspected patient serum and mixed gently for 2 to 3 minutes. The result was interpreted as score 1 (negative = no agglutination), score 2 (inconclusive = little agglutinate), score 3 to 5 (positive = large agglutinate), according to the score card provided with the kit. Serum containing *leptospira* sp. served as positive control.

IgM-Enzyme Linked-Immunosorbent Assay (IgM ELISA)

The SERION ELISA *classic* test (Virion/Serion GmBH, Germany) was used in the Enzyme Linked-Immunosorbent Assay-IgM (IgM ELISA). Firstly, the RF-dilution buffer solution was prepared by mixing 200 µl of rheumatoid factor (RF) absorbent with 800 µl dilution buffer at a ratio of 1:4. Then, 100 µl of each patient serum sample was diluted in 1000 µl RF-dilution buffer at a 1:10 ratio and incubated at room temperature (~27°C) for 15 min to remove IgM rheumatoid factors in the serum.

Next, 100 µl of standard solution with 100 µl diluted serum sample were transferred to the microtiter wells and incubated at 37°C for 60 minutes in a moist chamber. Residual serum was removed from the wells by washing four times with the wash buffer. Next, conjugate solution (100 µl) containing anti-human IgM antibody conjugated to alkaline phosphatase was added to each well and incubated at 37°C for 30 minutes in a moist chamber. After incubation, the content in each well was washed four times with the wash buffer. Next, 100 µl of substrate solution containing p-nitrophenyl phosphate was added to each well and incubated at 37°C for 30 minutes in a moist chamber. Finally, 100 µl of stop solution containing sodium hydroxide was added and the enzyme substrate reaction was stopped prior to measurement.

The ELISA plate was read at 405 nm using an ELISA plate reader. Each set of test was performed with cut-off calibrators (standards) in duplicates and a negative control. The test was valid when the absorbance reading of the above met the specifications of the Serion ELISA. The result was interpreted by using an evaluation table. The results of serum samples in the IgM ELISA test was recorded as negative (<15 U/ml), inconclusive (15 to 20 U/ml) and positive (≥20 U/ml), according to the evaluation table provided by the manufacturer.

Microscopic Agglutination Test (MAT)

The MAT test was conducted at the Institute of Medical Research (IMR), Malaysia. MAT was done to detect agglutinating antibodies in patient serum. The procedure for the MAT test involved dilution of serum, screening and titration of serum samples. Panel batteries of 20 live serovars representing 13 serogroups that are endemic in Malaysia were used as antigens. Fourteen *Leptospira* reference cultures were obtained from the World Health Organization (WHO) Collaborating Centre, Queensland, Australia, with 6 additional local cultures. The WHO cultures included in the antigen panel were Serovar Australis, serovar Autumnalis, serovar Bataviae, serovar Canicola, serovar Celledoni, serovar Grippotyphosa, serovar Hardjoprajitno, serovar Icterohaemorrhage, serovar Javanica, serovar Pyrogenes, serovar Tarrasovi, serovar Djasiman, serovar Patoc and serovar Pomona. The local cultures were serovar Melaka (IMR LEP 1), serovar Terengganu (IMR LEP 115), serovar Sarawak (IMR LEP 175), serovar Copenhageni (IMR LEP 27), serovar Hardjobovis (IMR LEP 27) and serovar Lai (IMR LEP 22).

The test was performed according to the method described by Watt *et al.*, 1988. 100 µl of serum mixture [patient's serum+ Phosphate buffer saline (PBS)] with ratio 1:1 were added into microtiter plates and mixed

well. The microtiter plates were then incubated at 30°C for 2 hours. A loopful of the suspension in each well was transferred onto a slide and the leptospires density was observed under a dark field microscope at x100 or x200 magnification. The agglutination in wells with patient's serum was observed by observing free leptospires in each well and comparing this with the well containing the control. Positive agglutination of sera was defined as the approximate number of free leptospires with at least 50 percent of leptospires observed compared to one or more control serovars. WHO and local *Leptospira* sp. were serve as a control.

