UNIVERSITI PUTRA MALAYSIA

DETECTION OF LEPTOSPIROSIS IN A DOG SHELTER

BOO AO LIN

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DETECTION OF LEPTOSPIROSIS IN A DOG SHELTER

BOO AO LIN

A Project Paper submitted to the

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It is hereby certified that we have read this project paper entitled "Detection of Leptospirosis in A Dog Shelter" by Boo Ao Lin and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD 4999-Final Year Project.

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DEDICATIONS

ALMIGHTY GOD

FAMILY

To my parents and siblings who give me continuous support all the time

LECTURERS AND STAFFS

To my supervisor and co-supervisors for all their guidance and assistance

To all lecturers and staffs of Faculty of Veterinary Medicine, UPM, for all the dedications and contributions in veterinary education

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To my beloved classmates of batch 2012/2017

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LIST OF ABBREVIATIONS

µl	microliter
◦C	degree Celsius
CDC	Centers for Disease Control and Prevention
CFR	Case Fatality Rate
CFU	Colony forming units
ELISA	Enzyme-linked immunosorbent aasay
EMJH	Ellinhausen-McCullough-Johnson-Harris
IACUC	Institutional Animal Care and Use Committee
LPHS	Leptospirosis Pulmonary Haemorrhage Syndrome
LPS	lipopoysaccharide
MAT	Microscopic Agglutination Test
mL	milliliter
n	sample size
OIE	World Organisation for Animal Health
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
rpm	round per minute
sv.	serovar
UPM	Universiti Putra Malaysia

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakultin Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999- Projek Ilmiah Tahun Akhir

PENGESANAN LEPTOSPIROSIS DI DALAM SATU PUSAT PERLINDUNGAN ANJING

Oleh

Boo Ao Lin

2017

Penyelia: Dr. Lau Seng Fong

Penyelia Bersama: Dr. Khor Kuan Hua

Dr. Rozanaliza Radzi

Leptospirosis (Penyakit Kencing Tikus) adalah penyakit bakteria zoonotik yang dilaporkan di seluruh dunia dengan rekod lebih daripada satu juta kes manusia setiap tahun di dunia. Kajian yang dijalankan ke atas Leptospirosis dalam kalangan anjing adalah tidak mencukupi di Malaysia walaupun mereka mungkin mempunyai risiko yang tinggi untuk menyebar penyakit ini kepada manusia. Tujuan kajian ini adalah untuk mengesan Leptospirosis dan serovars yang terlibat dalam pusat perlindungan anjing di Johor, Malaysia. Sampel darah telah dikumpul daripada 73 anjing terdiri

daripada 50 anjing yang telah menerima vaksinsi dan 23 anjing yang tidak pernah menerima vaksinasi. Ujian serologi MAT (Microscopic Agglutination Test) telah digunakan untuk mengesani antibodi anti-leptospiral dalam serum sampel. Di titer antibody penentuan iaitu 1:80, dua daripada 73 anjing (2.7%) adalah positif kepada Leptospira borgpetersenii serovar javanica. Dua daripada 73 anjing (2.7%) adalah positif kepada L. interrogans serovar Icterohaemorrgahiae dan satu daripada 73 anjing (1.4%) menunjukkan titers antibodi terhadap L.interrogans sv. Australis. Kelaziman keseluruhan leptospirosis adalah 6.8% (n = 5/73) dalam 73 anjing yang telah disampelkan. Antara kelima-lima anjing yang positif, 80% adalah betina dan 20% adalah jantan, dan kesemuanya telah menerima vaksinasi. Dapatan kajian ini menunjukkan bahawa anjing berpotensi menyebarkan penyakit ini kepada manusia dan binatang lain. Justeru, kajian lanjutan untuk menyelidik peranan epidemiologi anjing dalam Leptospirosis adalah diperlukan.

Kata kunci: Leptospirosis, Penyakit Kencing Tikus, anjing, MAT, kelaziman, antibody

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999 Final Year Project.

DETECTION OF LEPTOSPIROSIS IN A DOG SHELTER

by

Boo Ao Lin

2017

Supervisor: Dr. Lau Seng Fong

Co-supervisors: Dr. Khor Kuan Hua

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Leptospirosis is a zoonotic bacterial disease of worldwide distribution with more than one million human cases reported annually in the world. Limited study has been conducted on canine leptospirosis in Malaysia despite they may have high risk of transmitting the disease to human. The purpose of this study was to detect the canine leptospirosis and possible serovars involved in a dog shelter in Johor, Malaysia. Blood samples were collected from 73 dogs consisted of 50 vaccinated dogs and 23 non-vaccinated dogs. Microscopic agglutination test (MAT) was used to screen the serum samples for anti-leptospiral antibodies. At the cut –off titer of 1:80, two out of 73 dogs (2.7%) were seropositive for Leptospira borgpetersenii serovar Javanica. Another two out of 73 dogs (2.7%) were seropositive for L.interrogans serovar

Icterohaemorrgahiae and one out of 73 dogs (1.4%) showed antibody titers against L.interrogans sv. Australis. The overall seroprevalence was 6.8% (n=5/73) in the 73 dogs studied. All seropositive dogs are vaccinated, consisting of 80% females and 20% males. The seropositive status of these shelter dogs showed that they could be potential disease disseminator to human and other animals warrant further investigation for their potential epidemiological role in leptospirosis.

Keywords: Leptospirosis, canine, MAT, seroprevalence, anti-leptospiral antibodies

1.0 INTRODUCTION

Leptospirosis is a zoonotic bacterial disease of worldwide distribution with more than one million human cases reported annually in the world. It is caused by a spirochete of the *Leptospira* genus which belongs to the family *Leptospiraceae*, order Spirochaetales. Leptospires comprise of both saprophytic and pathogenic species. Among the 300 serovars classified based on the expression of the surface-exposed epitopes in a mosaic of the lipopolysaccharide (LPS) antigens, 250 are pathogenic (Adler & Moctezuma, 2010; Goris, 2016). This wide spread zoonosis is depicted by Alder and Moctezuma (2010) as disease incidence have been reported in all continents and virtually all mammalian species examined. This disease has been recognized as a re-emerging disease particularly in tropical countries and is significant for public health concerns due to its zoonotic risk. Besides, leptospirosis is a main cause of disease in production and companion animals such as dogs, cattle, swine, horses, deer and probably sheep (Ellis, 2015).

Natural reservoir hosts of the disease range from rodents, companion animals such as dogs, livestock such as cattle, pigs, and wild animals. Dogs are considered maintenance hosts for serovar Canicola, incidental hosts for other serovars. They are considered a potential source of infection to human due to the close association with people and their unsanitary habits (Phumoonna *et al*., 2009).

Leptospirosis is a systemic disease both in humans and domestic animals, predominantly dogs, cattle and swine. Clinical signs are variable in various kinds of animal species with most cases are subclinical and are related to host adapted serovars such as Canicola in dogs and Hardjo in cattle. According to Adler & Moctezuma (2010),

four syndromes have been recognized in dogs including icteric, hemorrhagic, uremic and reproductive (abortion and premature or weak pups).Typical leptospirosis in dogs may show signs such as fever, jaundice, vomiting, diarrhea, intravascular disseminated coagulation, uremia caused by renal failure, hemorrhages and death (Bolin, 1996).

