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DETECTION OF ANTIBODIES AGAINST *LEPTOSPIRA* SEROVARS IN DOGS AND CATS IN AN INDIGENOUS VILLAGE, BELUM, PERAK

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DETECTION OF ANTIBODIES AGAINST *LEPTOSPIRA* SEROVARS IN DOGS AND CATS IN AN INDIGENOUS VILLAGE, BELUM, PERAK

CHOONG JIA JIE

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It is hereby certified that we have read this project paper entitled "Detection of Antibodies Against Leptospira Serovars in Dogs and Cats in an Indigenous Village, Belum, Perak" by Choong Jia Jie and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 Final Year Project.

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LIST OF ABBREVIATIONS

µL	microliter
°C	degree Celsius
CDC	Centers for Disease Control and Prevention
ELISA	Enzyme-linked immunosorbent assay
EMJH	Ellinhausen-McCullough-Johnson-Harris
IACUC	Institutional Animal Care and Use Committee
LPHS	Leptospirosis Pulmonary Haemorrhage Syndrome
LPS	Lipopolysaccharide
MAT	Microscopic Agglutination Test
mL	millilitre
n	sample size
OIE	World Organisation for Animal Health
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pH	Potential Chain Reaction
rpm	round per minute
spp	species
UPM	Universiti Putra Malaysia

ABSTRAK

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

PENGESAHAN ANTIBODI TERHADAP SEROVAR *LEPTOSPIRA* DALAM KALANGAN ANJING DAN KUCING DI PERKAMPUNGAN ORANG ASLI, BELUM, PERAK

Oleh

Choong Jia Jie

2018

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Leptospirosis adalah penyakit muncul semula yang menjejaskan manusia dan haiwan. Walaupun dengan insiden yang tinggi, kajian telah dijalankan di Malaysia adalah kurang terutamanya di kawasan pedalaman seperti kampung orang asli di Belum. Wabak jangkitan herpangina virus pada tahun 2015 yang mengorbankan hampir separuh bilangan daripada anak-anak orang asli di kampung itu telah membangkitkan kebimbangan kesihatan awam. Bidang kajian yang terletak di Tiang River, Belum, Perak. Hakikat badan air (tasik buatan manusia), hidupan liar seperti tikus dan tingkah laku orang asli meletakkan mereka dalam keadaan berisiko tinggi daripada berdedah dengan wabak leptospirosis di kawasan itu. Dalam kajian ini, 40 sampel (37 anjing dan 3 kucing) dikumpul untuk menentukan kewujudan jangkitan leptospiral dari anjing dan status leptospirosis kucing di kawasan tersebut dan mengenal pasti serovar yang dominan. Ujian aglutinasi mikroskopik (MAT) digunakan untuk menentukan kewujudan antibodi anti-leptospiral dalam sampel serum yang dikumpul. Panel ujian termasuk 12 serovar leptospira: *Canicola*, *Pomona*, *Icterohaemorrhagiae*, *Grippotyphosa*, *Australis*, *Pyrogenes*, *Lai*, *Celledoni*, *Bataviae*, *Javanica*, *Hardjo* dan *Copenhageni*. Tiga daripada 37 anjing (8.1%) dan satu daripada 3 kucing (33%) menunjukkan seropositive untuk *Leptospira* pada titisan penentuan 1: 100 dengan mengikuti standard World Organisation for Animal Health (OIE). *Celledoni* 5.4% (n = 2/37) didapati sebagai serovar yang paling dominan, diikuti oleh Australis 2.7% (n = 1/37) dan Lai 2.7% (n = 1/37). 33% (n = 1/3) sebagai satu-satunya serovar utama bagi kucing yang dkaji. Penemuan ini memaklumkan bahawa risiko zoonosis yang berpotensi tinggi untuk menyebarkan leptospirosis dari haiwan kepada orang asli. dan juga kewujudan *leptospira* dalam persekitaran hidup mereka. Kajian dan penyiasatan yang melanjutkan diperlukan untuk menilai epidemiologi leptospirosis di kawasan tersebut.

Kata kunci: Leptospirosis, seroprevalensi, MAT, asli, terpencil, epidemiologi

ABSTRACT

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

DETECTION OF ANTIBODIES AGAINST *LEPTOSPIRA* SEROVARS IN DOGS AND CATS IN AN INDIGENOUS VILLAGE, BELUM, PERAK

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Leptospirosis is a re-emerging disease that affecting both human and animals. Despite the high incidence, limited study has been conducted in Malaysia especially in indigenous population. A recent outbreak of herpangina viral infection in year 2015 that wiped out almost half of the population of the indigenous children in the village has raised the public health concern. The targeted area of this study located at Tiang River, Belum state, Perak. Presence of large water bodies (man-made lake), exposure to the wildlife such as rats and living in vicinity with ownerless dogs and cats predispose this indigenous people to leptospirosis in the area. In this study, 40 samples (37 dogs and 3 cats) were collected to detect the presence of leptospiral infection of canine and feline leptospirosis status in the area and identify the predominant serovars. Microscopic agglutination test (MAT) was used to determine the presence of anti-leptospiral antibodies in the serum samples collected. The testing panel included 12 common serovars of *leptospira*: *Canicola*, *Pomona*, *Icterohaemorrhagiae*, *Grippotyphosa*, *Australis*, *Pyrogenes*, *Lai*, *Celledoni*, *Bataviae*, *Javanica*, *Hardjo* and *Copenhageni*. Three out of 37 dogs (8.1%) and one out of three cats (33%) were seropositive for Leptospira at the cut-off titer of 1:100 by following World Organisation for Animal Health (OIE) standard. *Celledoni* 5.4% (n=2/37) was found to be the most predominant serovar, followed by Australis 2.7% (n=1/37) and Lai 2.7% (n=1/37) among the dogs studied while Lai 33% (n=1/3) was found in cat as the only predominant serovar. These findings alerted that potential zoonosis risk of spreading leptospirosis from the animals to the indigenous people and also presence of *leptospira* in their living environment. Further study and investigation are crucial to evaluate the epidemiology of leptospirosis in the area.

Keywords: Leptospirosis, seroprevalence, MAT, indigenous, isolated, epidemiology

1.0 INTRODUCTION

Leptospirosis is recognized as a re-emerging zoonotic disease and raised public health concern at recent due to its increasing incidence in worldwide distribution particularly in developing tropical countries like Thailand, India and Malaysia (Tangkanakul *et al*, 2005; Vijayachari *et al*, 2008; Suut *et al*, 2016). This incidence is related to the climatic conditions and geographical topography in the countries that maintain the reservoir vectors in the environment which eventually lead to higher risk of exposure to human and domestic animals (Pappas *et al*, 2008). The exposure is higher when raining season comes into effect. The disease is caused by infection with pathogenic spirochaete bacteria of the genus Leptospira and can survive for months in water and moist soil (Alexander, 1975).