Next, a full titration was done for serums that showed positive results for specific serovar. Titration was performed using five different serum dilutions with the ratios of 1: 50, 1: 100, 1: 200, 1: 400 and 1: 800 and was incubated at 30°C for 2 hours. A loopful of the suspension in each well was transferred onto a slide and the density of leptospires was observed under dark field microscope at x100 or x200 magnification. The slides were interpreted as described earlier (Watt *et al.*, 1988). The titre was calculated as the reciprocal of the highest dilution of serum which showed at least 50 percent of agglutination of the free leptospires as compared to the control wells. Results of samples in the MAT test were recorded as positive (MAT titer of ≥ 1: 400), inconclusive (MAT titer of 1: 400 and negative (no agglutination).

Statistical Analysis

For accuracy, data from IgM LAT, IgM ELISA and MAT assay were entered and analyzed by using Microsoft Excel 2010. Sensitivity, specificity, positive predictive values (PPV) and negative predictive value (NPV) of IgM LAT and IgM ELISA were calculated based on the MAT cutoff of ≥ 1: 400 dilutions, using standard equations according to Sekhar *et al.* (2000) and Brownlow *et al.* (2015).

RESULTS

A total of 140 serum samples collected from leptospirosis-suspected patients from October 2015 to February 2016 were subjected to testing using IgM LAT, IgM ELISA and MAT for the comparison study. For IgM LAT, 21% of samples (n = 29) were positive, 18% (n = 25) inconclusive and 61% (n = 86) negative. Meanwhile, for IgM ELISA, 6% of samples (n = 8) were positive, 6% (n = 8) inconclusive and 88% (n = 124) negative. Using MAT, it was found that 11% (n = 16) of samples were positive, 47% (n = 65) inconclusive and 42% (n = 59) negative (Table 1). Overall, IgM LAT showed more positive results [n = 29 (21%)] in the presence of *Leptospira* IgM antibodies, as compared to IgM ELISA and MAT which only gave [n = 8 (6%)] and [n = 16 (11%)], respectively (Table 1).

Since MAT is used as a gold standard (Sekhar *et al.*, 2000; Brownlow *et al.*, 2014), comparative accuracies of IgM ELISA and IgM LAT were calculated based on the results from MAT. Only positive and negative (n = 75) MAT results were used in the calculations while inconclusive results (n = 65) were excluded. However, inconclusive results in IgM LAT and IgM ELISA tests were considered as positive results, according to Goris *et al.* (2013). The sensitivity of a test is

Table 1. Comparison of IgM LAT, IgM ELISA, MAT assays

Diagnosis assay	Percentage presence (n = 140)		
	Positive	Inconclusive	Negative
IgM LAT	29 (21%)	25 (18%)	86 (61%)
IgM ELISA	8 (6%)	8 (6%)	124 (88%)
MAT	16 (11%)	65 (47%)	59 (42%)

IgM LAT: IgM latex agglutination test, IgM ELISA: Enzyme Linked-Immunosorbent Assay-IgM, MAT: Microscopic agglutination test. Positive: *Leptospira* IgM presence; Inconclusive: uncertain *Leptospira* IgM presence; Negative: *Leptospira* IgM not present.

Table 2. Comparison of IgM LAT and IgM ELISA against MAT as the gold standard

Screening test	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Accuracy (%)
IgM LAT	68.8	57.6	30.6	87.2	63
IgM ELISA	37.5	89.8	50.0	84.1	64

IgM LAT: IgM latex agglutination test, IgM ELISA: Enzyme Linked-Immunosorbent Assay-IgM, MAT: Microscopic agglutination test. Positive predictive value: TP/(TP + FP). Negative predictive value: TN/(TN + FN). TP: True positive. FP: False positive. TN: True negative. FN: False negative. Accuracy (%) = [Sensitivity (%) + Specificity (%)]/2.

defined as the ability of the test to diagnose the illness in early stage. On the other hand, the specificity of the test is related to the ability of the test to indicate whether certain individual is infected or not by *Leptospira* sp. The positive predictive value of a test is defined as the proportion of patients with a positive test result after MAT test (true positive [positive leptospirosis after IgM ELISA, IgM LAT and MAT]) against total group of subjects with a positive result (true positive + false positive [positive leptospirosis IgM ELISA or IgM LAT but negative on gold standard MAT]). The negative predictive value of a test is defined as the proportion of patients with a negative test result after MAT test (true negative [negative leptospirosis after IgM ELISA or IgM LAT and negative after gold standard MAT) against total group of subjects with a negative test result (true negative + false negative [negative leptospirosis on IgM ELISA or IgM LAT but positive on gold standard MAT]). Diagnostic accuracy measures tell us about the ability of a test to discriminate between or predict disease and health (Eusebi, 2013: Akobeng, 2007). As compared to MAT, the sensitivity, specificity, positive predictive value, negative predictive value and accuracy of IgM LAT were 68.8%, 57.6%, 30.6%, 87.2% and 63%. For IgM ELISA, the values were 37.5 %, 89.8%, 50.0 %, 84.1% and 64% respectively (Table 2). As can be seen, the sensitivity of IgM LAT (68.8%) was higher than IgM ELISA (37.5%). However, IgM ELISA showed higher specificity (89.8%) compared to IgM LAT (57.6%). Nevertheless, both tests showed almost the same percentage of accuracy (63% = IgM LAT; 64% = IgM ELISA).

