LABLINK MEDICAL LABORATORY TESTS FOR THE DIAGNOSIS OF LEPTOSPIROSIS

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1. Introduction

Leptospirosis occurs worldwide but is most common in temperate or tropical climates. The incidence of leptospirosis in Malaysia is estimated to be between 2-5 cases per 100, 000 population. It is an occupational hazard for many people who work outdoors or with animals, including farmers, veterinarians, meat workers, dairy farmers, and military personnel. Infection is a recreational hazard for campers, or those who participate in outdoor sports in contaminated areas, and has been associated with swimming, wading, and white-water rafting.

Leptospirosis is a bacterial disease that affects humans and animals. It is caused by bacteria of the genus *Leptospira.* In humans, it causes a wide range of symptoms, including high fever, severe headache, chills, muscle aches, vomiting and may include jaundice, red eyes, abdominal pain, diarrhea, or a rash. Clinical diagnosis of leptospirosis is always problematic. Many leptospirosis symptoms can be mistaken for indicators of other diseases such as dengue, dengue haemorrhagic fever, malaria, chikungunya, rickettsial diseases and melioidosis, while some infected individuals may exhibit no symptoms at all.

If the disease is not treated, the patient could develop kidney damage, meningitis, liver failure, and respiratory haemorrhage. The illness lasts from a few days to three weeks or longer and is treated with antibiotics. In rare cases, death occurs. Outbreaks of leptospirosis are usually caused by exposure to water contaminated with the urine of infected animals. Many different kinds of animal carry the bacterium; they may become sick but sometimes have no symptoms. *Leptospira* organisms have been found in cattle, pigs, horses, dogs, rodents, and wild animals, including marine mammals. Humans become infected through contact with water, food, or soil containing urine from these infected animals. This may happen by swallowing contaminated food or water or through skin contact, especially with mucosal surfaces such as the eyes or nose, or with broken skin.

2. Diagnosis

Diagnosis of leptospirosis may be accomplished by direct detection of the organism or its components in blood, CSF, peritoneal fluid, urine, or tissues, by isolation of leptospires in cultures, or by detection of specific antibodies in serum or plasma. The collection of appropriate specimens and selection of tests for diagnosis depend upon the timing of collection and the duration of symptoms.