Clinical leptospirosis in dogs is more common than in cats (André-Fontaine, 2006: Arbour *et al*., 2012). Due to this reason, studies on canine leptospirosis is more advanced than feline leptospirosis in both local and global perspective. However, both dogs and cats can shed leptospirosis in their urine without having any clinical presentations of the disease (Rojas *et al*., 2010; Fenimore *et al*., 2012; Llewellyn *et al.,* 2013; Rodriguez *et al.,* 2014). Based on the literature, the most common serovars that affecting dogs include Canicola, Icterohaemorrhagiae, Pomona, Grippotyphosa and Australia (Koteeswaran, 2006). In cats, the most common serovars include Canicola, Gripotyphosa and Pomona (Larsson *et al*, n.d; Jamshidi *et al*, 2009). Despite the development of histological lesions in the kidneys and liver had been reported in experimental infected cats, clinical signs are rarely showed up in nautral infection (Jamshidi *et al*, 2009). It is also stress that cats can excrete potential zoonotic leptospires in their urine up to 3 months following the experimental infection (Willoughby *et al*, 2004).

There was no publication regarding the prevalence of Leptospirosis among isolated group of dogs and cats in indigenous village at the moment. The contribution towards the lack of study on the disease prevalence in the area probably due to unreachable rural area, lack of diagnostic tools and lack of public awareness (Suut *et al*, 2016). The targeted area (Belum indigenous village) for this study located at the

border of Thailand on the north, the state of Kelantan to the east, and Sungai Gadong in the west. The landscape of the area consists of mainly reserved forest, small areas of grassland, and abandoned agricultural lands, and a large man-made lake, Tasik Temengor (World Wildlife Fund - Malaysia). The only human population living in Belum are indigenous people and mostly consists of Jahai community. The presence of wildlife, domestic animals and water bodies have placed high risk factors of harbouring Leptospirosis in the area and hence pose a potential spread of the disease to the indigenous communities. The recent study among rural communities in East Malaysia reported that a seroprevalence of human leptospirosis was 37.4% (n=508) (Suut *et al*, 2016).

The need of studying zoonotic diseases in Belum indigenous village was further supported by the incidence of Jahai community's children were succumbed to a mysterious illness in the year 2015 (R.AGE, 2015; The Star Online, 2015). The final diagnosis was herpangina viral infection which weaken the immune system of the children (The Star Online, 2015). Poor sanitation, unbalanced diets and lack of medical facilities were believed to be the contributing factors to the incidence. Public health concern was raised and study on both human and domestic leptospirosis was initiated by Penang Medical College (PMC) and Universiti Putra Malaysia (UPM) supported by Malaysia One Health University Network (MyOHUN) in the year 2016.

According to Bahaman (1988), a total of 37 serovars had been isolated from both human and animals while rats had been described as the major reservoir of leptospires in Malaysia. The recent studies showed that the seroprevalence of Leptospirosis in non-human primates from Sarawak, Malaysia was 66% (Robertson *et al*, 2014). In addition, another seroprevalence study of Leptospirosis was done on wildlife in Sarawak showed that 80% of monkeys, 44% of rats, 20.8% of bats, 100% of squirrels and 100% of mongoose reacted against one or more serovars of Leptospira (Thayaparan, 2014).

The studies on both canine and feline leptospirosis among domestic population in Malaysia is still not sufficient to date, not to mention the isolated population in indigenous village. The objective of this study was to detect canine and feline

leptospirosis in the isolated population. The findings would provide information on the disease status of Leptospirosis and some insight of epidemiology of the disease in part of the remote rural area of Malaysia.

2.0 LITERATURE REVIEW

2.1 Epidemiology of Leptospirosis

Leptospirosis is listed as one of re-emerging infectious zoonotic disease worldwide because of the increasing incidence at both developed and developing countries (Meites *et al.*, 2004). This has been demonstrated by the large outbreaks occurred in countries such as Nicaragua, Brazil, India, Southeast Asia, Malaysia and in the United States (WHO, 2000; CDC, 2000; Evans et al., 2000). From the history, the cause of Weil's disease in human population of Japan was leptospirosis where it was more frequent among coal miners (Faine et al., 1999). The disease is more spread in tropical than temperate regions due to the sustainable survivability of the agent in warm and humid conditions (Zavitsanou & Babatsikou, 2008). There is also a strong association between periods of high rainfall and the occurrence of leptospirosis (Sullivan, 1974). *Leptospira* has been isolated from over 150 mammalian species (Ko *et al.*, 2009) but large majority of infections remains undetected since leptospirosis is a zoonosis of versatile and non-specific clinical presentations (Peter, 1982). Direct or indirect contact with urine, blood or tissue from an infected animal containing virulent *leptospiras* is the main mode of transmission in human and domestic animals (Koutis, 2007).

Leptospirosis is known to cause by spirochetes of the genus *Leptospira* which are thin, motile with a hook shape at the end. *Leptospira* demonstrates a surface morphology that mimics both the Gram-negative and Gram-positive bacteria. Presence of double membrane layers constitution supports Gram-negative bacteria whereas attachment of peptidoglycan to the inner membrane support the statement of Gram-positive nature (Vijayachari *et al.*, 2008). With that morphological similarities, *leptospira* is susceptible to any antibiotics that used to treat Gram-negative and Gram-

positive bacteria (Vijayachari *et al.*, 2008). *Leptospira spp*. can exist as saprophytic, pathogenic and intermediate species in the nature (Levett, 2001). About half of the pathogenic species belong to *L. interrogans* or *L. borgpetersenii* which causes the major leptospirosis outbreak (Victoriano *et al.*, 2009); saprophytic species such as *L. biflexa*, live in environment, water and soil and do not infect animals (Victoriano *et al.*, 2009); and intermediate species to these two groups also have been isolated from human and animals (Perolat *et al.*, 1998; Ganoza *et al.*, n.d.). Intermediate *Leptospira* which include *L. inadai*, and *L. wolffii* cause only mild and self-limiting disease without any fatality (Lehmann, 2014). According to Sykes *et al.* (2010) and Picardeau (2012), the determinant of pathogenicity among the species of *leptospira* are based on the differences in carbohydrate component of the bacterial lipopolysaccharide (LPS). LPS is the outer surface membrane of *Leptospira* sp and responsible for their serovar specificity (Cerqueira & Picardeau, 2009).

