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The importance of using a right test method in diagnosing leptospirosis

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Abstract. Leptospirosis is a common febrile illness in Malaysia. The disease is caused by pathogenic bacteria called leptospires that are transmitted directly or indirectly from animals to humans via contaminated water or soil. It is a potentially serious but treatable disease. Its symptoms may mimic those of other unrelated febrile illnesses such as dengue, influenza, meningitis, hepatitis or viral haemorrhagic fevers. The spectrum of the disease is extremely wide, ranging from subclinical infection to a severe syndrome of multiorgan infection with high mortality. The diagnosis requires high suspicion with history of exposure to water or environment possibly contaminated with infected animal urine. This is a case of a 13 year-old-girl with no known medical illness, and a history of exposure to outdoor activities. However, paired sera for leptospirosis serology was not diagnostic. She then developed septic shock on day 14 of illness. But due to high suspicion of leptospirosis, antibiotic therapy was upgraded to ceftriaxone and samples were sent for further testing which revealed that leptospires were detected in the urine, using molecular technique. She improved after treated as leptospirosis.

INTRODUCTION

Leptospirosis in humans is a common zoonotic disease found worldwide, especially in tropical and temperate climate countries (Yaakob *et al.*, 2015). The causative organism are spirochetes from genus *Leptospira*. They are thin and highly motile, with a distinctive hook or question-mark shape (Yaakob *et al.*, 2015). The genus *Leptospira* can be divided into pathogenic and non-pathogenic (or saprophytic) strains (Musso & La, 2013). The former is known as *Leptospira interrogans*, meanwhile the latter is known as *Leptospira biflexa*. The species are then divided to more than 200 known serovars of *L. interrogans* and more than 60 serovars of *L. biflexa* based on the variability of surface antigens (Musso & La, 2013). These serovars are further organized into serogroups of *L. interrogans* and serogroups of *L. biflexa*.

Leptospirosis is characterised by wide clinical variability, ranging from a mild flu-like illness to an acute life threatening condition, but only patients with the symptomatic forms of the disease are hospitalised (Panwala *et al.*, 2015). Leptospirosis is a common cause of acute febrile illness in tropical countries and must be differentiated from other infection like typhoid, malaria, dengue, influenza and viral hepatitis (Rafizah *et al.*, 2012).

The World Health Organisation (WHO) stated that the global prevalence of leptospirosis is more than one million cases per year (WHO, 2003). The case fatality rate (CFR) ranges between five to 72% worldwide. It was estimated that, as low as 0.1 to 1 per 100,000 population living in the temperate climates are affected compared to those living in tropical climates with a higher incidence of 10 or more people per 100,000

population (Victoriano *et al.*, 2009). In Malaysia, the incidence is increasing in trend. This partly because since December 2010, leptospirosis has been gazetted as a notifiable disease under Prevention and Control of Infectious Disease Act 1988 (MOH, 2011). According to Malaysia Ministry of Health (MOH), the number of leptospirosis cases has increased dramatically from 2010 to 2015 with 22,566 cases been reported and 296 fatalities seen in the age group between 20 and 60 years (MOH, 2011).

Most leptospiral serovars have their primary reservoir in wild mammals, which continually reinfect domestic populations. The most important reservoirs are rodents, and rats are the most common. Urinary shedding of organisms from infected animals is the most important source of these bacterial pathogens (Sandra *et al.*, 2018). Contact with the organism via infected urine or urine-contaminated media results in human infection. The resulting leptospiraemia can spread to any part of the body but particularly affects the liver and kidney (Sapian *et al.*, 2012). Human infections may be acquired through occupational, recreational, or avocational exposures. Occupation is a significant risk factor for humans (Levett, 2001). In June 2010, an outbreak of melioidosis and leptospirosis co-infection was reported in Lubuk Yu, Pahang, Malaysia, following a search and rescue operation of a drowned victim (Sapian *et al.*, 2012). Lubuk Yu is a natural recreational forest with waterfall and stream. It is situated about 130 km from Kuantan, the capital state of Pahang with only basic facilities available which include toilets, changing rooms and prayer room without treated water supply.