Canine leptospirosis was first described in 1899. The causative agents which are most common in clinical cases of canine leptospirosis are *Leptospira* interrogans and the serovars Icteroahemorrhagiae and Canicola were described in 1960 (Carrasco, 2015). The incidence of infection seems to have reduced with the widespread use of the bivalent vaccines containing these two common serovars. However, there is increased incidence of the disease reported in the past 20 years and the most prevalent serovars nowadays are *L kirschneri* serovar Grippotyphosa, *L interrogans* serovar Pomona and *L. interrogans* serovar Bratislava (Carrasco, 2015). Species such as: *L. hebdomadis, L. autumnalis, L. australis, L. medanesis, L. bataviae* and *L. sejroe* also have been observed in studies from other countries (Robert, 1955).

To date, studies on canine leptospirosis among dog population in Malaysia is still not adequate which leads to limited knowledge on the prevalence and epidemiology of canine leptospirosis in Malaysia. The purpose of this study was to detect the canine leptospirosis and possible serovars involved in a dog shelter in Johor, Malaysia. The findings would provide information on the disease status of leptospirosis in local dog population in Malaysia and provide some insight into the epidemiology of leptospirosis in Malaysia.

2.0 LITERATURE REVIEW

2.1 Epidemiology and Transmission of Leptospirosis

Leptospirosis is a transmissible disease of animals and humans caused by infection with any of the pathogenic members of the genus *Leptospira* (OIE, 2016). Levett and Haake (2010) explained that "Leptospira" derives from the Greek *leptos* (thin) and Latin *spira* (coiled). Leptospires are long (6-20 μm) and thin (< 0.2 μm) coiled bacteria which belongs to the family *Leptospiraeae,* order Spirochaetales. The pointed end of the cells is usually bent into a characteristic hook and *Leptospira* appear as actively motile spirochetes under darkfield microscopy by rotation of two axial flagella (Levett & Haake, 2010). There are 22 species in the genus *Leptospira* (11 pathogenic), approximately 38 serogroups (31 pathogenic) and more than 250 serovars (Xu *et al*., 2014). Xu *et al*. (2014) depicted that *L.interrogans* serovars Canicola, Icterohemorrhagiae and Pomona and *L.kirschneri* serovar Grippotyphosa are the most common pathogenic leptospires in dogs. Lig proteins are the key virulence determinants which exclusively present in pathogenic *Leptospira* species (Xu *et al*., 2014). Leptospires are obligate aerobes with an optimum growth temperature of 28-30˚C (Thayaparan *et al*., 2013).

There are two types of mammalian hosts in Leptospirosis, namely primary (reservoir) host and incidental host. The primary hosts harbor the organisms in the renal tubules without overt clinical signs with continuous shedding of organisms in the urine. The urine containing the leptospires may contaminate the environment such as soil and water which facilitate disease transmission via direct contact with the urine, blood, or

infected animal tissue or indirect contact with the contaminated environment. The renal carrier state is therefore important for the persistence and epidemiology of leptospirosis (Adler & Moctezuma, 2010). On the other hand, the incidental hosts often develop clinical signs and either clears the organisms or die when they are infected with a specific serovar that is not adapted to live chronically in this species of mammal. Clinical disease of leptospirosis in humans and domestic animals such as dogs, cattle and swine is characterized by systemic signs such as fever, renal and hepatic insufficiency, pulmonary manifestations and reproductive failure. Each serovar is adapted to one or more reservoir hosts. Dogs serve as the reservoir hosts only for the pathogenic *L. interrogans* serovar canicola (Goldstein, 2010). Other mammals such as rodents, skunks, raccoons, farm animals and deer serve as reservoir hosts for other serovars. For instance, mice serve as reservoirs for serovar Ballum and Icterohaemorrhagiea and rats for Copenhageni (Bharti *et al*., 2003). Other host-adapted serovars include Bratislava in horses and pigs, Hardjo in cattle and Australis and Pomona in pigs (Grooms, 2006). These host-adapted serovars usually do not cause signs in their reservoir hosts, but remain in the kidney tubules, becoming an important source of infection for humans and other susceptible animals.

Leptospirosis is endemic in tropical regions where the humid and warm environmental conditions favor the survival of Leptospires (Goris, 2016). Following their excretion into the environment, the bacteria are able to survive for weeks to months in water or moist soil. In tropical countries, floods and rain are considered as one of the main risk factors (Mwachui *et al*., 2015). Several environmental conditions favor the survival of Leptospires including neutral to basic pH, non-concentrated urine, and ambient

temperatures of 0˚C-25˚C (Goldstein, 2010). Leptospires remain viable in alkaline urine but do not survive well in acidic urine, resulting in animals whose diet produces alkaline urine such as herbivores play a relatively more important role as shedders than are producers of acidic urine (Adler & Moctezuma, 2010).

Mode of transmission of leptospirosis involves direct and indirect transmission. Direct transmission occurs via direct contact with urine, blood, or infected animal tissue. Indirect transmission is more common especially in tropics where most cases are acquired by this route (Levett & Haake, 2010). It happens through exposure to a contaminated environment such as water or soil contaminated with the urine of infected animals.

2.2 Clinical Features and Pathogenesis of Leptospirosis

Canine leptospirosis is associated with acute multisystemic febrile illness with manifestation of clinical signs such as fever, anorexia, coagulopathies, hepatic disease, and renal failure (Goldstein, 2010). Goris (2016) has elucidated the pathogenesis of Leptospirosis in which the Leptospires first penetrate the wounded skin and mucous membranes, followed by multiplication, crossing of the tissue barriers and later, are disseminated to all organs via blood. Binding factor H allows the survival of pathogenic *Leptospira* in the non-immune host by inhibiting the complement system (Goris, 2016). Levett (2004) described the clinical presentation of leptospirosis as biphasic. The infection is initiated by an acute bacteremia phase which lasts for one week, followed by the immune or convalescent stage in which antibodies production and leptospires excretion via urine occurs (Levett & Haake, 2010).Goris stated that the first symptoms

such as high fever, muscle pain and headache occur usually after one to two weeks. Once antibodies appear in the immune stage, the *Leptospira* disappear from the blood, but persist in several organs such as liver, lung, kidney, heart and brain (Goris, 2016). Damage to the endothelium of small blood vessels which is the primary lesion leads to localized ischemia in organs, resulting in renal tubular necrosis, hepatocellular and pulmonary damage, meningitis, myositis and placentitis (Adler & Moctezuma, 2010). In severe cases, hemorrhage, jaundice and platelet deficiency can occur. According to Goris (2016), post-mortem findings showed multi-organ involvement such as acute tubular damage, interstitial nephritis, and hepatocellular necrosis.

2.3 Laboratory Diagnosis

Tests that are designed for Leptospirosis detection can detect either anti-leptospiral antibodies, or the agent including: leptospires, leptospiral antigens or leptospiral nuclei acid (OIE, 2016).

Identification of the agent:

Leptospires can be isolated from samples such as organs and body fluids by culture, but it is time-consuming as an incubation period of minimum 16 weeks is necessary. Therefore, it is not considered useful as a routine test for diagnosis of individual patients, but remains important for epidemiological purposes (Adler & Moctezuma, 2010).

Immunochemical tests (immunofluorescence and immunohistochemistry) is useful to demonstrate leptospires when a rapid diagnosis is required but these tests are unable to identify infecting serovar, and less suitable for diagnosing chronic carrier state because these are dependent on the number of organism present in the samples.

Demonstration of leptopsiral nuclei acid is based on polymerase chain reaction (PCR) assays which has high sensitivity, but PCR does not identify the infecting serovar (OIE, 2016).There are two categories of assays: detection of genes that are universally present in bacteria such as gryB, rrs and secY and detection of genes present exclusively in pathogenic *Leptospira* such as ligA, ligB, lipL21, lipL32, lipL41 (Thaipadunpanit, 2011).