The transmission of leptospirosis is cycled between maintenance host, the reservoirs, the environment and the human population (Waitkins, 1987). Large number of domestic mammals such as cattle, pigs, and dogs serve as a source of permanent maintenance hosts or reservoirs for over 250 known serovars of the genus *Leptospira* (Anon, 2003; WHO, 1999) and rodents are known to be the most important sources of human leptospirosis (Anon 2003) and serovar *Icterohaemorrhagiae* has been associated and shed by rodents. (Matthias and Levett 2002). Domestic animals mainly the dogs despite vaccinated against *leptospira* are the significant reservoir of leptospirosis resulted in domestic transmission to human (Feigin *et al.*, 1973). The clinical presentation of leptospirosis in both human and domestic animals is characterized by systemic signs such as fever, jaundice, renal and hepatic insufficiency, pulmonary distress and reproductive problems. Leptospirosis in dogs is caused primarily by pathogenic *L. interrogans* and *L. kirschneri* and served as the reservoir hosts only for pathogenic *L. interrogans* serovar *canicola* (Goldstein, 2010). In canine leptospirosis, *L. icterohaemorrhagiae* infection causes severe liver involvement making icterus as primary clinical manifestation, while *L. canicola* infection is manifested by renal failure (Sullivan, 1974).

Feline leptospirosis appears mostly subclinical and less frequently show serological evidence in surveillance study. The serovars *L. Canicola, L. Grippotyphosa*, and *L. Pomona* have been isolated from cats. Experimental study of feline leptospirosis showed that the pathogenesis is similar to canine leptospirosis but rarely develop into clinical signs even with histological lesions detected in both liver and kidneys (Jamshidi *et al.*, 2009). This poses a higher zoonosis risk compared to dogs as subclinical cats are known to shed leptospires in their urine up to months. Other domestic animals include pigs are commonly infected with serovars *L. Pomona, L. Tarassovi, L. Grippotyphosa, L. Bratislava, L. Sejroe, L. Icterohaemorrhagiae* and *L. Canicola* (Faine, 1994). Adult pigs are usually become chronic carriers. In cattle, they are mostly infected with serovars *L. Hardjobovis*, *L. Pomona*, and *L. Grippotyphosa* according to Faine (1994). Leptospirosis in cattle can be resulted in unapparent or subclinical but may result in acute febrile illness (fever, haemoglobinuria, anaemia and icterus) or severe complications such as abortion (Sullivan, 1974).

Climate, humidity and pH of water have the vital role in spreading Leptospirosis among human and animals. Tropical countries such as Malaysia, Thailand, and India which are warm, humid and high rainfall throughout the year are at high risk for exposure to *leptospira* in the environment (Benacer *et al.*, 2016). Nevertheless, temperate countries also experience Leptospirosis incidence when seasonal changes occur especially during warm climate (Goldstein, 2010). According to WHO (2006), free *leptospira* can survive up to 180 days in water with saturated soil and surface water and this facilitate the survivability of *leptospira* in area of country like Malaysia where flooding and copious rainfall are common.

2.2 Pathogenesis and Clinical Features of Leptospirosis

Clinical signs of leptospirosis vary among species and depend on the type of serovars that infecting the animals (Goldstein *et al.,* 2006), host immunity and other secondary diseases. Recently, the emergence of leptospiral pulmonary haemorrhage syndrome (LPHS) as a life-threatening canine leptospirosis in certain area of Europe had raised concern (Schweighauser & Francey 2008a, Kohn *et al*., 2010, Sykes *et al*.,

2011, Tangeman & Littman, 2013). The incubation period of Leptospirosis can be as rapid as 1 day and experimental induced animals start showing sign at 7th day (Saravanan *et al.*, 1999, Greenlee *et al*., 2005). The period varies depending on the infecting dose, the strains and the host immunity.

Leptospirosis affects multiple systems of the animals, targeting mostly the kidneys and liver, but also affecting other organs such as lungs, spleen, endothelial cells, retina, skeletal and heart muscles, meninges, pancreas and the genital tract (Schuller *et al*., 2015). *Leptospires* first get infected into the animals by penetrate the wounded skin and mucus membrane, followed by replication and causing bacteraemia, later disseminated to organs through hematogenous route. Werts *et al* (2001) explained that the early onset of leptospirosis is not sudden due to the low endotoxic properties of leptospiral lipopolysaccharides (LPS). During the early infection, the ability of *leptospires* binding to the inhibitors of complement activation on their surface allow them to evade the host immune response (Meri *et al*., 2005, Barbosa *et al*., 2009).

The damages done by Leptospirosis include tubular cell necrosis causing interstitial nephritis, hepatocellular necrosis causing cholestatic hepatitis, pulmonary oedema and haemorrhage, pancreatitis, meningitis, myocarditis, uveitis and bleeding disorders (Nally *et al*., 2004, De Brito *et al*., 2006, Ranawaka *et al*., 2013, Shah *et al*. 2010). Consequently, this lead to clinical manifestations such as fever, tachypnoea or dypnoea, polyuria polydipsia, vomiting, anorexia, lethargy, epistaxis and haematuria (Rentko *et al*., 1992, Birnbaum *et al*., 1998, Mastrorilli *et al*., 2007).

The clinical pathology of infected animals often shown thrombocytopenia, leukopenia (acute), leucocytosis (subacute), increased alanine transaminase (ALT), aspartate transaminase (AST), azotemia, electrolyte imbalance and increased bilirubin (Nelson & Couto, 2014). Urinalysis revealed reduced urine concentrating ability, haematuria, mild proteinuria and glucosuria due to the damage done by *leptospira* in the kidneys.