DISCUSSION

IgM LAT successfully detected 29 samples (21%) as positive with *Leptospira* sp., compared to MAT [n = 16 (11%)] and IgM ELISA [n = 8 (6%)] (Table 1). This indicated that IgM LAT has the ability to detect *Leptospira* sp. in patient's serum earlier as compared to the other two methods. This is in contrast with other findings which indicated that IgM LAT, IgM ELISA and MAT have almost the same percentage of *Leptospira* sp. detection (Shekatkar *et al.*, 2010). However, IgM LAT is usually considered as one of the best methods for rapid detection of *Leptospira* sp. in early stages of infection, compared to IgM ELISA and MAT, which are usually used in the late stages of infection (Panwala *et al.*, 2015). Another study conducted by Brownlow *et al.* (2014) gave similar results with the present study after IgM LAT successfully detected more positive samples than IgM ELISA (87/168 samples vs. 66/168 samples). Brownlow *et al.* concluded that IgM LAT is a suitable method for the rapid detection of *Leptospira* sp., when compared to IgM ELISA, particularly in endemic regions. (Brownlow *et al.*, 2014).

On the other hand, both immunoassays tested (IgM LAT, IgM ELISA) have more than 50% negative detection (IgM LAT = 61%; IgM ELISA = 88%) when compared to MAT which had only 42% (Table 1). Since MAT is considered to be the gold standard, (Blacksell *et al.*, 2006; Panwala *et al.*, 2015; Rosa *et al.*, 2017; Dittrich *et al.*, 2018) any rapid method for *Leptospira* sp. detection requires further confirmation by MAT to reduce misdiagnosis, since the rapid methods sometimes fail to

detect or distinguish certain serovars or serogroups of *Leptospira* sp. (van Eys *et al*., 1991; Hartskeer *et al*., 2004). Table 1 also shows that MAT has the highest inconclusive result at 47% compared to IgM LAT (18%) and IgM ELISA (6%). This indicated that MAT should not be used alone to detect *Leptospira* sp. Furthermore, previous researchers usually combine the MAT method with other methods such as IgM ELISA, IgM LAT, Check-Point assay (CP), detection of specific genes using PCR or real-time PCR for *Leptospira* sp. detection (Merien *et al*., 2005; Ahmed *et al*., 2009; Shekatkar *et al*., 2010; Bourhy *et al*., 2011; Panwala *et al*., 2015).