Serological tests:

The most commonly used method for leptospirosis diagnosis is serological tests. Microscopic agglutination test (MAT) is the standard serological test which is useful for diagnosing acute infection by a four-fold antibody titer rise in paired serum samples. However, MAT is less sensitive in diagnosing chronic or endemic infection as the antibody titer may below the minimum significant titers of 1/100. The advantage of MAT is its specificity for serovars, or at least serogroups, but it cannot identify between antibodies resulting from infection or vaccination (Adler & Moctezuma, 2010). Moreover, live cultures of *Leptospira* serovars which are prevalent in a particular geographical area are necessary to achieve maximum reliability of the test.

Enzyme-linked immunosorbent assays (ELISAs) is also useful for anti-leptospiral antibodies detection. It is used for detection of recent infections and herd health screening. However, cross-reactivity between vaccine and tested serovars may present. Although ELISA obviates the need for maintenance of live cultures, its sensitivity and specificity do not match those of the MAT. Therefore, reliance on ELISA alone is not recommended (Adler & Moctezuma, 2010).

2.4 Seroprevalence of Canine Leptospirosis Worldwide

Leptospirosis in dogs is prevalent worldwide and as well as a cause of canine disease, it presents a zoonotic risk to human contacts (Klaasen & Adler, 2015). An increasing prevalence of seropositive dogs was reported as in publications from various countries, such as Switzerland (Major *et al*., 2014), Canada (Alton *et al*., 2008), United States of America (Moore *et al*., 2006).

Traditionally, *Leptospira interrogans* serovars Canicola and Icterohaemorrhagiae are thought to be the predominant serovars for majority cases of canine leptospirosis (Goldstein, 2010). The widespread usage of a bivalent vaccine containing the bacterins of these serovars has reduced the incidence of classic canine leptospirosis caused by these two serovars. In the last decade, canine leptospirosis has re-emerged in many countries and leptospires from other serogroups (Grippotyphosa, Pomona, Sejroe, Australis) have been confirmed as the causative agents (Geisen *et al*., 2007; Stokes *et al*., 2007). Changes in the infecting serovars are probably associated with the re-emergence of canine leptospirosis (Alton *et al*., 2008).

Numerous studies on the seroprevalence of canine leptospirosis and the predominant serovars have been conducted in different countries and some of the findings of the recent publications are shown in Table 1.

TABLE I SEROPREVALENCE OF CANINE LEPTOSPIROSIS IN VARIOUS COUNTRIES (*Serogroups)

Geographic region	**Country**	**Number of sera, % positive**	**Predominant *serogroups/serovars detected**	**Screening Test (cut-of titer)**	**Publicatio-ns**
Europe	Ireland	464, 45.3	*Ballum *Australis *Pomona *Sejroe	MAT, 1:10	Schller *et* al., 2015
	Serbia	1045, 5.45	Icterohaemorrhagiea Pomona Canicola Grippotyphosa	MAT, 1:100	Vojinović *et al*., 2015

	Croatia	151, 37.7	Pomona Grippotyphosa Icterohemorrhagiae Australis Saxkoebing Hardjo	MAT, 1:100	Majetic *et al*., 2012
Asia, South Pacific	Kerala, India	205, 71.12	Autumnalis Australis Pomona Grippotyphosa Canicola	MAT, 1:100	Ambily *et al*., 2012
	China	314, 7.3	Not applicable	ELISA	Shi *et al*., 2011
	Iran	93, 6.5	Grippotyphosa Icterohaemorrhagiea Hardjo Canicola	MAT, 1:100	Hayatrohi *et al*., 2014
	New Zealand	466, 14.2	Copenhageni Hardjo	MAT, 1:100	O'Keefe *et al*., 2011

South America	Trinidad	207, 15.5	Copenhageni Icterohaemorrhagiae Mankarso	MAT, 1:20	Suepaul *et al*., 2014
	Brazil	282,7.1	Copenhageni Bratislava Canicola Grippotyphosa	MAT, 1:100	Lavinsky *et al*., 2012
	Chile	247, 25.1	*Canicola	MAT, 1:100	Lelu *et al*., 2015
	Columbia	83,22.9	*Icterohaemorrhagiae *Louisiana *Tarassovi	MAT, 1:100	Romero-Vivas *et al*., 2013
North America	United States of America	33119, 8.1	Autumnalis Grippotyphosa Pomona Bratislava	MAT, 1:1600	Gautam *et al*., 2010
	Mexico	92, 8.6	Canicola	MAT, 1:100	Cruz-Romero *et al*., 2013

Africa	Zimbabwe	250, 15.6	Not applicable	ELISA (ImmunoComb ®), 1:400	Dhliwayo *et al*., 2012
	Sudan	62, 74.2	*Canicola *Icterohemorrhagiae	MAT, 1:40	Roqueplo *et al*., 2014
	Gabon	255, 24.7	Grippotyphosa Australis		
	Ivory Coast	158, 53.8	Grippotyphosa Icterohaemorrhagiae Sejroe		

2.5 Seroprevalence of Canine Leptospirosis in Malaysia

The first case of leptospirosis in domestic animals in Malaysia was reported in dog by Fletcher (1928). The serovar identified by Fletcher (1928) was *Leptospira interrogans* serovar Hebdomadis. The study performed by Wisseman *et al*. (1955) revealed that the prevalence in dogs was the second highest (42%) in domestic species after pigs in a survey of animals in Malaysia. Smith *et al*. (1961) reported that 18% of the dog sera examined was positive to leptospirosis. Joseph (1979) reported five out of nine dogs submitted to Veterinary Research Institute, Ipoh showed positive titers to leptospirosis and one of three dogs found on a pig farm with suspected leptospirosis had positive titers to serovars Bataviae, Grippotyphosa, Cynopteri and Canicola.

Since then, investigation on leptospirosis in dog population in Malaysia is limited until recent years, the potential of dogs as a source of leptospirosis infection for human are recognized once again as dogs have become popular pets in Malaysia and develop a close association with humans. Several studies have been carried out by researchers in Malaysia to investigate the seroprevalence of leptospirosis in different dog population from various regions of Malaysia and also, the common infecting serovars. Based on a study by Phumoonna *et al*. (2009), the prevalence of leptospirosis in the stray dogs in Malaysia is 83% as determined by ELISA while 33% as determined by MAT, with MAT titers of ≥100. They also found that *Leptospira interrogans* serovar Pomona was found to be the most predominat serovar in the dogs. Lau *et al*. (2016) found that the prevalence of leptospirosis in dogs in Klang Valley, Malaysia was 7% (n=4/57) and the serovars detected were *Leptospira* Canicola and *Leptospira* Icterohemorrhagiae. According to a study conducted by Khor *et al.* (2016), the seroprevalence of canine leptospirosis among shelter dogs was 3.8% (n=80) where three of the samples showed positive results towards serovar Bataviae using MAT with the titre of 1:80. In a recent study by Wong (2016), she found that the seroprevalence of leptospirosis among working dogs in Malaysia was 3.1% using MAT with the cut off titer of 1:80. The findings are shown in Table 2.