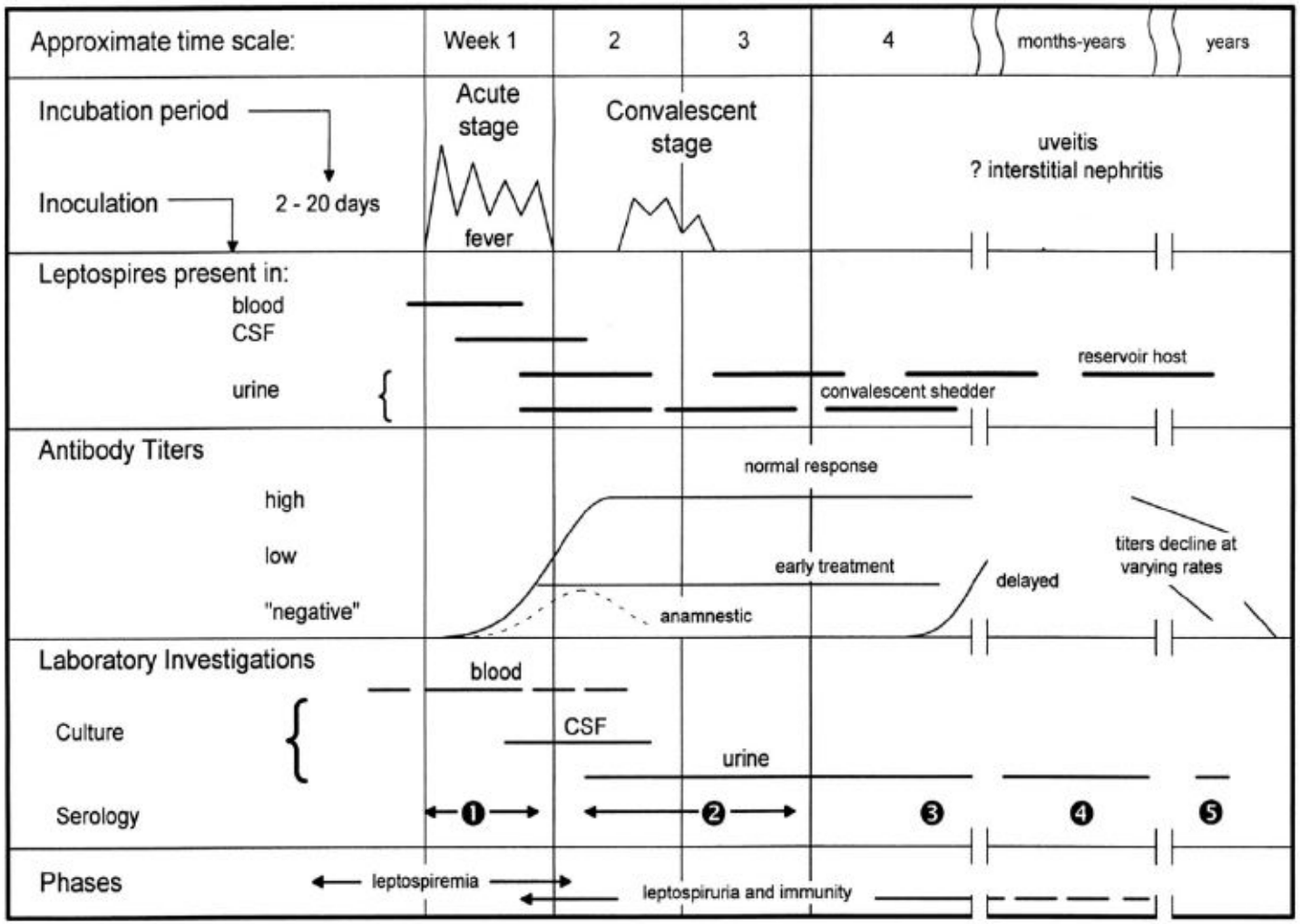


Figure 1: Biphasic nature of leptospirosis and relevant investigations at different stages of disease.

2.1 Leptospiral DNA PCR

Leptospiral DNA is practically extracted as amplified PCR products from various specimens such as plasma, serum, urine, aqueous humor, CSF and a number of post-mortem organs. Many PCR assays have been described, which target a number of different genes. Assays developed for diagnostic use can be considered in two broad categories, targeting either housekeeping genes, such as *rrs*, *gyr*B, or *sec*Y, or pathogen-specific genes such as *lipL*32, *Lig*, or *lfb* 1. Example of these two types of quantitative assay have been evaluated in a large case-control study in a high-prevalence population in Thailand which have discovered to the earlier reports that PCR detection in blood samples collected at admission to hospital is more sensitive than culture, but serology using the microscopic agglutination test (MAT) ultimately has detected more cases.

Leptospiral DNA PCR and PCR for Tropical Fever Panel are two types of PCR assays for detection of Leptospiral DNA that are already available at Lablink Molecular Diagnostic Laboratory. Both tests are strongly recommended during the acute septicaemic and immune phase. Plasma or CSF is the most preferred sample for Leptospiral DNA PCR during acute septicaemic phase, in patients without any prior antibiotic therapy, whilst whole blood is for PCR for the Tropical Fever Panel. However, early morning first void urine is the preferred sample for both Leptospiral DNA PCR and PCR for Tropical Fever Panel, if the patient presented during the immune phase as well as in acute septicaemic phase with prior antibiotic therapy (Refer Table 1, Page 11).

A limitation of PCR-based diagnosis of leptospirosis is the current inability of PCR assays to identify the infecting serovar. While this is not significant for individual patient management, the identity of the serovar has both epidemiological and public health value. Serovar identification requires isolation of the infecting strain from patients or carrier animals.

2.2 Culture

Culture of leptospires requires specialized media. Leptospires can be recovered from humans during the acute septicaemic phase of the illness and during the immune phase. Leptospiremia occurs during the first stage of the disease, beginning before the onset of symptoms and has usually declined by day 7 of the acute illness. Timing of culture of different specimens depends upon an accurate date of onset of symptoms, so a careful history is essential. Blood culture should be taken as soon as possible after the patient's presentation. Four to five ml of blood are inoculated into 5-10 ml heparin tube at the bedside. Multiple cultures yield higher recovery rates, but this is rarely possible. Leptospires have been shown to survive in commercially available blood culture media for periods of time ranging from 48 h to 4 weeks. Blood cultures with no growth can be used to inoculated leptospiral culture medium.