Table 2 shows that the IgM LAT has a higher sensitivity (68.8%), compared to IgM ELISA (37.5%), but lower specificity (57.6% vs. 89.8% respectively). This indicated that IgM LAT is more sensitive than IgM ELISA in the early stages of infection (within 7 days). Other studies did show that IgM ELISA was more sensitive (>80%) (Niloofa *et al*., 2015; Desakorn *et al*., 2012; Ooteman *et al*., 2006), but only for sample collected after 7 days of infection. Therefore, any test using samples collected before 7 days of infection will show reduced sensitivity of detection when using IgM ELISA (Bajani *et al*., 2003). However, IgM ELISA also requires reconsideration as an early *Leptospira* sp. screening method. There have been several reports indicating that IgM ELISA shows low sensitivity (<65%) using samples collected after 9 days of infection, with sensitivity increasing using samples collected after 15 days of infection (Reller *et al*., 2011; Signorini *et al*., 2013). Furthermore, Sekhar *et al* (2000) also showed high numbers of false negative results during tests that lowers the sensitivity of IgM ELISA (Sekhar *et al*., 2000). In the present study, the IgM LAT detection kit has a sensitivity of 68.8% which is lower than 90% (Table 2), as compared to other reports (Effler *et al*., 2002; Niloofa *et al*., 2015). There are several possible explanations for the variation in screening test sensitivity observed among studies. For example, test sensitivity may be affected by the prevalence of the different infecting serovars that varies geographically. Diagnostic tests should use broad-reacting antigens to detect a patient's immune response to the infecting leptospires. The screening test sensitivity also depends on the ability of test antigens to detect antibodies produced against the site-specific leptospiral serovars. The characteristics of the leptospiral antigen may also differ from one place to another (Effler *et al*., 2002; Wuthiekanun *et al*., 2007). Therefore, laboratories need to validate the performance of screening tests in the setting in which they are to be used. In addition, leptospirosis patients might have co-infections or cross reactive antibodies of other diseases such as dengue, malaria and others and this may affect the sensitivity of the test (Musso and La Scola, 2013). However, the specificity of the IgM ELISA (89.8%) was shown to be higher than IgM LAT (57.6%) (Table 2). This result was congruent with several previous findings which stated that IgM ELISA had high specificity properties up to 88%, compared to other rapid screening tests (including IgM LAT) which shows specificity below 70% (Cumberland *et al*., 1999; Akobeng *et al*., 2007; Panwala *et al*., 2015). Table 2 shows that both tests have almost same accuracy values with IgM LAT at 63% and IgM ELISA at 64%. This indicated that both tests have a moderate accuracy and may require an additional test since factors such as specific serovars or cross reactive antibody reactions may decrease or affect the detection accuracy of the tests (Panwala *et al*., 2015).

In conclusion, IgM LAT may be selected as a suitable rapid screening test for early diagnosis of leptospirosis because more serum samples were identified as positive by IgM LAT (n = 29/140) compared to IgM ELISA (n = 8/140) and MAT (n = 16/40). Furthermore, IgM LAT has almost twice the sensitivity (68.8%) of the IgM ELISA (37.5%) with MAT used as the gold standard. However, IgM ELISA can be used as a confirmation test for the presence of *Leptospira* sp. due to its high specificity properties (89.8%) compared to IgM LAT (57.6%). However, both tests have a moderate accuracy percentage (which is 63% for IgM LAT and 64% for IgM ELISA). This is probably affected by several factors such as different serovars and locations. This indicated that the accuracy of rapid tests such as IgM LAT and IgM ELISA is largely

dependent on the presence of anti-*Leptospira* antibodies that may affect the sensitivity and specificity of the tests. Furthermore, the available diagnostic tests are not always serovar-specific because cross-reactivity against different serovars may occur between organisms in the same serogroup and remains to be explored. Molecular tests using PCR may be considered as alternative supporting methods if immuno-rapid test failed to produce positive results. With the current robust leptospiral research output, we may see the development of simple and inexpensive diagnostic systems in the near future that are appropriate for highly endemic, resource-poor areas, as well as the application of state-of-the-art technologies for vaccine development.

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REFERENCES

Adler, B. (Editor). (2015). Leptospira and Leptospirosis, 1st Edition. Berlin, Germany: Springer-Verlag Berlin Heidelberg.

Ahmed, A., Engelberts, M.F., Boer, K.R., Ahmed, N. & Hartskeerl, R.A. (2009). Development and validation of a real-time PCR for detection of pathogenic *leptospira* species in clinical materials. *PLoS One* **4**(9): e7093.

Akobeng, A.K. (2007). Understanding diagnostic tests 1: sensitivity, specificity and predictive values. *Acta Paediatrica* **96**(3): 338-341.

Bahaman, A.R. (1991). Leptospiral infection in domestic animals in malaysia: its importance, epidemiology and control. International Symposia on Veterinary Epidemiology and Economics proceedings, ISVEE 6: Proceedings of the 6th International Symposium on Veterinary Epidemiology and Economics, Ottawa, Canada, Poster session, p 608, Aug 1991

Bahaman, A.R. & Ibrahim, A.L. A review of leptospirosis in Malaysia. *Veterinary Research Communications* 1988 **12**: 179-189.

Bajani, M.D., Ashford, D.A., Bragg, S.L., Woods, C.W., Aye, T., Spiegel, R.A., Playkatis, B.D., Perkins, B.A., Phelan, M., Levett, P.N. & Weyant, R.S. (2003). Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. *Journal of Clinical Microbiology* **41**(2): 803-809.