CASE REPORT

A 13 year-old-girl with no known medical illness, presented to a clinic with fever for 3 days associated with myalgia, anorexia and vomiting. Full blood count (FBC) done showed thrombocytopenia and after hydration with 1 pint of fluid, she was referred to the nearest hospital. Dengue combo test revealed NS1 antigen and dengue antibodies were not detected. She was then discharged but instructed for repeated FBC at the nearest clinic. Her platelet levels were normalising with no further appointment for follow up. Later, 8 days after the presentation, she went to a private general practitioner presented with lethargy and dehydration. She was rushed to the emergency department via ambulance and was triaged at the red zone. She was hypotensive, tachycardic with fever. She was then admitted to the ward and intravenous (IV) co-amoxiclav was commenced. Upon admission, white cell count (WCC) 5.4 x 10^3/UL, haemoglobin (Hb) 11.6 g/dL, haematocrit (HCT) 37.4%, platelet 242 x 10^3/UL with renal profile and liver function test within normal range. Blood for culture and sensitivity test was withdrawn before commencement of the antibiotic.

On day 4 of admission, she developed septic shock with impending respiratory failure. At this point, full blood count, renal profile and liver function tests were all deranged. WCC, Hb, HCT and platelet dropped to 1.7 x 10^3/UL, 9.6 g/dL, 30.3% and 84 x 10^3/UL respectively. Her sodium and potassium levels were below normal level with increase in aspartate transaminase above normal range. Dengue combo test were negative for NS1 antigen and antibodies. She was electively intubated and transferred to the intensive care unit (ICU). Upon further questioning, her mother mentioned that she had history of jungle trekking at Hutan Simpan Universiti Kebangsaan Malaysia (UKM) 10 days prior to the first presentation to the clinic. At this point, she is treated as leptospirosis. Antibiotic was upgraded to IV ceftriaxone and a single inotrope was added to support the blood pressure. Her blood culture test sent from admission revealed no growth after 5 days incubation. Leptospirosis serology sent from the ICU was also negative. However, due to high suspicion and strong history of exposure, serum and urine samples were sent for leptospirosis molecular testing. Leptospires were detected both in the urine and serum samples using a Taqman qualitative polymerase chain reaction (qPCR) assay which was established by Stoddard *et al.* The primers and probe sequences used in this

case are as follows: LipL32-45F (5'-AAG CAT TACCGC TTG TGG TG-3'), LipL32-286R (5'-GAA CTCCCA TTT CAG CGA TT-3') and the probe, LipL32-189P (FAM-5'-AA AGC CAG GAC AAG CGCCG-3'-BHQ1 (Stoddard *et al.*, 2009), respectively. Further confirmation with 16S rDNA sequence of the positive urine sample showed 100% agreement to *Leptospira interrogans*. Therefore, the treatment of leptospirosis was continued and completed for two weeks. On the other hand, subsequently, repeat serology and microscopic agglutination test (MAT) were sent. However, the paired sera sent for MAT showed the first sample with titre of 1:200 (local serovar IMR 175) while the second sample titre was less than 1:50.