Other samples that may be cultures during the first week of illness include CSF and peritoneal dialysate. Urine should be cultured from the beginning of the second week of symptomatic illness. The duration of urinary excretion varies, but may be several weeks. Survival of leptospires in voided human urine is limited, so urine should be collected into sterile phosphate buffered saline. Contamination of urine culture is a major problem and the use of selective media containing 5-fluorouracil or other anti-microbial agents is strongly recommended. Cultures are incubated at 28-30 degree Celcius and examined weekly by dark field microscopy, for up to 13 weeks.

Isolated leptospires are identified either by serological methods, or more recently, by molecular techniques. Traditional methods relied on cross-agglutinin absorption. Monoclonal antibodies are available for identification of many, but not all, serovars. Molecular methods for identification and subtyping have been studied extensively. Increasingly, sequence-based identification of leptospira is becoming the standard and this can be performed on the products of diagnostic PCR. Pulse-field gel electrophoresis has been shown to identify most serovars, but complements, rather than replaces, serological identification. Identification of serovars by whole genome sequencing will likely become standardized in near future.

Leptospiral culture is not available at Lablink Medical Laboratory. The test is outsourced to Leptospiral Reference Laboratory at Institute for Medical Reasearch, Kuala Lumpur.

2.3 Serological diagnosis

Most cases of leptospirosis are diagnosed by serology and PCR, because capacity for culture is limited. IgM antibodies are detectable in the blood 5-7 days after the onset of symptoms. Serological methods can be divided into those which are genus specific and those which are serogroup-specific. The use of agglutination test was described soon after the first isolation of the organism and the microscopic agglutination test remains the definitive serological investigation in both human and animals.

2.3.1 Microscopic Agglutination test (MAT)

Microscopic Agglutination Test (MAT) is only available at Leptospiral Reference Laboratory. In the MAT, patients' sera are reacted with live antigen suspensions of leptospiral serovars. After incubation, the serum/antigen mixtures are examined microscopically for agglutination and the titers are determined. The MAT can be complex test to control, perform, and interpret. Live cultures must be maintained of all the serovars required for the use as antigens. The range of antigens used should include serovars representative of all serogroups and locally common serovars. A wide range of antigens is used in order to detect infections with uncommon, or previously undetected, serovars. The MAT is a serogroup-specific assay and cannot be relied upon to detect the infecting serovar.

The MAT is read by dark field microscopy. The endpoint is the highest dilution of serum in which 50% agglutination occurs. Because of the difficulty in detecting when 50% of the leptospires are agglutinated, the end point is determined by the presence of approximately 50% free, un-agglutinated leptospires, by comparison with the control suspension. Considerable effort is required to reduce the subjective effect of observer variation, even within laboratories.

Interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serogroups, especially in acute-phase samples. Patients often have similar titers to all serovars of an individual serogroup, but 'paradoxical' reactions, in which the highest titers are detected to a serogroup unrelated to the infecting one, may also occur. The broad cross-reactivity in the acute phase, followed by relative serogroup specificity in convalescent samples, results from the detection in the MAT of both IgM and IgG antibodies.

Paired sera are required to confirm a diagnosis with certainty. A fourfold or greater rise in titre between paired sera confirms the diagnosis, regardless of the interval between samples. The interval between first and second samples depends very much on the delay between onset of symptoms and presentation of the patient. If symptoms typical of leptospirosis are present, then an interval of 3-5 days may be adequate to detect rising titers. However, if the patient present earlier in the course of the disease, or if the date of onset is not known precisely, then an interval of 10-14 days between samples is more appropriate. Less often, seroconversion does not occur with such rapidity, and a longer interval between samples (or repeated sampling) is necessary. MAT serology is insensitive in the early acute-phase specimens. Moreover, patients with fulminant leptospirosis may die before seroconversion occurs.