Basker, P., Kannan, P., Kolandaswamy, K.G. (2014). Study on the prevalence of leptospirosis among fever cases reported from private clinics in the urban areas of Villupuram district, Tamil Nadu, India. *Osong Public Health and Research Perspectives* **5**(1): 54-67.

Benacer, D., Woh, P.Y., Mohd Zain, S.N., Amran, F. & Thong, K.L. (2013). Pathogenic and saprophytic *Leptospira* species in water and soils from selected urban sites in peninsular Malaysia. *Microbes and Environmets* **28**: 135-140.

Blackmore, D.K., Bahaman, A.R. & Marshall, R.B. (1982). The epidemiological interpretation of serological responses to leptospiral serovars in sheep. *New Zealand Veterinary Journal* **30**: 38-42.

Blacksell, S.D., Smythe, L., Phetsouvanh, R. Dohnt, M., Hartskeerl, R., Symonds, M., Slack, A., Vongsouvath, M., Davong, V., Lattana, O., Phongmany, S., Keolouangkot, V., White, N.J., Day, N.P.J. & Newton, P.N. (2006). Limited Diagnostic Capacities of Two Commercial Assays for the Detection of *Leptospira* Immunoglobulin M Antibodies in Laos. *Clinical and Vaccine Immunology* **13**: 1166-1169.

Bourhy, P., Bremont, S., Zinini, F., Giry, C. & Picardeau, M. (2011). Comparison of real-time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. *Journal of Clinical Microbiology* **49**: 2154-2160.

Brownlow, T., Kavanagh, O., Logan, E., Hartskeerl, R., Savage, R., Palmer, M., Krahl, M., Mackie, D. & Ellis, W. (2014). 'Leptorapide' – a one-step assay for rapid diagnosis of human leptospirosis. *Epidemiology and Infection* **142**: 1182-1187.

Brown, P.D., Gravekamp, C., Carrington, D.G., Van de Kemp, H., Hartskeerl, R.A., Edwards, C.N., Everard, C.O., Terpstra, W.J. & Levett, P.N. (1995). Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. *Journal of Medical Microbiology* **43**: 110-114.

Desakorn, V., Wuthiekanun, V., Thanachartwet, V., Sahassananda, D., Chierakul, W., Apiwattanaporn, A., Day, N.P., Limmathurotsakul, D. & Peacock, S.J. (2012). Accuracy of a commercial IgM ELISA for the diagnosis of human leptospirosis in Thailand. *The American Journal of Tropical Medicine Hygiene* **86**: 524-527.

Dikken, H. & Kmety, E. (1978). Serological typing methods of leptospires. *Methods in Microbiology* **11**: 259-307.

Dittrich, S., Boutthasavong, L., Keokhamhoung, D., Phuklia, W., Craig, S.B., Tulsiani, S.M., Burns, M., Weier, S.L., Dancel, D.A.B., Davong, V., Vongsouvath, M., Mayxay, M., Phetsouvanh, R., Newton, P.N. & Woods, K. (2018). A prospective hospital study to evaluate the diagnostic accuracy of rapid diagnostic tests for the early detection of leptospirosis in Laos. *The American Journal of Tropical Medicine and Hygiene* **98**: 1056-1060.

Effler, P.V., Bogard, A.K., Domen, H.Y., Katz, A.R., Higa, H.Y. & Sasaki, D.M. (2002). Evaluation of eight rapid screening tests for acute leptospirosis in Hawaii. *Journal of Clinical Microbiology* **40**: 1464-1469.

Eugene, E.J., Handunnetti, S.M., Wickramasinghe, S.A., Kalugalage, T.L., Rodrigo, C., Wickremesinghe, H., Dikmadugoda, N., Somaratne, P., De Silva, H.J. & Rajapakse, S. (2015). Evaluation of two immunodiagnostic tests for early rapid diagnosis of leptospirosis in Sri Lanka: a preliminary study. *BMC Infectious Diseases* **15**: 319.

Eusebi, P. (2013). Diagnostic accuracy measures. *Cerebrovascular Diseases* **36**: 267-272.