TABLE II RECENT STUDIES OF SEROPREVALENCE OF LEPTOSPIROSIS IN VARIOUS TYPES OF DOG POPULATION IN MALAYSIA

Type of dog population studied	Seroprevalence (%)	Serovars detected	Serological tests (cut-off titer)	Publication
Stray dog	83 (n=142) 33 (n=142)	Pomona	ELISA MAT , 1:100	Phumoonna *et al*., 2009
Stray dog & pet dog	7 (n=57)	Canicola. Icterohemorrhagiae	MAT, 1:80	Lau *et al*., 2016
Shelter dog	3.8 (n=80)	Bataviae	MAT, 1:80	Khor *et al*., 2015
Working dog	3.1 (n=96)	Australis, Bataviae, Javanica	MAT, 1:80	Wong, 2016

2.6 Seroprevalence of Leptospirosis among Dog Population in Shelters

Due to close association with human, dogs seem to play an important role in possible transmission of zoonotic infection, especially in relation to stray dogs. This is because stray dogs seem to have a higher chance of exposure with the contaminated environment when they wander on the streets, scavenging garbage and possibly hunting rodents for food (de Paula Dreer *et al*., 2013).

Studies have been conducted in several countries on the prevalence of canine leptospirosis among dog population from animal shelter as shown in Table 3. Zwijnenberg *et al*. (2008) reported a seroprevalence of 1.9% among 956 shelter dogs in

Australia recruited in the study. Oliveira *et al*. (2012) also detected leptospires using PCR from 20% of the 65 urine samples collected from shelter dogs in Brazil and 53.8% of sera samples were seropositive when tested with MAT. The most prevalent serovars detected were Canicola, Icterohaemorrhagiae and Copenhageni. In another study (de Paula Dreer *et al*., 2013), 20% out of 175 serum samples obtained from stray dogs in Brazil were found to be seropositive to leptospirosis when analyzed with MAT. The serovars detected in the study included Canicola, Bratislava, Tarassovi, Hardjo and Pyrogenes. In addition to the mentioned studies, Vojinović *et al*. (2015) revealed a seroprevalence of 5.45% among 1045 serum sample obtained from 11 shelters in Serbia. The predominant serovars were found to be Icterohaemorrhagiae, Pomona, Canicola and Grippotyphosa. In dog shelters in Mexico, 8.6% of 92 dogs were found to be seropositive for leptospirosis by Cruz-romero (2013). Not to forget that the most recent findings reported by Khor *et al*. (2016), a seroprevalence of 3.8% has been reported among 80 dogs from animal shelter in Malaysia with identification of serovar Bataviae.

These findings indicated that stray dogs may contribute to the spread and maintain of *Leptospira* spp. and could serve as a potential source of zoonotic infection to humans since the apparently healthy dogs are actively shedding leptospires to the environment (Khor *et al*., 2016; Oliveira *et al*., 2012).

TABLE III SEROPREVALENCE OF CANINE LEPTOSPIROSIS REPORTED IN DOG SHELTERS IN VARIOUS COUNTRIES

Country	Number of sera, Seroprevalence (%)	Serovars detected	Screening test (cut-off titer)	Publications
Australia	956, 9.5	Copenhageni	MAT, 1:50 (non-vaccinated) 1:100 (vaccinated)	Zwijnenberg *et al*., 2008
Brazil	65, 53.8 65, 20 65, 7.7	Canicola Icterohaemorrhagiae Copenhageni	MAT, 1:100 PCR (urine) PCR (blood)	Oliveira *et al*., 2012
Brazil	175, 20	Canicola Bratislava Tarassovi Hardjo Pyrogenes	MAT, 1:100	de Paula Dreer *et al*., 2013
Mexico	92, 8.6	Canicola Icterohaemorrhagiae Autumnalis Ballum	MAT, 1:100	Cruz-Romero *et al*., 2013

		Grippotyphosa		
Serbia	1045, 5.45	Icterohaemorrhagiae Pomona Canicola Grippotyphosa	MAT , 1:100	Vojinović *et al*., 2015
Malaysia	80, 3.8	Bataviae	MAT , 1:80	Khor *et al*., 2016

2.7 Public Health Concerns

Leptospirosis, a zoonotic bacterial disease appears as an emerging public health problem globally. Costa *et al*. (2015) estimated that there were annually more than one million cases and 58, 9000 deaths due to leptospirosis globally which place leptospirosis as a leading zoonotic cause of morbidity and mortality in human. However, this disease is often under-reported due to its nonspecific clinical manifestation and lack of an adequate diagnostic test (Costa *et al*., 2015).

Southeast Asia is an endemic area for leptospirosis in which the important serovars of public health significance include Icterohaemorrhagiae, Autumnalis and Canicola (Victoriano *et al*., 2009). In Malaysia, leptospirosis has been recognized as a reemerging disease. The high humidity and warm temperatures in Malaysia allow the long survival time of *Leptospira* in the environment probably facilitate the spread of leptospirosis especially during period of heavy rainfall (Lau *et al*., 2010). The reported

cases of leptospirosis in Malaysia have increased from 248 cases in 2004 to 3604 cases in 2012 and the Ministry of Health Malaysia has gazette leptospirosis as a notifiable disease since 2010 (Benacer *et al*., 2016).

There are a wide variety of clinical symptoms shown by human infected with leptospirosis and often the signs are non-specific such as fever, severe headache, myalgia, jaundice, chills, nausea, vomiting and abdominal pain. Weil's disease refers to hepato-renal disorders associated with bleeding tendency which are caused by serovars Icterohaemorrhagiae and Copenhageni and the case fatality rate (CFR) ranges from 2% to 40% (Goris,2016). According to Goris (2016), another emerging severe complication of leptospirosis is Leptospirosis Pulmonary Haemorrhage Syndrome (LPHS) with a CFR up to 80%.

Leptospira spp. infects wide range of animal species including wild and domestic animals. Dogs are found to be the most important reservoir for leptospirosis after rat (Meeyam *et al*., 2006). As dogs are closely associated with human, the risk of zoonosis transmission between dogs and human is possible since apparent healthy dogs can be potential carriers of the leptospires and continuously disseminating the bacteria in the environment.

3.0 MATERIALS AND METHODS

3.1 Sample Collection:

Prior to sample collection, consent was obtained from the animal shelter in Pekan Nanas, Johor. This study was conducted with the approval from the Institutional Animal Care and Use Committee (IACUC) (UPM/IACUC/AUP-R091/2016).

A cross-sectional study design was used in this study. The animals were restrained and 3-5 mL of blood was collected into plain blood tubes via cephalic or saphenous venipuncture by veterinarian. Information of the dogs including name, sex, breed, age, vaccination status and medical history were recorded.

3.2 Transportation and Storage of Samples:

All blood tubes containing the blood samples were stored in a polystyrene ice box with ice packs of temperature of 4˚C. The blood samples were immediately sent to Bacteriology Laboratory in Faculty of Veterinary Medicine, University Putra Malaysia. Centrifugation of the blood samples was performed at 4000 rpm for 5 minutes, followed by isolation and transferring of the blood sera into 1.5 mL Eppendorf tubes. After that, the blood sera were stored at -20˚C for further analysis using Microscopic Agglutination Test (MAT).