The MAT is the most appropriate test to employ in epidemiological sero-surveys, since it can be applied to sera from any animal species, and because the range of antigens utilized can be expanded or decreased as required. It is usual to use a titer ≥ 100 as evidence of past exposure. However, conclusions about infecting serovars cannot be drawn without isolates; MAT data can give only a general impression about which serogroups are present within a population.

Microscopic Agglutination Test is not available at Lablink Medical Laboratory. The test is outsourced to Leptospiral Reference Laboratory at Institute for Medical Reasearch, Kuala Lumpur.

2.3.2 Other Serological test

Because the complexity of the MAT, rapid screening tests for leptospiral antibodies in acute infection have been developed. IgM antibodies become detectable during the first week of illness, allowing the diagnosis to be confirmed and treatment to be initiated while it is likely to be most effective. IgM detection has repeatedly been shown more sensitive than MAT when the first specimen is taken early in the acute phase of the illness.

Detection of IgM using ELISA has been employed widely, most often using antigen prepared from cultures of L biflexa, although pathogenic species have also been used. Several products are available commercially. Recombinant antigens have also been employed, but none has been evaluated widely. Specificity of IgM detection by ELISA is affected by the antigen used in the assay, by the presence of antibodies due to previous exposure (in endemic regions) and by the presence of other diseases.

At Lablink Medical Laboratory, there are three types of rapid leptospiral antibody test kit being used, which include Leptospiral IgM Duo, Leptospiral Rapid IgM and IgG and Leptorapide IgM Latex Agglutination. However, there are significant limitations to early diagnosis using any serological test and testing of a second sample should be considered mandatory. Moreover, confirmation of rapid diagnostic test results by a reference test has been recommended.

3. List of tests available at Lablink Medical Laboratories as at 1st January 2016.

3.1 Leptospira Rapid IgM Duo – Available at Lablink KPJ APSH, KPJ ISH, KPJ Perdana.

Type of specimen: Serum for cases with fever more than 5-7 days.

3.2 Leptospira Rapid IgG and IgM – Available at Lablink KPJ Selangor, KPJ Kajang, Nilai Medical Center, Taiping medical Center, KPJ Kuantan, KPJ JSH, and KPJ Sabah.

Type of specimen: Serum for cases with fever more than 5-7 days.

3.3 Leptorapide IgM Latex Agglutination – Available at Lablink KPJ Tawakkal, KPJ Sentosa, KPJ Damansara, KPJ Rawang and KPJ Klang.

Type of specimen: Serum for cases with fever more than 5-7 days.

3.4 Leptospiral DNA PCR – Available at Molecular Diagnostic Laboratory (MDL) at Lablink Central.

Type of specimen:

3.4.1 Blood in EDTA (Plasma): Only for cases with fever less than 7 days, also known as acute septicaemic phase, without prior antibiotic therapy.

3.4.2 Urine, Early Morning first void: Only for cases with fever less than 7 days, also known as acute septicaemic phase, with prior antibiotic therapy; or for cases with fever more than 7 days, also known as immune phase, with or without antibiotics.

3.5 PCR for Tropical Fever Panel – Available at Molecular Diagnostic Laboratory (MDL) at Lablink Central.

Type of Specimen:

3.5.1 Whole blood: Only for cases with fever less than 7 days, also known as acute septicaemic phase, without prior antibiotic therapy.

3.5.2 Urine, Early morning first void: Only for cases with fever less than 7 days, also known as acute septicaemic phase, with prior antibiotic therapy; or for cases with fever more than 7 days with or without prior antibiotic therapy.

4. List of tests referred to outsource laboratory

4.1 MAT – Outsource test to Institute for Medical Research (IMR)

Type of specimen: Serum for MAT; send only if Leptospiral rapid test is positive or equivocal. Paired sera is required; the second specimen should be collected within 7-10 days interval.