Faine, S. & Stallman, N. (1982). Amended descriptions of the genus *Leptospira Noguchi* 1917 and the species *L. interrogans* (Stimson 1907) Wenyon 1926 and *L. biflexa* (Wolbach and Binger 1914) Noguchi 1918. *International Journal of Systematic and Evolutionary Microbiology* **32**: 461-463.

Faine, S., Adler, B. & Palit, A. (1974). Chemical, serological and biological properties of a serotype-specific polysaccharide antigen in Leptospira. *Australian Journal Experimantal Biology Medical Science* **52**: 311-319.

Goris, M.G.A., Leeflang, M.M.G., Loden, M., Wagenaar, J.F.P. & Klatser, P.R. (2013). Prospective evaluation of three rapid diagnostic tests for diagnosis of human leptospirosis. *PLoS Neglected Tropical Disease* **7**(7): e2290.

Hartskeerl, R.A., Goris, M.G., Brem, S., Meyer, P., Kopp, H., Gerhards, H. & Wollanke, B. (2004). Classification of Leptospira from the eyes of horses suffering from recurrent uveitis. *Journal of Veterinary Medicine B, Infectious Diseases and Veterinary Public Health* **51**: 110-115.

Jain, M., Nigam, R. & Malik, R. (2015). A review of laboratory techniques for detecting leptospirosis. *International Journal of Current Pharmaceutical Research* **7**(3): 4-8.

Levett, P.N. & Branch, S.L. (2002). Evaluation of two enzyme-linked immunosorbent assay methods for detection of immunoglobulin M antibodies in acute leptospirosis. *The American Journal of Tropical Medicine and Hygiene* **66**(6): 745-748.

Levett, P.N., Branch, S.L., Whittington, C.U., Edwards, C.N. & Paxton, H. (2001). Two methods for rapid serological diagnosis of acute leptospirosis. *Clinical and Diagnostic Laboratory Immunology* **8**(2): 349-351.

Levett, P.N. (2001). Leptospirosis. *Clinical Microbiology Review* **14**(2): 296-326.

Loong, S.K., Khor, C.S., Chen, H.W., Chao, C.C., Ling, I.S.C., Abdul Rahim, N.F., Hassan, H., Nellis, S., Ching, W.M., Abu Bakar, S. (2018). Serological evidence of high Leptospira exposure among indigenous people (Orang Asli) in Peninsular Malaysia using a recombinant antigen-based ELISA. *Tropical Biomedicine* **35**: 1-9.

Merien, F., Portnoi, D., Bourhy, P., Charavay, F., Berlioz-Arthaud, A. & Baranton, G. (2005). A rapid and quantitative method for the detection of *Leptospira* species in human leptospirosis. *FEMS Microbiology Letters* **249**: 139-147.

Mgode, G.F., Machang'u, R.S., Mhamphi, G.G., Katakweba, A., Mulungu, L.S., Durnez, L., Leirs, H., Hartskeerl, R.A. & Belmain, S.R. (2015). *Leptospira* serovars for diagnosis of leptospirosis in humans and animals in Africa: Common *Leptospira* isolates and reservoir hosts. *Plos Neglected Tropical Diseases* **9**(12): e0004251.

Ministry of Health (2015). Epidemiology and Current Situation of Leptospirosis in Malaysia. Dr. Zainudin AW Persidangan Kesihatan Persekitaran Pihak Berkuasa Tempatan 2015 8-9 September 2015, WP Labuan.

Mohamed-Hassan, S.N., Bahaman, A.R., Mutalib, A.R. & Bejo, S.K. (2010). Serological prevalence of leptospiral infection in wild rats at the National Service Training Centres in Kelantan and Terengganu. *Tropical Biomedicine* **27**: 30-32.

Mohamed-Hassan, S.N., Bahaman, A.R., Mutalib, A.R. & Khairani, B.S. (2012). Prevalence of Pathogenic Leptospires in Rats from Selected Locations in Peninsular Malaysia. *Research Journal of Animal Sciences* **6**: 12-25

Mullan, S. & Panwala, T.H. (2016). Polymerase Chain Reaction: An important tool for early diagnosis of leptospirosis cases. *Journal of Clinical and Diagnostic Research* **10**: 8-11.

Musso, D. & La Scola, B. (2013). Laboratory diagnosis of leptospirosis: A challenge. *Journal of Microbiology, Immunology and Infection* **46**(4): 245-252.