2.3 Seroprevalence of Canine and Feline Leptospirosis in Worldwide

To date, numerous studies on seroprevalence of canine and feline leptospirosis was reported in publications from various countries around the world. The findings were tabulated in table 1 and 2 as below:

TABLE 1 : SEROPREVALENCE OF CANINE LEPTOSPIROSIS IN VARIOUS COUNTRIES

No	Area and Country	Seroprevalence (%) and sample size (n)	Predominant serovars detected	Serology test	Publication
1	Kerala, India	71.12, 146	Autumnalis Australis Pomona Grippotyphosa Canicola	MAT, 1:100	Ambily *et al.,* 2012
2.	Curitiba, Southern Brazil	38.9, 90	Icterohaemorrhagiae Canicola	MAT, 1:100	Martins *et al*., 2010
3	Michigan, United States	24.9 1241	Grippotyphosa Bratislava Canicola Icterohaemorrhagiae	MAT, 1:200	Stokes *et al.,* 2007
4	Trinidad, South America	23.9, 419	Mankarso Icterohaemorrhagiae	MAT, 1:100	Adesiyun *et al.,* 2006
5	Mashhad, Iran	14.38, 292	Canicola Pomona	MAT, 1:100	Kamrani & Sardari, 2003
6	KwaZulu Natal, Eastern Cape, Western Cape, Gauteng (South Africa)	4.7, 530	Canicola Pyrogenes	MAT, 1:100	Gatley, 2009
7	Yucatan, Mexico	35, 400	Canicola Icterohaemorrhagiae	MAT, 1:100 ELISA 1.34.	Jimenez-Coello *et al*., 2008

8	Ontario, Canada	22, 1136	Not stated	MAT, 1:100	Prescott, 2008
9	Saint Kitts, West Indies	73.2, 101	Autumnalis Icterohaemorrhagiae Canicola Djasiman	MAT, 1:100	Pratt *et al*., 2017
10	Ireland, Europe	45.3, 463	Ballum Australis Pomona Sejroe	MAT, 1:100	Schller *et al.,* 2015

TABLE 2 : SEROPREVALENCE OF FELINE LEPTOSPIROSIS IN VARIOUS COUNTRIES

No	**Area and Country**	**Seroprevalence (%) and sample size (n)**	**Predominant serovars detected**	**Serology test**	**Publication**
1	Saint Kitts, West Indies	6.9, 103	Pomona Bataviae Ballum	MAT, 1:100	Betance *et al*., 2015
2	Chile, South America	8.1, 124	Bataviae Autumnalis Canicola	MAT, 1:100	Azócar-Aedo *et al.,* 2014
3	Tehran, Iran	27, 111	Canicola Hardjo Icterohaemorrhagiae Grippotyphosa	MAT, 1:100	Jamshidi *et al.,* 2009
4	Worcester County, Massachusetts	4.8, 63	Autumnalis	MAT, 1:100	Markovich *et al.,* 2012
5	Belgrade, Serbia	26.7, 161	Australis Pomona Canicola Pyrogenes Bratislava	MAT, 1:100	Sonja *et al.,* 2014
6	Southern Taiwan	9.3, 233	Javanica Icterohaemorrhagiae	MAT, 1:100	Chan *et al.,* 2014

			Australis Pyrogenes		
7	Greece, Balkans	33.3, 99	Rachmati Bratislava Ballum	MAT, 1:50	Mylonakis *et al.,* 2005

2.4 Leptospirosis status in Malaysia

Leptospirosis is an endemic and re-emerging disease in tropical countries which including Malaysia (Thayaparan *et al.,* 2014). From the statistics of Ministry of Health Malaysia, from 2011 to 2015, reported cases of leptospirosis had increased from 2,268 to 8,291 which about 2.5 folds. The tropical rainforest in Malaysia possess a great bed for harbouring *leptospiras* particularly among wild animals such as bats, squirrels, rats and primates. Transmission is easily occurring between animal to animal and animal to human through various routes: water bodies, excreta contamination, and direct contact. Rats are considered the most important carrier, reservoir and source of leptospirosis in the country and they are present abundance in the environment (Mohamed-Ha *et al.,* 2012). A research done by Mohamed-Ha *et al* (2012) using Polymerase Chain Reaction (PCR) to detect pathogenic *leptospires* in rats from various states in Malaysia revealed a prevalence of 8.6%. The various states covered in the research include National Service Training Campsites, oil palm estates and Royal Belum Rainforest.

The favourable environmental conditions (moisture, pH of the soil and temperature) in Malaysia have greatly increase the survivability of *leptospires* in the environment and increases the likelihood of leptospirosis (Bahaman, *et al.,* 2010). Due to this, the prevalence of leptospiral infection depends on the environmental factors that facilitate the survival of *leptospires* outside the host and the susceptibility of human and animals towards the agent (Bejo, *et al.,* 2004). According to Bejo *et al* (2004), the longest survival time of pathogenic serovars *Hardjo* was 11 days in river water at pH 6.7 to 7.3 under shaded area. Based on the research done by Bahaman *et al* (2010), various samples (water and soils) were collected from the environment in Kelantan and Terengganu showed a leptospiral prevalence of 10.34% using PCR. The

research proposed that an outbreak can potentially occur from the environment if proper preventive measure is not initiated.

Malaysia with a geographical area consists of Peninsular Malaysia, Sabah, and Sarawak, has reported a rising incidence of leptospirosis over the past few years. A review of human leptospirosis in Malaysia at year 2014 showed an incidence rate of 25.94 per 100 000 population (Abdul Wahab, n.d.). With all the incidence, leptospirosis thus prompting to be included in the country's notifiable disease list starting in the year 2010 whereby confirmed cases must be notified to relevant health district office. Various prevalence studies had been done in Malaysia and is tabulated in table 3:

TABLE 3 : PREVALENCE OF LEPTOSPIROSIS IN MALAYSIA

No	**Location**	**Species**	**Prevalence (%) and sample size (n)**	**Predominant serovars detected**	**Type of tests used**	**Publications**
1	Bako National Park & Matang Wildlife Center (Sarawak)	Non-human primates	66.6, 12	Lai Lepto175	MAT, 1:100	Thayaparan *et al.*, 2014
2	Kuching (Sarawak)	Small rodents, bats and squirrels	47, 155	Lepto 175 Icterohaemorrhagiae Patoc Australis	MAT, 1:50	Thayaparan *et al.*, 2015
3	Various states in Peninsular Malaysia	Rats	8.6, 488	Icterohaemorrhagiae	PCR	Mohamed-Hassan *et al.,* 2012
4	Rejang Basin (Sarawak)	Human	37.4, 508	Djasiman Shermani Pomona	MAT, 1:100	Suut *et al.,* 2016
5	Kota Bharu (Kelantan)	Human	24.7, 296	Bataviae Patoc 1	MAT, 1:100	Shafei *et al.,* 2012

6	Klang Valley (Selangor)	Dogs	7, 57	Canicola Icterohaemorrhagiae	MAT, 1:80	Lau *et al.,* 2016

2.5 Diagnosis of Leptospirosis

Diagnosis of Leptospirosis can be performed by clinical presentations, clinicopathologic findings, diagnostic imaging and further confirmed by demonstrating the leptospiral agents, antigens and antibodies using several tests which include polymerase chain reaction (PCR), microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA). Few other rapid serologic tests such as indirect hemagglutination assay (IHA), IgM dipstick assay (LDS), and IgM dot-ELISA dipstick test (DST) are commercially available for leptospirosis diagnosis (Bajani *et al.,* 2003).