4.2 Leptospiral Culture – Outsource test to Institute for Medical Research (IMR).

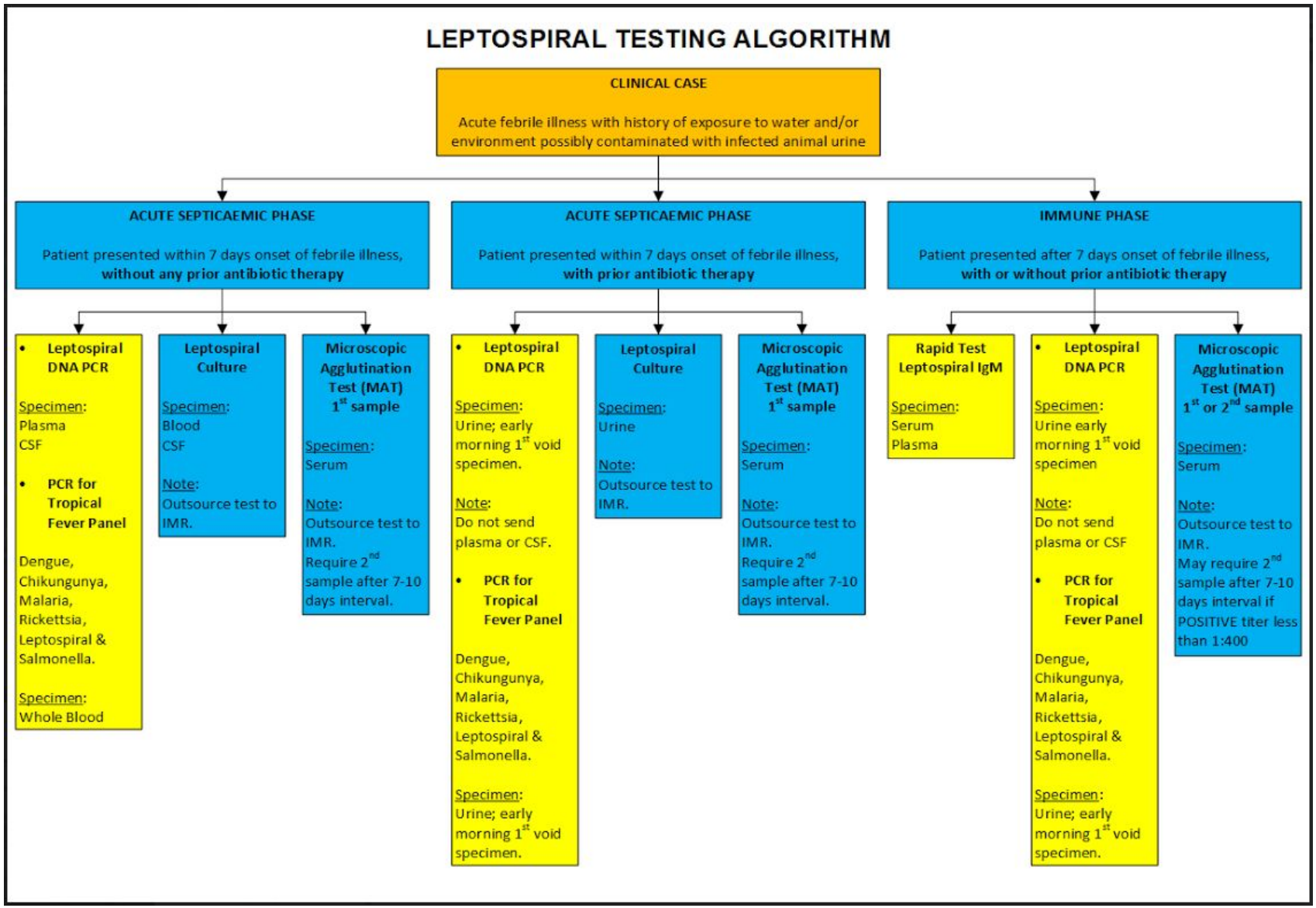
Type of specimen: Blood or CSF in EDTA tube for cases with fever less than 7 days, and urine in sterile container during immune phase, or acute septicaemic phase with prior antibiotic therapy.

Table 1 List of Leptospiral assays available at Lablink Medical Laboratories as at January 2016.