New Straits Times Online. (2015). http://www.nst.com.my/node/69967. Accessed 2 Feb 2015

Niloofa, R., Fernando, N., De Silva, N.L., Karunanayake, L., Wickramasinghe, H., Dikmadugoda, N., Premawansa, G., Wickramasinghe, R., De Silva, H.J. & Premawansa, S. (2015). Diagnosis of leptospirosis: comparison between microscopic agglutination test, IgM ELISA and IgM rapid immunochromatography test. *PloS one* **10**(6): e0129236.

Ooteman, M.C., Vago, A.R. & Koury, M.C. (2006). Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. *Journal of Microbiological Methods* **65**(2): 247-257.

Reller, M.E., Bodinayake, C., Nagahawatte, A., Devasiri, V., Kodikara-Arachichi, W., Strouse, J.J., Flom, J.E., Dumler, J.S. & Woods, C.W. (2011). Leptospirosis as frequent cause of acute febrile illness in Southern Sri Lanka. *Emerging Infectious Diseases* **17**: 1678-1684.

Rosa, M.I., dos Reis, M.F., Simon, C., Dondossola, E., Alexandre, M.C., Colonetti, T. & Meller, F.O. (2017). IgM ELISA for leptospirosis diagnosis: a systematic review and meta-analysis. (2017). *Ciencia & Saude Coletica* **22**: 4001-4011.

Panwala, T., Rajdev, S. & Mulla, S. (2015). To evaluate the different rapid screening tests for diagnosis of leptospirosis. *Journal of Clinical and Diagnostic Research* **9**(2): 21-24.

Paster, B., Dewhirst, F., Weisburg, W., Tordoff, L., Fraser, G., Hespell, R., Stanton, T., Zablen, L., Mandelco, L. & Woese, C. (1991). Phylogenetic analysis of the spirochetes. *Journal of Bacteriology* **173**(19): 6101-6109.

Sehgal, S., Murhekar, M. & Sugunan, A. (1995). Outbreak of leptospirosis with pulmonary involvement in North Andaman. *The Indian Journal of Medical Research* **102**: 9-12.

Sehgal, S.C. (2006). Epidemiological patterns of leptospirosis. *Indian Journal of Medical Microbiology* **24**: 310-311.

Sekhar, W., Soo, E., Gopalakrishnan, V. & Devi, S. (2000). Leptospirosis in Kuala Lumpur and the comparative evaluation of two rapid commercial diagnostic kits against the MAT test for the detection of antibodies to *Leptospira interrogans*. *Singapore Medical Journal* **41**(8): 370-375.

Shekatkar, S.B., Harish, B.N., Menezes, G.A. & Parija, S.C. (2010). Clinical and serological evaluation of Leptospirosis in Puducherry, India. *Journal of Infection in Developing Countries* **4**(3): 139-143.

Signorini, M.L., Lottersberger, J., Tarabla, H.D. & Vanasco, N.B. (2013). Enzyme-linked immunosorbent assay to diagnose human leptospirosis: a meta-analysis of the published literature. *Epidemiology Infectious* **141**(1): 22-32.

Victoriano, A., Smythe, L.D., Gloriani-Barzaga, N., Cavinta, L.L., Kasai, T., Limpakarnjanarat, K., Ong, B., Gongal, G., Hall, J. & Coulombe, C. (2009). Leptospirosis in the Asia Pacific region. *BMC Infectious Diseases* **9**(1): 1.

van Eys, G.J., Gerritsen, M.J., Korver, H., Schoone, G.J., Kroon, C.C. & Terpstra, W.J. (1991). Characterization of serovars of the genus Leptospira by DNA hybridization with hardjobovis and icterohaemorrhagiae recombinant probes with special attention to serogroup sejroe. *Journal of Clinical Microbiology* **29**: 1042-1048.

Watt, G., Alquiza, L., Padre, L., Tuazon, M. & Lauglin, L. (1988). The rapid diagnosis of leptospirosis: a prospective comparison of the dot enzyme-linked immunosorbent assay and the genus specific microscopic agglutination test at different stages of illness. *The Journal of Infectious Diseases* **154**(4): 840-842.

Wuthiekanun, V., Sirisukkarn, N., Daengsupa, P., Sakaraserane, P. & Sangkakam, A. (2007) Clinical diagnosis and geographic distribution of leptospirosis, Thailand. *Emerging Infectious Diseases* **13**: 124-126.