3.3 Microscopic Agglutination Test (MAT):

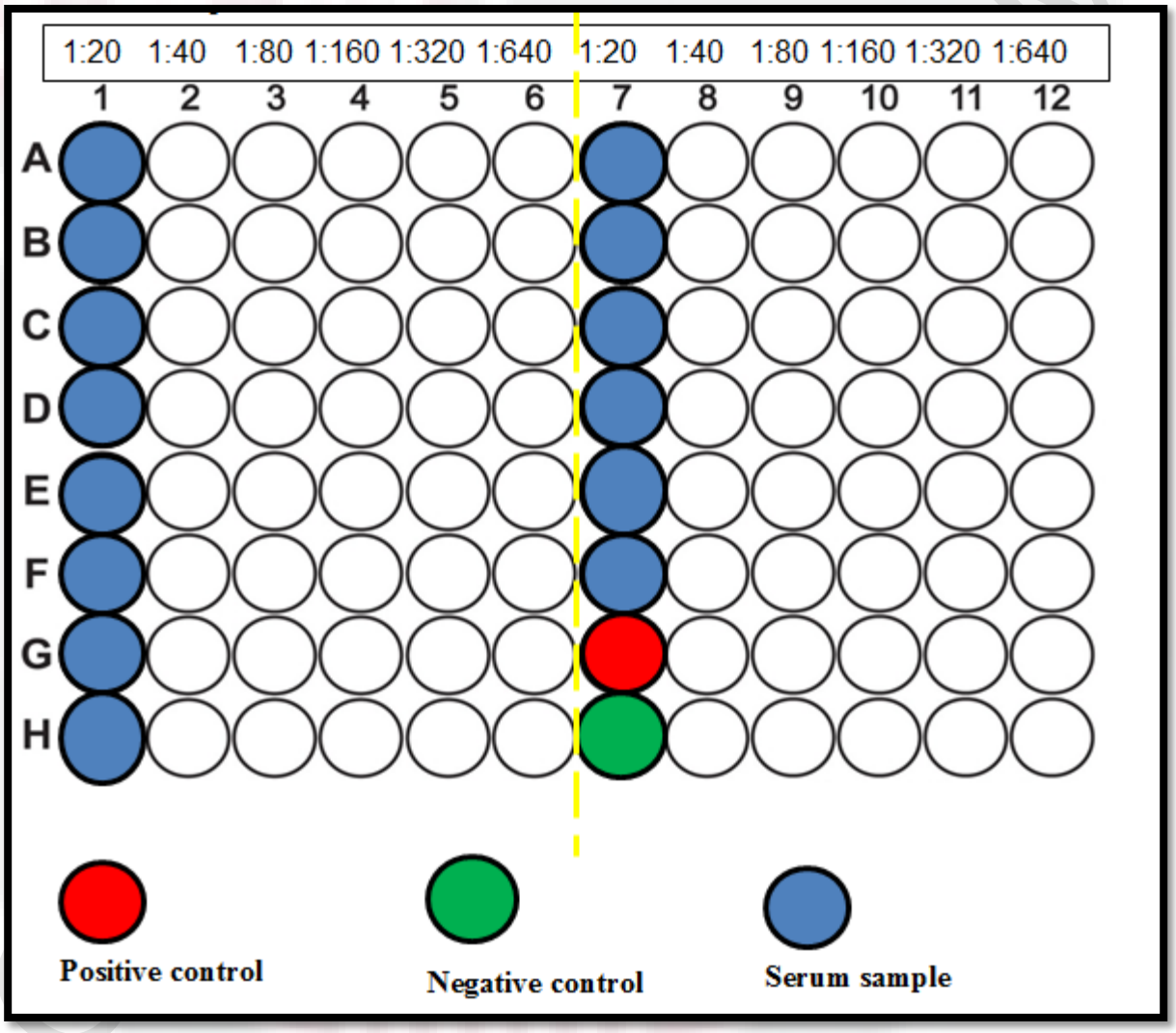
Ten serovars of live leptopsires which include Canicola, Icterohemorrhagiae, Pomona, Lai, Grippotyphosa, Celledoni, Javanica, Bataviae, Australis and Pyrogenes were subcultured using Ellinhausen-McCullough-Johnson-Harris (EMJH) medium. The EMJH mediums were then incubated at 30 ˚for 7-10 days. The densities of the leptospires antigen were examined under dark field microscopy and estimated using 0.5 MacFarland standards (1.5 x 10 10^8 CFU/mL) before being used for MAT.

The microtiter plate which has 8 rows and 12 columns was divided into half to accommodate 16 samples per plate as shown by Figure 1. For 73 samples, about 5 microtiter plates were required for each serovar including one positive and one negative control. Sterile 96-wells microtiter plates containing positive control, negative control and serial dilution of the samples were prepared as follow:

1. Each well was filled with 50 µl phosphate buffer saline (PBS) of pH 7.2.
2. Additional 40 µl of PBS were then added to wells of column 1 and column 7.
3. Next, 10 µl of serum sample were added to wells of column 1 and column 7.
4. Serial dilution was performed for each serum sample respectively by pipetting 50 µl of mixtures from wells of column 1 to column 6. The last 50 µl of mixtures was discarded.
5. Serial dilution was repeated for sera samples in column 7 by pipetting 50 µl of mixtures from wells of column 7 until column 12 as stated in step 4.

6. For the positive control well, step 1 and 2 were repeated. After that, 10 µl of hyperimmune serum were added. Serial dilution was performed as shown in step 4.
7. For negative control well, step 1 was repeated.
8. 50 µl of live antigens were added to all wells including wells of positive and negative control.
9. The microtiter plates were covered and the mixtures were then mixed thoroughly by using incubator shaker for 5 minutes.
10. All the microtiter plates were incubated at 37˚C for 2 hours.
11. After incubation, one drop of the mixtures from the negative control and positive control wells were place onto the glass slide for dark field microscopic examination. Then, repeat the same steps for the other wells to examine for any antibody-antigen agglutination under dark field microscopy. The cutoff point was set at 1:80 in which the sample was recorded as positive if at least 50% agglutination occurs at this point and the endpoint dilution is determined.
12. All the results were recorded.

FIGURE I STERILE 96-WELLS MICROTITER PLATE CONTAINING SERUM SAMPLE, POSITIVE CONTROL AND NEGATIVE CONTROL



4.0 RESULTS

In this study, 73 dogs were sampled randomly from the shelter, which consists of 31 males and 42 female dogs. Out of the 73 dogs, 23 are not vaccinated and; 50 are vaccinated. The last vaccination was on May 2016 and doxycycline was administered for one month when new dogs are introduced.

Of the 73 serum samples, the seroprevalence of Leptospirosis was 6.8% (*n*=73) where 5 of the samples showed positive results at a cut-off titer of 1:80. Of the 5 seropositive sera samples, one reacted with serovar Australis with a titer of 1:80; two reacted with serovar Javanica with titers of 1:160; two were seropositive for serovar Icterohaemorrhagiae at titers of 1:80. On the other hands, all the samples showed negative results towards the other seven serovars. All of the seropositive samples were obtained from vaccinated dogs in the shelter. 80% of seropositive dogs were females; 20% were males.