LIST OF LEPTOSPIRAL ASSAYS AVAILABLE AT LABLINK MEDICAL LABORATORIES AS AT JANUARY 2016

INFECTIOUS DISEASE SEROLOGY		KUALA LUMPUR				SELANGOR					NS		PERAK			PP	PHG	JOHOR					KEL	SRW		SBH		
NO	LIST OF TESTS	LabLink Molecular Diagnostic Lab	KPJ Tawakkal Specialist Hospital	KPJ Sentosa Medical Centre	KPJ Ampang Puteri Specialist Hospital	KPJ Damansara Specialist Hospital	KPJ Rawang Specialist Hospital	KPJ Klang Specialist Hospital	KPJ Selangor Specialist Hospital	KPJ Kajang Specialist Hospital	KPJ Seremban Specialist Hospital	Nilai Medical Centre	KPJ Ipoh Specialist Hospital	Taiping Medical Centre	Sri Manjung Medical Centre	KPJ Penang Specialist Hospital	Kuantan Specialist Hospital	KPJ Johor Specialist Hospital	KPJ Puteri Specialist Hospital	Kluang Utama Specialist Centre	KPJ Pasir Gudang Specialist Hospital	KPJ Bandar Maharani Specialist Hospital	KPJ Perdana Specialist Hospital	Kuching Specialist Hospital	Sibu Specialist Hospital	KPJ Sabah Specialist Hospital	Damai Specialist Hospital	Outsource to IMR
38	Leptospira Serology																											
	a. Leptorapide - Latex Agglutination																											
	b. Leptospira Rapid IgG and IgM																											
	c. Leptospira Rapid IgM Duo																											
	Leptospira DNA PCR																											
	PCR for Tropical Fever Panel																											
	Microscopic Agglutination Test																											
	Leptospiral Culture																											
			Leptospiral Serology																									
			Leptospiral PCR																									
			MAT and Leptospiral Culture																									

5. Table 2 : Lablink Medical Laboratory – Leptospiral Testing Algorithm



6. Table 2 : List of Lablink Leptospiral Tests

No.	Test / Objective	Specimen	Container	Transportation Requirement	Method	Note
6.1	**Leptospira IgM Duo Rapid** **Objective:** Rapid, semi-quantitative detection of IgM antibody against leptospira at titres of 50 and 200.	Venous Blood (Serum, Plasma and whole blood)	Plain Tube or Plain Tube with Serum Separator. OR Li-heparin tube OR EDTA tube OR Li- heparin with plasma separator	Ambient, or 3 days at 2–8 °C	Immuno-chromatographic Test (ICT)	**Note 1:** The test sensitivity and specificity were satisfactory when those samples tested positive for both titres were consider true positive. **Note 2:** For those sample positive at only 50 cut off titre, a repeat test with a second sample need to be done at least 2-7 days after the first sample. **Note 3:** Test efficiencies where results showing positive on IgM 50 and 200 titres. Sensitivity: 86% Specificity: 89% NPV: 88% PPV: 87% **Note 4 : Test Limitation** Cross reactivities with other causes of acute febrile illnesses. Dengue (10 samples) – 10% Typhus (5 samples) – 0% Syphilis (5 samples) – 20% Scrub typhus (10 sample) – 20% Melioidosis (5 sample) – 0%

No.	Test	Specimen	Container	Transportation Requirement	Method	Note
6.2	**Leptospira IgM and IgG Rapid** **Objective:** Rapid, qualitative screening for IgM and IgG antibodies against leptospira. **Note 1:** IgM cut off titre at 50. **Note 2:** MAT titre level < 50 indicate negative; and > 400 on a single sample indicate positive; and 4 fold increase for paired sera within 2-7 days.	Venous Blood (Serum, Plasma and whole blood)	Plain Tube or Plain Tube with Serum Separator. OR Li-heparin tube OR EDTA tube OR Li- heparin with plasma separator	Ambient, or 3 days at 2–8 °C	Immuno-chromatographic Test (ICT)	**Note 1:** Sensitivity: 87.7% Specificity: 62% NPV: 79% PPV: 75% **Note 4 : Test Limitation** Cross reactivities with other causes of acute febrile illnesses. Dengue (10 samples) – 40% Typhus (5 samples) – 0% Syphilis (5 samples) – 60% Scrub typhus (10 sample) – 60%