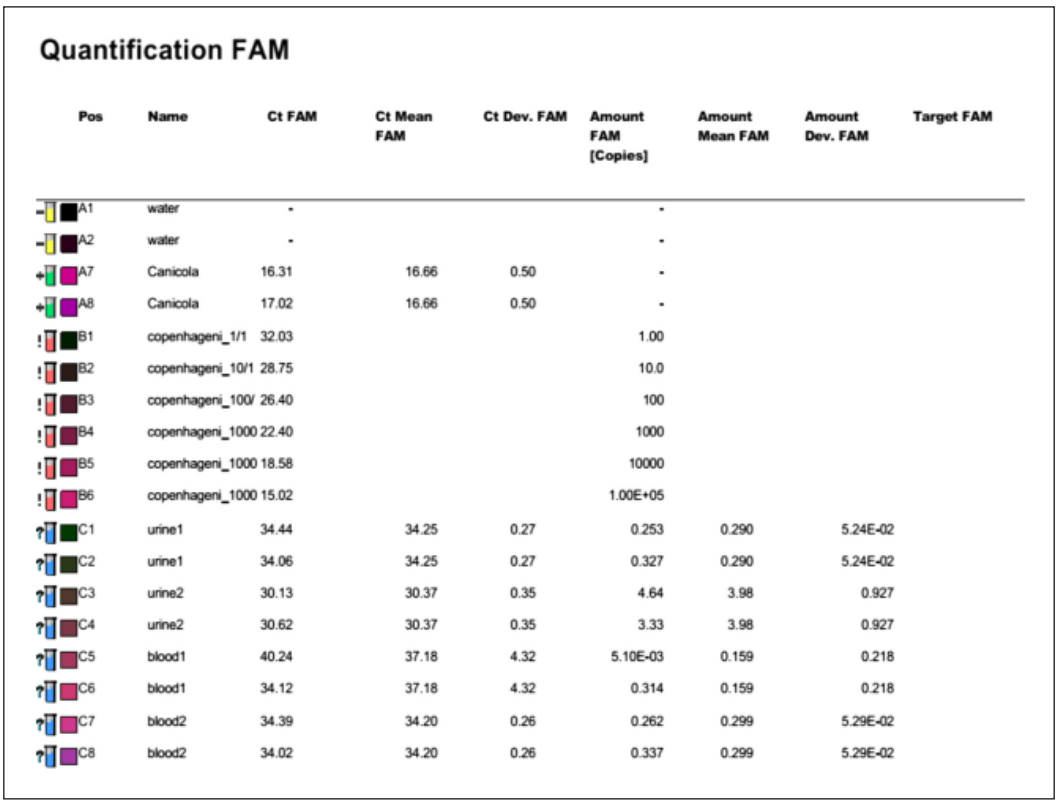
She was extubated after one day in ICU and to complete the antibiotics for 2 weeks. She was discharged well and her follow up to the outpatient clinic showed normal blood parameters with no further follow up required.

DISCUSSION

Leptospirosis is a febrile disease with varying disease spectrum caused by *Leptospira interrogans*. Numerous animals, primarily mammals, are sources of human infection. Rodents are the most important and widely distributed reservoirs of leptospires. In chronic infections, leptospires are localised in the kidneys, usually without detectable clinical manifestations (Musso & La, 2013). The mode of transmission is via contact with environmental surface water contaminated with urine of chronically infected mammals (Tan *et al.*, 2016).

The patient had history of jungle trekking at Hutan Simpan UKM, which exposed her to contaminated water and soil. However, leptospirosis was not included as initial diagnosis due to several reasons. Firstly, because poor history taking to begin with during clerking as no recent history of traveling or outdoor activities were inquired. More important reason is that leptospirosis mimics other acute febrile illnesses such as dengue and influenza, which are commoner in Malaysia. Moreover, when the WCC was not elevated, together with thrombocytopaenia. However, if proper history taking of jungle trekking was taken, leptospirosis would become one of the differential diagnosis and appropriate treatment could have been commenced earlier. Literature showed that FBC is non-specific, i.e. WCC usually is normal or only slightly elevated in most leptospirosis cases. (Silva *et al.*, 2014). Meanwhile, thrombocytopenia occurs in more than 50% of patients (Silva *et al.*, 2014; Daher *et al.*, 2010). Other blood parameters which may lead to the diagnosis include elevated bilirubin, aminotransferases, creatinine kinase and haematuria (Budihal & Perwez 2014). De Silva *et al.* also stated that anaemia, thrombocytopaenia and low haematocrit levels were associated with leptospirosis severity (Silva *et al.*, 2014), which present in this case on day 4 of admission. Due to this non-specific diagnosis, clinical history and physical examination cannot be emphasised enough, particularly when initial dengue test was negative.

On the other hand, tests used in microbiology laboratories for diagnosis include microscopy, culture, serology and molecular techniques. In this case, initial leptospirosis serology testing was negative even though tested on the 14th day of illness. This could be due to the low sensitivity of the testing kit used for the test. The test used in most government hospitals is latex agglutination method. In many reviews, the sensitivity of latex agglutination method ranges from 25 to 85% (Panwala *et al.*, 2015; Dittrich *et al.*, 2018; Sakhaee *et al.*, 2016) as compared to other method such as enzyme immunoassay (EIA), which has higher sensitivity (84–92%) (Goarant *et al.*, 2013; Niloofa *et al.*, 2015; Simon *et al.*, 2017). However, molecular test using real-time or quantitative polymerase chain reaction (qPCR) revealed positive result from both serum and urine samples. As in this case, the leptospires were detected in very high copies in the urine sample but low copies from the serum sample at day 11 of illness as shown in Figure 1. The presence of leptospires were expected in the urine sample during the second week of illness after onset. Urine is the appropriate sample