Pathogenic leptospiral infection lead to wide range of clinical manifestations from subclinical to severe and potentially can cause lethal (Schuller *et al.,* 2015). The clinical manifestations often include fever, anorexia, vomiting, lethargy, dypnoea/tachypnoea, jaundice, epistaxis, melena and polyuria polydipsia. For clinicopathologic findings, common haematological abnormalities revealed mild to moderate anemia, leukopenia (acute), leucocytosis and thrombocytopenia (Nelson & Couto, 2014; Schuller *et al.,* 2015). Leukaemoid reaction is reported in the case of acute leptospiral infection (Kohn *et al*., 2010). The most common biochemical abnormalities reported are increased blood urea nitrogen (BUN) and creatinine, elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP), and hyperbilirubinaemia. Electrolyte imbalance, such as hypo- and hyperkalaemia, hyper- and hypophosphataemia, hyponatraemia and hypochloraemia, are also commonly reported. Urinalysis from leptospiral infected animals revealed isosthenuria, hyposthenuria, glucosuria, proteinuria, pyuria and hematuria (Rentko *et al*., 1992, Adin & Cowgill, 2000, Goldstein *et al*., 2006, Mastrorilli *et al.,* 2007). On the other hand, thoracic radiograph findings showed mild interstitial pattern or alveolar pattern (Baumann and Fluckiger 2001). The most common abdominal ultrasonographic examination findings revealed renomegaly,

hepatomegaly and splenomegaly (Forrest, 1998; Rentko *et al*., 1992; Birnbaum *et al*., 1998; Adin & Cowgill, 2000; Mastrorilli *et al*., 2007; Kohn *et al*., 2010).

The gold standard diagnosis for confirmation of leptospirosis is microscopic agglutination test (MAT) and most widely used in case of acute leptospirosis. MAT is a serological testing to detect antibodies titer against *leptospira* using subculture live antigen from different serovars. Serial dilution of patient serum is made, and MAT is done to determine the ability of the serum to agglutinate the live leptospiral serovars in vitro (Schuller *et al.,* 2015). The serovars selected for the testing panel are based on the endemicity of the serovars in the area, the most common serovars included in the panel are *Canicola*, *Pomona*, *Icterohaemorrhagiae*, *Grippotyphosa*, *Pyrogenes*, *Australis* and *Autumnalis*. According to OIE (2014), positive cut-off point is set when 50% of agglutination of antibody-antigen reaction are observed in serial dilution of 1:100 under dark field microscopy. Seropositive serum can result from active infection, previous infection/exposure and vaccination. Hence, a paired serum or retesting in 2 to 4 weeks are recommended in seronegative patient (Nelson & Couto, 2014) or to confirm an infection.

PCR assay is developed as a rapid and direct diagnostic tool for leptospirosis. It gives a high sensitivity and specificity at early infection of the disease. Samples such as urine, blood and body fluids are used to demonstrate the DNA materials of *leptospira* by using PCR. *Leptospires* can be identified in blood for the first 5 to 10 days after infection and thereafter in urine (Greenlee *et al.,* 2005). Blood and urine specimens should not be tested by pooling, as this will potentially decrease the sensitiveness of the specimens. The targeted genes described in canine leptospirosis are lipL32/hap1 gene and 23S rDNA (Branger *et al*., 2005; Stoddard *et al.,* 2009; Rojas *et al*., 2010; Harkin *et al.,* 2003). However, PCR is not able to identify the type of serovars infected by the patient and hence MAT remains the preferred confirmatory test for leptospirosis in general.

Detection of antileptospiral immunoglobulins (IgM or IgG) via ELISA is getting popularity and becoming more commercially available. ELISA test kits can provide results within minutes but facing the same limitation as in MAT that early infection

(absence of antibodies) is not applicable or false positive due to vaccination. Re-testing of negative serum is advisable.

2.6 Potential Risk of Leptospirosis among Indigenous People

Public health concerns on leptospirosis are raised due to the increasing incidence in the countries worldwide (Vijayachari *et al.*, 2008). According to WHO, estimation concluded that 0.1 to 1 per 100 000 people living in temperate climates are affected each year, 10 or more per 100 000 people living in tropical climates and in the endemic area, the incidence is increased to 100 per 100 000 people. The disease prevalence is often under-reported due to several reasons: nonspecific clinical manifestation, presence of other endemic diseases and lack of diagnostic tests.

In Malaysia, leptospirosis has been recognized as a re-emerging disease and recorded a total of 10 684 clinical human cases from year 2010 to 2013 (Suut, 2016). The situation is further highlighted in rural area as such in indigenous communities due to extreme poverty, vulnerability and poor health conditions. Royal Belum, Kelantan state of Malaysia, bordered by Thailand on the north are inhabited by a group indigenous community known as Jahai tribe is the example of circumstances stated. The potential risk of zoonosis to the Jahai Community are the land concessions for plantation and logging activities, resources overexploitation, tourism, unbalanced diets and poor health care facilities (United Nations Human Rights Council, 2016). The public health concern is further raised in this group of people when a recent disease outbreak occurred in Sungai Kejar of Royal Belum where almost 50% of Jahai children were wiped out (The Star, 2015).

The presence of unknown origin of feral dogs and cats in the Jahai village further possess a threat of spreading zoonotic diseases to the population. Sharing the common source of untreated water among the community and the feral animals is a risk factor of transmitting water-borne zoonoses such as leptospirosis. Close bordering to Thailand where leptospirosis is endemic possess another risk to the Jahai community. In year 2001, 2002 and 2003, Thailand reported a total case of 10217, 6864, and 4958 cases respectively (Tangkanakul *et al.*, 2005). Living at close proximity to other wildlife such as rats, squirrels and bats also facilitate the possible

transmission of *leptospire* to the community. Bats and rats are popular for harbouring leptospiral serovars in their bodies and transmit to other species including humans (Roth, 1964; Faine *et al*., 1999; Richardson and Gauthier, 2003; Matthias *et al*., 2005; Vashi *et al*., 2010).

More researches need to be done to evaluate the current zoonosis status in the indigenous area. Necessary measures need to be taken to enhance the health care, access to health facilities, food subsidy, clean and treated water, population control of the feral dogs and cats and reduce exploitation of land use to prevent further zoonotic outbreak among the indigenous people in the future.