FIGURE II SEROPREVALENCE OF LEPTOSPIROSIS FROM THE BLOOD SAMPLE OBTAINED FROM 73 SHELTER DOGS AND TESTED AGAINST 10 LEPTOSPIRAL SEROVARS USING MAT WITH THE CUT-OFF TITER OF 1:80

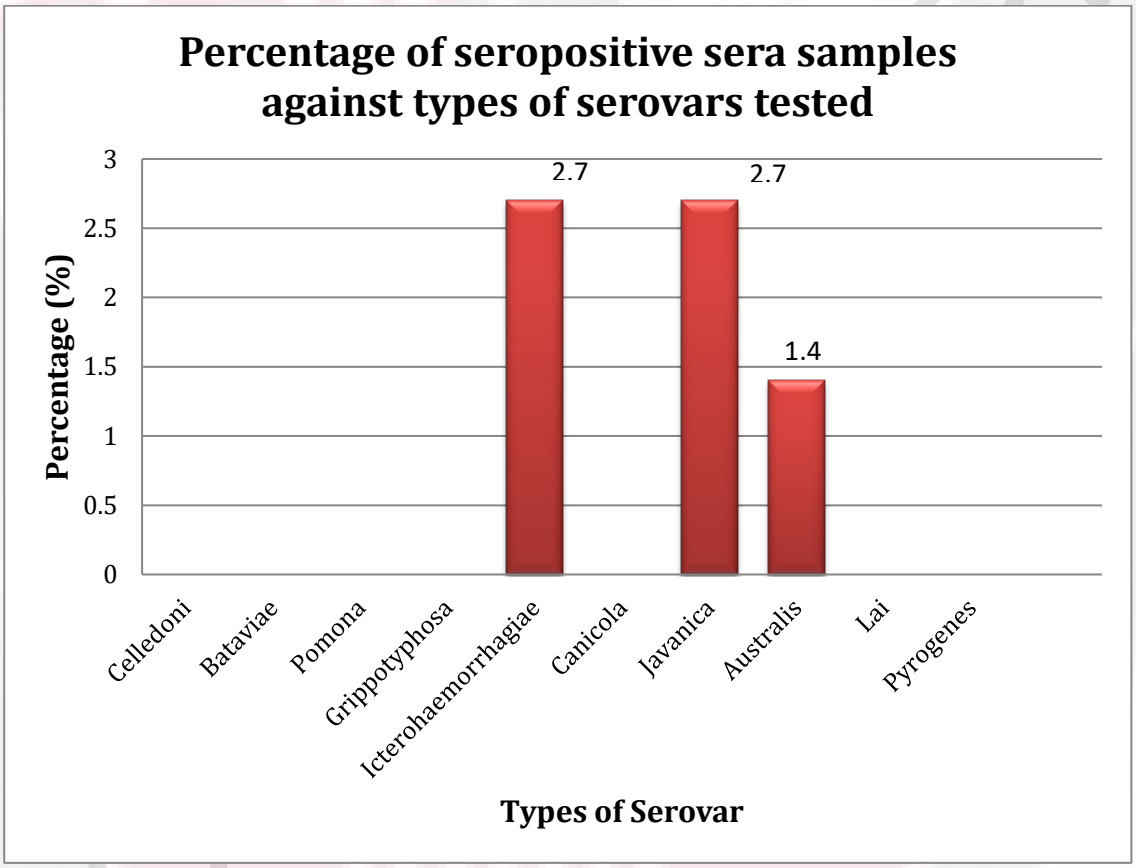
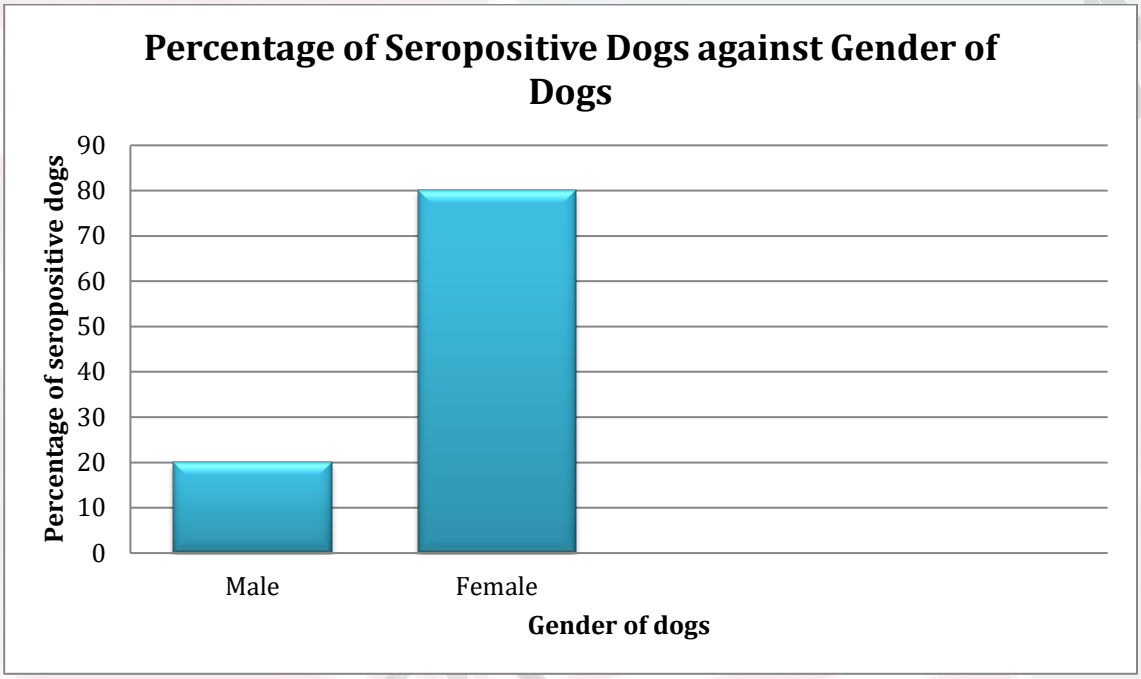


FIGURE III PERCENTAGES OF SEROPOSITIVE DOGS AGAINST GENDER



5.0 DISCUSSION

In the present study, the seroprevalence is 6.8%. The seroprevalence of anti-*Leptospira* spp. antibodies in dogs reported in other studies in Malaysia ranges from 3.1 to 33% tested which corroborates with our current findings. The present work found that predominat serovars of *Leptospira* spp. among this shelter dog population studied were Javanica (2.7%), Icterohaemorrhagiae (2.7%) and Australis (1.4%), which shares some similarities with several previous studies in Malaysia (Lau *et al*., 2016; Wong, 2016). However, these findings are in contrast with the studies by Phumoonna *et al*. (2009) and Khor *et al*. (2016) in which serovar Pomona and serovar Bataviae were reported to be the predominant serovars respectively.

The differences in the findings could be attributed to the different areas of sampling as the circulating serovars could be significantly varies from one area to another area within a country. This is evident in the studies published by Zwijnenberg *et al*. (2008) in which seroprevalence of canine leptospirosis varies from 0 to 2.8% in dog population from different geographical region in Australia and variation in serovars detected was observed in different sampling area in Australia. Moreover, variation in seroprevalence and predominant circulating serovars were observed in the studies conducted in different region of Brazil which are Porto Alegre and Paraná, Brazil respectively (Oliveira *et al*., 2012; de Paula Dreer *et* al., 2013). Besides, this can be due to the animals in contact with the stray dogs in a particular area as different serovars are maintained by different maintenance hosts, for example serovar icterohaemorrhagiae in rats and serovar Pomona

in pigs. Furthermore, leptospirosis has been reported to be directly related to sanitation conditions, infrastructure deficiencies, and the presence of rodents in each region (de Paula Dreer *et al*., 2013). The origin of dogs could also related to the differences in findings as the dogs in this shelter are mainly abandoned or rescued dog that have owner previously and therefore, are assumed to have a better care and were relatively less exposed to unsanitary condition as compared to the stray dogs in Selangor which have a higher risk of exposure to rodents in unsanitary environment such as rubbish dump area and wet markets. Type of dog population is another contributing factor to the differences seen in the findings. This is because the dogs receive different preventive care, as exemplified by the working dog population which is subjected to a regular vaccination program as compared to the stray dogs that may have not been vaccinated at all.