No.	Test	Specimen	Container	Transportation Requirement	Method	Note
6.3	**Leptorapide IgM Rapid**	Venous Blood (Serum, Plasma and whole blood)	Plain Tube or Plain Tube with Serum Separator. OR Li-heparin tube OR EDTA tube OR Li- heparin with plasma separator	Ambient, or 3 days at 2–8 °C	Latex Agglutination Antigen bound latex beads	**Note 1:** Sensitivity: 90.91% Specificity: 95.24% Shortfall:- Visual reading Strictly on time

No.	Test	Specimen	Container	Transportation Requirement	Method	Note
6.3	Leptospiral DNA PCR	Plasma, or Urine (Preferably Early Morning first void) Note: Selection of specimen is depend on the duration of febrile illness and prior antibiotic therapy	EDTA tube, or Sterile Container	2 – 8 ° C (up to 10 days)	Real-Time PCR	**NOTE 1:** **Send PLASMA** **Acute Septicemic Phase;** Patient presented within 7 days onset of febrile illness, **without any prior antibiotic therapy.** **Note 2:** **Send URINE, Early Morning First Void** **Acute Septicemic Phase;** Patient presented within 7 days onset of febrile illness, **with prior antibiotic therapy.** **Immune Phase;** Patient presented after 7 days onset of febrile illness **with or without prior antibiotic therapy.** **Sensitivity: 85.71%** **Specificity: 100%** **PPV: 100%** **NPV: 95.83%** Note: Reference – Evaluation of GenoAmp Real-Time PCR Leptospirosis in human plasma and environmental samples. W.T. kang, H.Y.Tang, Muslim N.S., Lim L. S., Y.Y. Chan & Aziz M.N.

No.	Test	Specimen	Container	Transportation Requirement	Method	Note
6.4	PCR for Tropical Fever Panel Objective PCR for the detection of Dengue Virus RNA, Chikungunya Virus RNA, Plasmodium spp. DNA, Rickettsia spp. DNA, Leptospiral spp. DNA , & Salmonella spp. DNA	Whole Blood, or Urine (Early morning first void urine)	EDTA Tube, or Sterile Container	2-8° Celcius – Up to 10 day	Multiplex Real Time PCR	**NOTE 1:** **Send WHOLE BLOOD** **Acute Septicemic Phase;** Patient presented within 7 days onset of febrile illness, **without any prior antibiotic therapy.** **Note 2:** **Send URINE, Early Morning First Void** **Acute Septicemic Phase;** Patient presented within 7 days onset of febrile illness, **with prior antibiotic therapy.** **Immune Phase;** Patient presented after 7 days onset of febrile illness **with or without prior antibiotic therapy.**

No.	Test	Specimen	Container	Transportation Requirement	Method	Note
6.5	Microscopic Agglutination Test (MAT) Note: **OUTSOURCE** to IMR.	Serum	Plain Tube, or Plain Tube with serum separator	Ambient Temperature, or 2-8° Celcius – Up to 10 day	Agglutination Test using live antigen suspension of Leptospiral serovars.	**Note 1:** The MAT is the gold standard for sero-diagnosis of leptospirosis because of its unsurpassed diagnostic specificity. A wide range of antigens or a suspension live leptospiral serovars is used in order to detect infections with locally common serovars, as well as uncommon or previously undetected serovars. **Note 2:** A repeat convalescent sera showing a four fold rise or greater in titre are required to confirm a diagnosis with certainty if the first specimen titre is less than 1:400. **Note 3:** The test is ONLY available at Leptospiral Reference Laboratory.

No.	Test	Specimen	Container	Transportation Requirement	Method	Note
6.6	Leptospiral Culture	Blood, or CSF, or Other Sterile Body Fluids, or Tissue, or Urine	Heparin Tube, Sterile Container,	Ambient	Culture	**Note 1:** Timing of culture of different specimens depends upon an accurate date of onset of symptoms, so **a careful history is essential.** **Note 2:** Send Blood, CSF, Tissue & Peritoneal Dialysate Acute Septicemic Phase; Patient presented within 7 days onset of febrile illness, without any prior antibiotic therapy. **Note 3:** Send URINE, Early Morning First Void Acute Septicemic Phase; Patient presented within 7 days onset of febrile illness, with prior antibiotic therapy. Immune Phase; Patient presented after 7 days onset of febrile illness with or without prior antibiotic therapy.

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