3.0 MATERIALS AND METHODS

3.1 Sample Collection

Prior to sample collection, consent was obtained from Jahai community, Belum, Gerik District, Perak. This study was conducted by obtaining approval from Institutional Animal Care and Use Committee (IACUC) (UPM/IACUC/AUP-R067/2016).

Total of forty animals (37 dogs and 3 cats) were selected to represent the isolated populations in the indigenous village, Belum. The animals were manually restrained by the handlers for venipuncture blood collection. Jugular vein was selected as blood collection site and approximately 2mL of blood were withdrawn from each animal and place into plain tubes by veterinarians. Information from each animal such as species, sex, age and colour were recorded.

3.2 Transportation and Storage of Samples

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

3.3 Microscopic Agglutination Test (MAT)

Total of twelve serovars of *Leptospira* were selected to be tested using Microscopic Agglutination Test (MAT) which include *Canicola*, *Pomona*, *Icterohaemorrhagiae*, *Grippotyphosa*, *Australis*, *Pyrogenes*, *Lai*, *Celledoni*, *Bataviae*, *Javanica*, *Hardjo* and *Copenhageni*. Prior to testing, 1000µL of each *Leptospira* serovar (live antigen) was sub-cultured in Ellinhausen-McCullough-Johnson-Harris

(EMJH) medium and incubation at 30°C for 7 – 10 days. After the incubation period, the sub-cultured live antigens were examined under dark field microscopy and estimated using 0.5 MacFarland standards (1.5 x 10^8 CFU/mL) before being used for MAT.

Sterile 96-wells microtiter plate consist of 8 rows and 12 columns were used. The plate was divided into half to load 14 samples with 1 positive and 1 negative control. A total of 3 microtiter plates were required for 40 samples in each serovar. The serial dilution of the plates is up to 1:1600 and were prepared as the steps follow:

1. All wells of the microtiter plates were filled with 50µL phosphate buffer saline (PBS) of pH 7.2.
2. Additional 46µL of PBS were added to all wells of column 1 and column 7.
3. Next, 4µL of serum samples were added to wells of column 1 and column 7.
4. Serial dilution was done for each serum sample by pipetting 50µL of mixtures starting from wells of column 1 to column 6. The last 50µL of mixtures was discarded.
5. Step 4 was repeated for sera samples in column 7 and starting from wells of column 7 to column 12.
6. For the positive control well, step 1 and 2 were repeated followed by adding 10µL of hyperimmune serum. Serial dilution was performed as in step 4.
7. For negative control, step 1 was repeated.
8. 50µL of live antigens were added to all wells including wells of positive and negative control.
9. The microtiter plated were then covered and mixed in incubator shaker for 5 minutes.
10. All the microtiter plates were incubated at 37°C for 2 hours.
11. After incubation, one loop of the mixture was taken out using inoculation loop from the positive and negative control wells were placed onto glass side for dark field microscopic examination.

12. Step 11 was repeated for all other wells to examine for any antibody-antigen agglutination under dark field microscopy. The cut-off point was set at 1:100 (OIE Terrestrial Manual 2008) in which the sample was recorded as positive if at least 50% agglutination occurs, and the endpoint dilution is determined.
13. All the results were recorded.

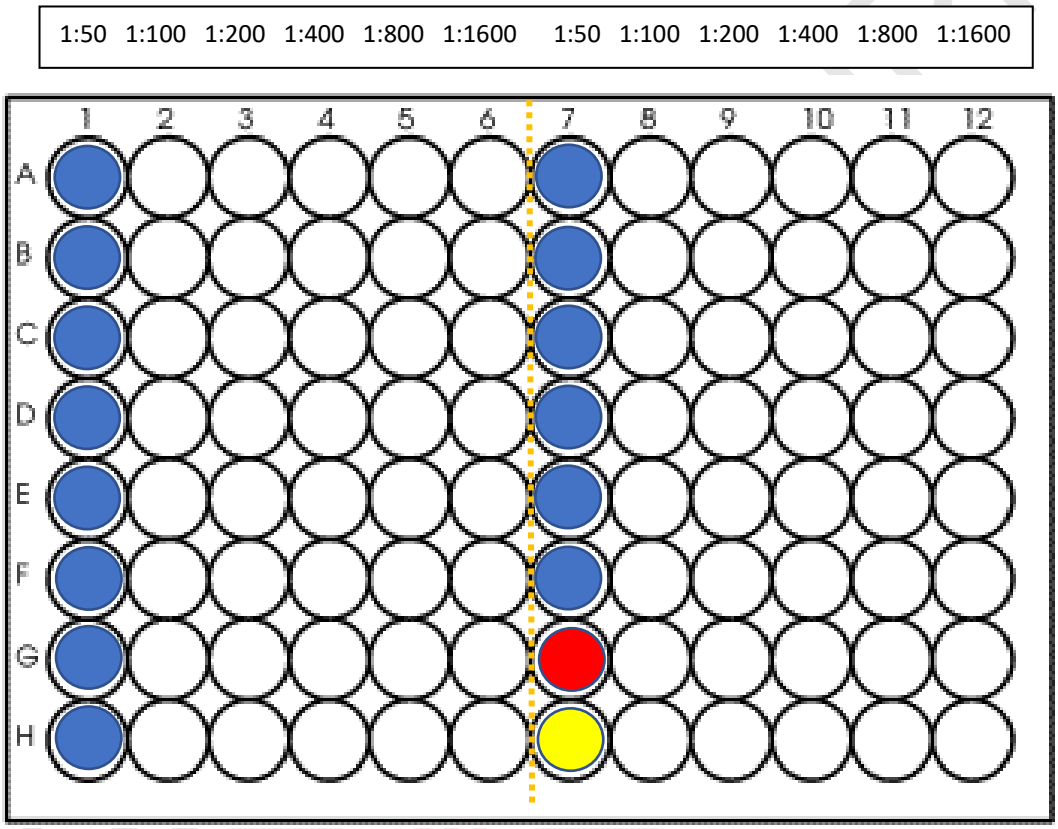

Figure 1 : Sterile 96-Wells Microtiter Plate Containing Serum Sample, Positive Control and Negative Control

4.0 RESULTS

In this study, forty (n=40) animals (37 dogs and 3 cats) were selected randomly from the isolated population of dogs and cats in indigenous village, Belum. The total estimated population for the dogs and cats in this village was approximately 60 dogs and 20 cats, respectively. All the selected animals were not known for their origin and exposure to *leptospira*. The dogs and cats were not claim (no ownership) by any indigenous people in the village and feed on scraps and sometimes fish offered by the villagers. The information of the animals was tabulated as follow:

TABLE 4 : DATA OF ANIMALS (DOGS AND CATS) COLLECTED

Number	**Species**	**Gender**	**Age**	**Colour**
1	Dog	F	Adult	Creamy
2	Dog	M	Adult	Creamy
3	Cat	F	Pregnant	Orange
4	Dog	M	Adult	Creamy
5	Dog	M	Adult	Creamy
6	Dog	M	Adult	Mixed
7	Dog	M	Adult	Creamy
8	Dog	M	Adult	Brown
9	Dog	M	Puppy	Brown
10	Dog	M	Adult	Mixed
11	Dog	F	Adult	Creamy
12	Dog	M	Adult	Creamy
13	Dog	M	Adult	Brown
14	Dog	M	Adult	Creamy
15	Dog	M	Adult	Brown
16	Cat	M	Adult	Orange
17	Dog	M	Adult	Creamy
18	Dog	M	Adult	Creamy
19	Dog	M	Puppy	Brown
20	Dog	M	Adult	Brown

21	Dog	M	Adult	Creamy
22	Cat	F	Adult	Mixed
23	Dog	M	Adult	Brown
24	Dog	M	Adult	Creamy
25	Dog	M	Adult	Brown
26	Dog	F	Puppy	Creamy
27	Dog	F	Puppy	Creamy
28	Dog	M	Puppy	Creamy
29	Dog	M	Adult	Brown
30	Dog	M	Adult	Creamy
31	Dog	M	Puppy	Mixed
32	Dog	F	Adult	Creamy
33	Dog	M	Adult	Creamy
34	Dog	M	Adult	Creamy
35	Dog	M	Puppy	Brown
36	Dog	F	Puppy	Creamy
37	Dog	F	Puppy	Creamy
38	Dog	M	Puppy	Brown
39	Dog	M	Adult	Brown
40	Dog	F	Adult	Creamy

The animals are categorized into dogs (37/40) and cats (3/40), adult and young and male and female. In the dog category, 73% (27/37) are adult and 27% (10/37) are puppy whilst in cat category, all the 3 cats are adult and 1 of them was pregnant. Of the 37 dogs, 78% (29/37) are male and the remaining 22% (8/37) are female whereas out of 3 cats, 33.33% (1/3) is male and 66.66% (2/3) are female.

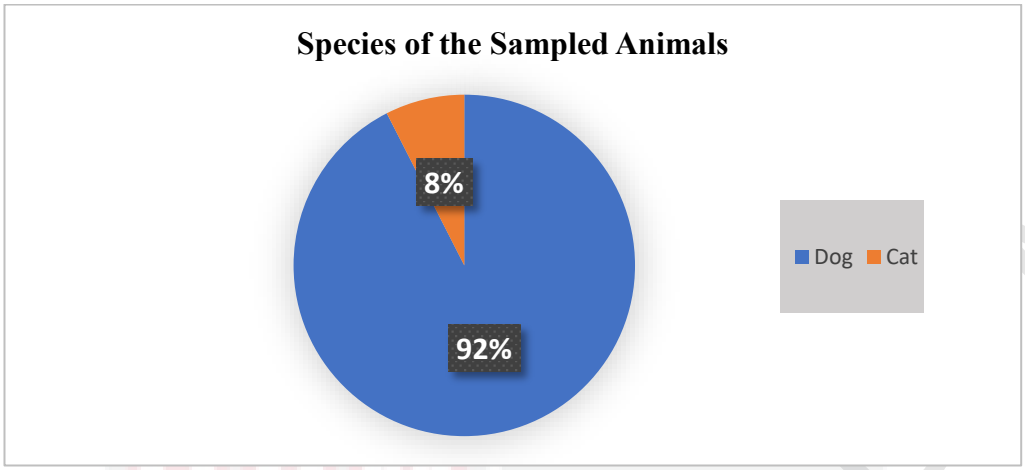

Figure 2 : Species of sampled animals (dogs and cats)