Besides, comparison is made with dog shelter from other countries, in which the seroprevalence ranges from 5.45% to 53.8%. The seroprevalence in this present work is slightly higher compared 5.45% in Serbia which is reported by Vojinović *et al*. (2015). The predominant serovars detected were Icterohaemorrhagiae, Pomona, Canicola and Grippotyphosa. On the other hands, dog shelters in Brazil showed higher seroprevalence which were 20% and 53.8% as reported by de Paula Dreer *et al*. (2013) and Oliveria *et al*. (2012) respectively. The serovars detected included Canicola, Bratislava, Tarassovi, Hardjo and Pyrogenes which showed significant variation with this present study. The differences between the findings can be due to variation in cut-off titer used for MAT. Cut-off titers of 1:100 were used in those studies, whereas a lower titer of 1:80 was used as the cut-off point in this present study. Choice of cut-off titer can be attributed to

different presumed level of exposure in the dog population studied which is originated from different geographical location. Based on a study by Petlanchanapong *et al*. (2009), the best cut-off titer to be used will be 1:100 in both high and low endemic areas for presumptive diagnosis of leptospirosis. Similarly, a titer of 1:100 is taken as a positive titer according to OIE (2016), but lower titer can be taken as evidence of previous exposure to *Leptospira* since MAT has a high specificity. Moreover, serosurvey in the asymptomatic high risk group should be done with MAT only and a titer of 1:50 can be used as cut off titer (Shivakumar & Krishnakumar, 2006). According to Khor *et al*. (2016), there is still inadequate documentation of local canine leptospirosis status in Malaysia which necessitates further investigation to determine an appropriate cut-off titer. Currently, the recommended cut-off titer of our local setting is 1:80. This cut-off titer seems to be appropriate as the sampled dog population appeared clinically health without any signs related to leptospirosis and their high risk status probably related to the environment and the endemic status of leptospirosis in Malaysia despite information on endemicity level of canine leptospirosis is scanty.

Besides, sample size may contribute to the differences among these findings. A larger sample size provides more accurate estimation of the seroprevalence of the population studied. A small sample size may overestimate or underestimate the seroprevalence especially when random sampling is not applied. As compared to the present study which includes 73 samples, studies in Serbia and Brazil collected much bigger sample size which were 1045 and 175 respectively (Vojinović *et al*., 2015; de Paula Dreer *et al*., 2013).

The variation as seen in the serovars detected can be affected by the number of serovars tested and the panel of serovars selected. Twenty two types of serovars were being tested in the study conducted by de Paula Dreer *et al*. (2013) which leads to detection of serovars not included in this present work such as Bratislava, Tarassovi and Hardjo. Similarly, among the thirteen types of serovars tested by Oliveria *et al*. (2012) successfully detected serovar Copenhageni which is not tested in this local study.

The types of reservoir hosts present in vicinity of the population being studied may influence the types of serovars detected. According to Bharti *et* al. (2003), the prevalence of different leptospiral serovars depends on the reservoir animals present and the serovars that they carry. In a study published by O' Keefe *et* al. (2011), the dogs from rural farming area in New Zealand are more likely to be seropositive for serovar Hardjo than urban dogs as they have more exposure with cattle which is the maintenance host of serovar Hardjo. Furthermore, Bharti *et* al. (2003) stated that a single species may carry different serovars in geographically distinct populations as illustrated by the small Indian mongoose (*Herpestes auropunctatus*), which maintains serovars Canicola in Trinidad (Everard *et al*., 1980), serovars Sejroe and Icterohaemorrhagiae in Hawaii (Tomich, 1979), serovars Icterohaemorrhagiae and Jules in Jamaica (Sulzer, 1975) and serovars Djatzi and Icterohaemorrhagiae in Puerto Rico (Alexander *et al*., 1963). Therefore, the animals in contact with the dog population of various countries can be a contributing factor to the variation in the serovars detected in respective countries.

Also, the types of screening test used influence the level of seroprevalence detected. This is especially true when we look at the study published by Oliveria *et al*. (2012) in which both Polymerase Chain Reaction (PCR) and MAT were used. The prevalence of leptospirosis detected varied significantly from 7.7% to 53.8%. In addition to that, the types of samples used can make a difference in the findings even if the same test is used. It is evident in the aforementioned study in which different prevalence was obtained although same PCR test was used which is 7.7% and 20% in blood and urine samples respectively. The variation observed is largely due to the fact that leptospirosis has a biphasic clinical presentation which is acute leptospiraemia phase and immune phase. Leptospiraemia lasts for short period of time about 3 to 10 days. Leptospires are found in the bloodstream during this acute stage in a decreasing number until 15 days after onset of symptoms (Picardeau, 2013). After that, the host enters the immune phase when antibodies production begins and clears the leptospires from the blood circulation. The immune stage occurs during the second week after onset of symptoms and lasts from 4 to 30 days (Picardeau, 2013). Shedding of the leptospires in the urine may occur 10-14 days after onset of symptoms (Picardeau, 2013; Picardeau *et al*., 2014) and persist for long period of time which in some case as long as three months after infection if inadequate treatment is provided according to CDC (2015). Therefore, MAT is suitable for detection of anti-leptospiral antibodies when the host is in the immune phase, whereas PCR detect the acute phase of leptospirosis when the leptospires is present in the blood circulation and the chronic stage when excretion of leptospires in the urine occurs. However, it is difficult to judge which phase the host is experiencing in real life situations

which makes selection of the most suitable screening test become complicated. Comparison of the results of both PCR and MAT to obtain a most comprehensive overview of the seroprevalence of a particular population maybe recommended if this option is economically and technically feasible.

In this present study, serovar Javanica is among the serovar detected that has the highest antibody titer which is 1:160 as compared to serovar Australis and Icterohaemorrhagiae. A titer of 1/100 or 1/200 may correspond to the beginning of leptospirosis, to a previous infection, or to vaccinal antibodies (Picardeau, 2013). Although all the seropositive dogs are vaccinated, the vaccine only contains serovars Pomona, Icterohaemorrhagiae, Canicola, and Grippotyphosa which does not confer protection against this serovar. This means the seropositivity for serovar Javanica and Australis could correspond to a previous infection or subclinical stage of the disease. It is therefore evident that these shelter dogs could be a potential source of transmission to human. According to Wilson *et al*. (2013), duration of immunity provided by multivalent vaccines lasts for 1 year which explains the absence of detectable antibody titer in the vaccinated dogs in the shelter. Presence of antibody titer against serovar Icterohaemorrhagiae can be related to recent infection or vaccination, whereas other vaccinated dogs which do not have detectable antibody titer may be related to non-updated vaccination schedule. A yearly booster vaccination may be required. However, absence of detectable levels of antibody is not necessarily correlated to susceptibility to infection as stated by Wilson *et al*. (2013).

The serovars detected in the present study include Javanica, Icterohaemorrhagiae and Australis. The presence of these serovars can be due to rats present in the shelter as rats are seen during sample collection. Besides, the dogs are possible to be infected before they are transported into the shelter as they originally come from stray dog population. The rodents and stray dogs share similar habitats for food foraging such as wet market as it is an ideal place for them as there are plenty of leftovers. When the stray dogs roam around freely, they are at high risk of coming into contact with infected rodents or environment contaminated with urine when they scavenge for food in the garbage area. This is supported by the studies published by Benacer *et al*. (2016) which found that *L. borgpetersenii* serovar Javanica and serovar Bataviae was one of the predominant circulating serovars among urban rat population in Pennisular Malaysia. Furthermore, rodents are natural reservoirs of leptospires especially serovar Icterohaemorrhagiae and are considered the carriers for pathogenic *Leptospira* serovars. More than half of the serovars identified in Malaysia which consists of 37 *Leptopspira* serovars from 13 different serogroups are found to be carried by rodents (Benacer *et al*., 2016). *L .interrogans* sv. Icterohameorrhagiae and sv. Canicola are used to be the dominant serovars reported. In this present study, although sv. Icterohaemorrhagiae is detected in two dogs tested; none of the samples has showed antibody titers towards sv. Canicola. This result can be related to the widespread use of the vaccine that leads to low prevalence of the serovar (Zwijnenberg *et al*., 2008; Carrasco, 2015).