Quantification FAM

Pos	Name	Ct FAM	Ct Mean FAM	Ct Dev. FAM	Amount FAM [Copies]	Amount Mean FAM	Amount Dev. FAM	Target FAM
A1	water	-			-			
A2	water	-			-			
A7	Canicola	16.31	16.66	0.50	-			
A8	Canicola	17.02	16.66	0.50	-			
B1	copenhageni_1/1	32.03			1.00			
B2	copenhageni_10/1	28.75			10.0			
B3	copenhageni_100/	26.40			100			
B4	copenhageni_1000	22.40			1000			
B5	copenhageni_1000	18.58			10000			
B6	copenhageni_1000	15.02			1.00E+05			
C1	urine1	34.44	34.25	0.27	0.253	0.290	5.24E-02	
C2	urine1	34.06	34.25	0.27	0.327	0.290	5.24E-02	
C3	urine2	30.13	30.37	0.35	4.64	3.98	0.927	
C4	urine2	30.62	30.37	0.35	3.33	3.98	0.927	
C5	blood1	40.24	37.18	4.32	5.10E-03	0.159	0.218	
C6	blood1	34.12	37.18	4.32	0.314	0.159	0.218	
C7	blood2	34.39	34.20	0.26	0.262	0.299	5.29E-02	
C8	blood2	34.02	34.20	0.26	0.337	0.299	5.29E-02	

Figure 1. PCR result of serum and urine samples.

to be tested at this time (Levett, 2001). However, low copy numbers were also being detected in the serum sample, indicating the high sensitivity of the test method. PCR detects DNA in blood in the first five to ten days after the onset of the disease and up until the 15th day (Musso & La, 2013). The bacterial load in serum ranges from 10^5 to 10^9 leptospires/L. Molecular method is more sensitive and specific in diagnosing leptospirosis compared to latex agglutination, allowing early accurate diagnosis. Even though in this case it is not particularly used in diagnosis, it is important to confirm the diagnosis, thus leading to continuation of appropriate antibiotic for the patient. Therefore, patient was discharged well after completion of the antibiotic.

Nevertheless, microscopic agglutination test (MAT) remains the gold standard for leptospirosis diagnosis. This test is not only to detect leptospira, but also enables its identification. Despite having positive result for molecular testing, using qPCR method, the paired sera of MAT did not show positivity or increase in antibody levels; either by seroconversion or four-fold rise in the titre. Even using single sera, it is still not diagnostic as in the MOH guidelines, the titre must be of 1:400 or more to diagnose as leptospirosis (MOH, 2011). MAT will be detected positive from day 10 to 12 after the onset of illness, however the test could remain negative sometimes after antibiotics administration during the early phase of the disease. This may be in fact the reason why MAT did not show four-fold rise in the convalescent sample. MAT was reported to have a sensitivity of 41% during the 1st week, 82% during the 2nd to 4th week, and 96% beyond the 4th week of illness (Musso & La, 2013). Currently, 24 live serovars were used in leptospirosis diagnosis at our reference laboratory including 5 of local strains while the remaining are of WHO strains. A limited serovars panel used may be the reason for

false negative results. This happens because the infective serovar was not available or identified in the reference laboratory.

CONCLUSION

Diagnosis of leptospirosis requires proper history and high suspicion from the treating physicians, supported by laboratory diagnosis. Apart from proper history taking and physical examination, laboratory tests may aid in the diagnosis. Molecular method is more sensitive and specific in diagnosing leptospirosis compared to latex agglutination or MAT, allowing for early and more importantly accurate diagnosis and hence appropriate treatment. Molecular testing is superior in the diagnosis, which it can detect even after one week of illness, i.e. post bacteraemic phase showing good detection rate. Best sample is urine as observed in this case. However, as this test is expensive, it can be used as a second line method if serology is negative in high suspicion index. Nevertheless, serology still has epidemiological value and MAT remains the gold standard of diagnosis.

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