Most of the seropositive samples are obtained from female dogs (4/42) compared to male dogs (1/31). This is in agreement with the findings published by Zwijnenberg *et*

al. (2008) in which higher number of female dogs were associated with leptospirosis seropositive status. However, the findings of higher number of male dogs showed antibodies against leptospirosis published by Kikuti *et al.* (2012) and Majetić *et al*. (2012) who explained that this could probably related to the tendency to roam more in male, are in contrast with this present work. Miller *et al*. (2007) related the predilection for seropositive status in male dogs to their tendency in sniffing which increased the likelihood of exposure to contaminated urine.

There are about 3000 dogs in the animal shelter in this current study. The dogs come from strays population all around Malaysia especially Johor area. Some of the dogs may be pet dogs that are being abandoned by their owner and become strays dog. The vaccination status of these dogs could be difficult to be ascertained, instead most of the time it remains as an assumption only. The seropositive dogs against serovar Javanica and Australis could have experienced natural infection because there is no leptospira vaccine against serovar Javanica and Autralis currently in Malaysia. The seropositive dogs could be in the beginning of infection or chronic renal carriers of leptospires because they appeared clinically healthy during sample collection. For the dogs that showed antibody titer to serovar Icterohaemorrhagiae, it is hard to confirm whether it is related to natural infection or post-vaccination titer because the last vaccination they received was in May 2016 and the vaccine practiced in Malaysia contains serovar Icterohaemorrhagiae with duration of immunity according to Wilson *et* al. (2013). The personnel of the shelter claimed that doxycycline was given for one month for any new coming dogs. However, the efficacy of such treatment remain doubted as shedding of leptospires in urine still

persist despite antibiotic treatment is administered as reported by Juvet *et al*. (2011) in a case report. Screening test is difficult to be a routine practice because it is not economical for shelter with such huge population of dogs and the operation of this shelter depends on the donation of public. Furthermore, when the dog population is so large in the shelter, it is quite hard for the animal handlers to notice clinical signs in the infected dogs especially when the signs are mild or non-specific. This can contribute to the possible spread of the disease in the dog population as the dogs may remain as carrier even after the clinical sign has resolved.

The animal shelter is located at Pekan Nanas, Johor. The shelter is quite remote and isolated which is in close proximity to oil palm plantation. It is therefore not surprised that rats are observed in the shelter as rats are known to be important vertebrate pest of oil palm in Malaysia (Phua *et al*., 2017). Also, garbage was seen scattered around the areas surrounding the shelter and improper food storage in the shelter can be the contributing factors for the breeding and spreading of rats. Rodents are known to be the natural reservoirs and potential lifelong carriers of leptospires. Thus, the seropositive status against different leptospiral serovars among the dogs tested can be related to direct contact with the infected rats or indirect contact with the urine containing leptospires. Besides, the low-lying area surroundings the shelter tends to accumulate puddles of water which creates a wet and humid environment. When this factor couples with the warm climate in Malaysia, it becomes a favorable environment for the survival of leptospires.

6.0 CONCLUSION

The overall seroprevalence was 6.8% (n=5/73) in the 73 dogs studied. The predominant leptospiral serovars detected were *L. borgpetersenii* serovar Javanica., *L.interrogans* serovar Icterohaemorrgahiae and *L.interrogans* sv. Australis. These findings showed that the vaccination scheme adopted from United States of America may not protect the local dog population from infection.

7.0 RECOMMENDATIONS

In future study, the sample size should be increased to represent the overall population. Also, education campaign for the personnel of the shelter is recommended to raise their awareness on leptospirosis, identification of the clinical signs of the disease and proper hygiene practice.

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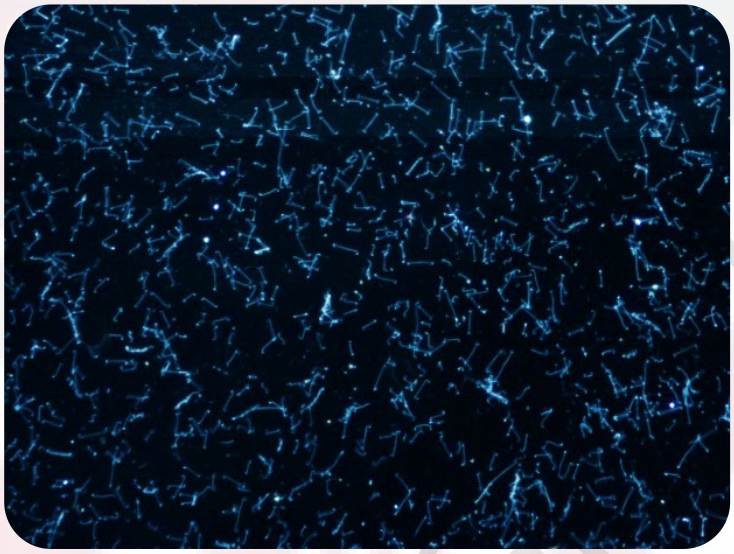
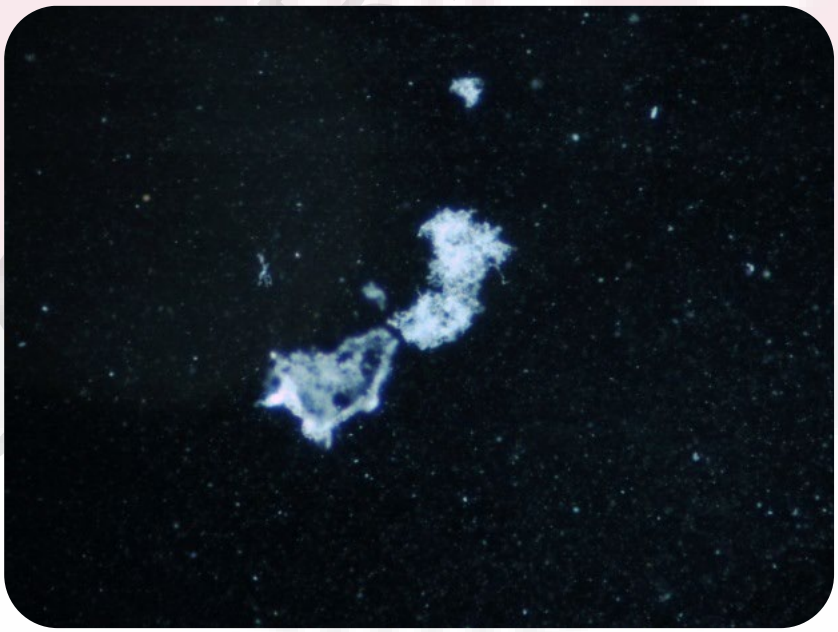
9.0 APPENDICES



Appendix 1 Negative MAT result shows >50% free living leptospires under dark field microscopy at 200x magnifications



Appendix 2 Positive MAT result shows antibody-antigen agglutinations under dark field microscopy at 200x magnifications