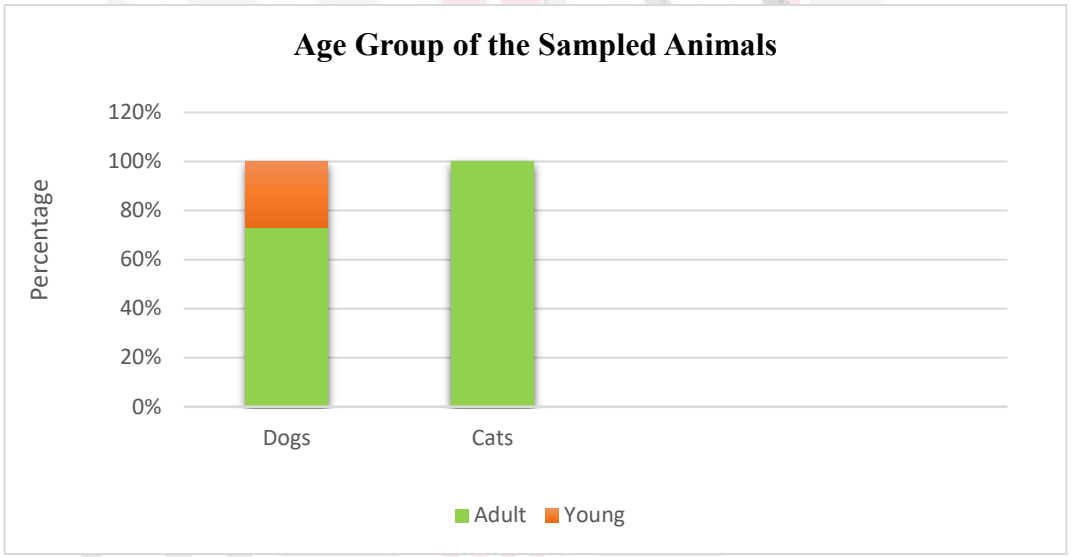

Figure 3 : Age group of sampled animals

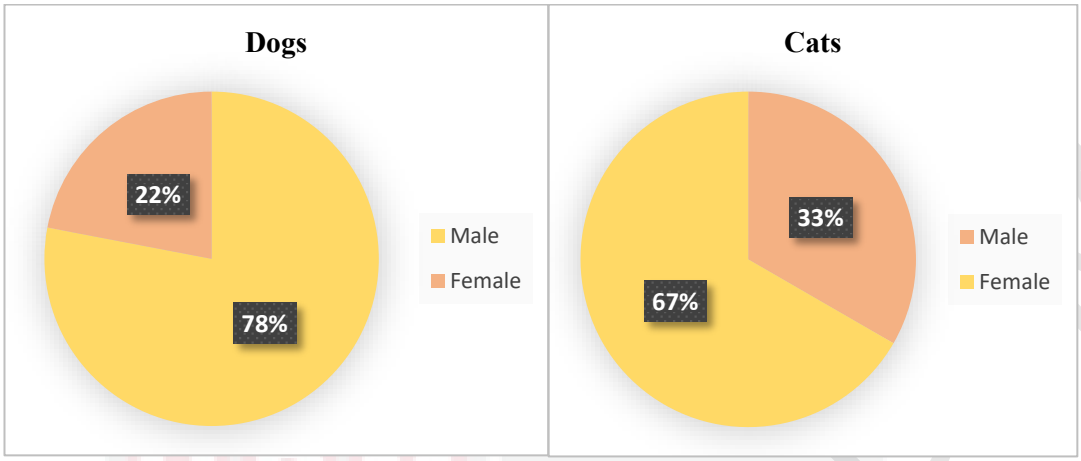

Figure 4 : Gender group of sampled animals

4.1 Microscopic Agglutination Test (MAT) Results

Out of 37 dog's serum samples, the seroprevalence of Leptospirosis was 8.1 % (3/37) where 3 of the samples showed positive results at a cut-off titer of 1:100. Of the 3 samples (number 6, 10 and 40), sample number 6 reacted with serovar Celledoni with a titer of 1:200; sample number 10 reacted to both serovars Celledoni and Lai with titers of 1:400 and 1:100 respectively; sample number 40 was seropositive for serovar Australis with a titer of 1:200. All the seropositive dogs were adult with 67% were male (2/3) and 33% was female (1/3). Besides the seropositive dogs, some samples (number 12, 29 and 32) also showed low titer (1:50) of MAT which were insufficient to qualify as seropositive. Both sample number 12 and 29 showed low titer toward serovar *Icterohaemorrhagiae*; number 32 showed low titer to both serovars *Celledoni* and *Copenhageni*.

For cat's serum samples, the seroprevalence of Leptospirosis was 33% (1/3) where 1 of the samples showed positive titer of 1:400 against serovar *Lai*. The seropositive cat was female adult (pregnant).

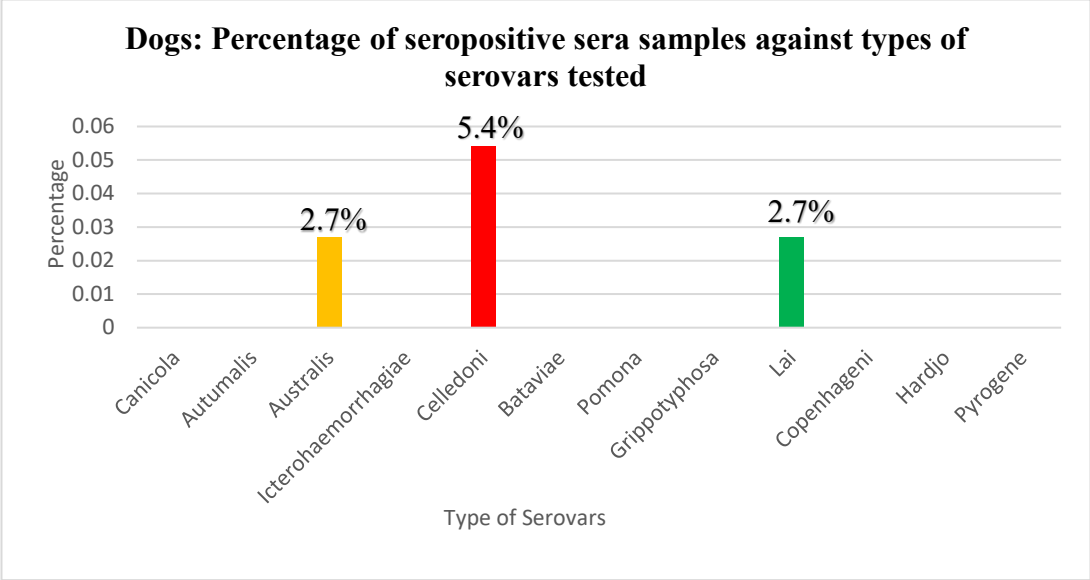

Figure 5 : Seroprevalence of Leptospirosis of dog's serum samples collected

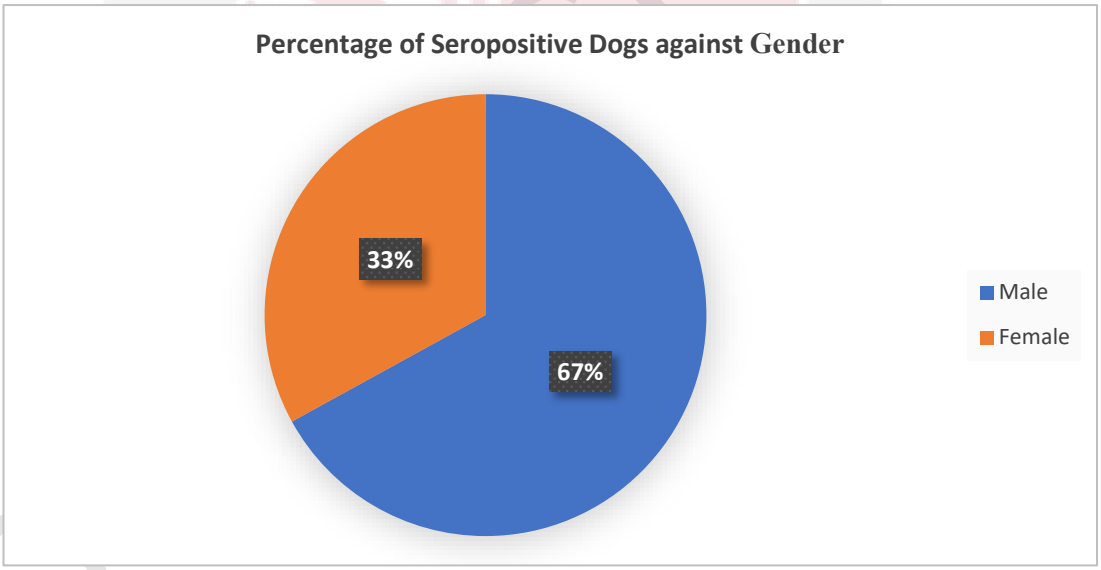

Figure 6 : Percentage of Seropositive Dogs against Gender

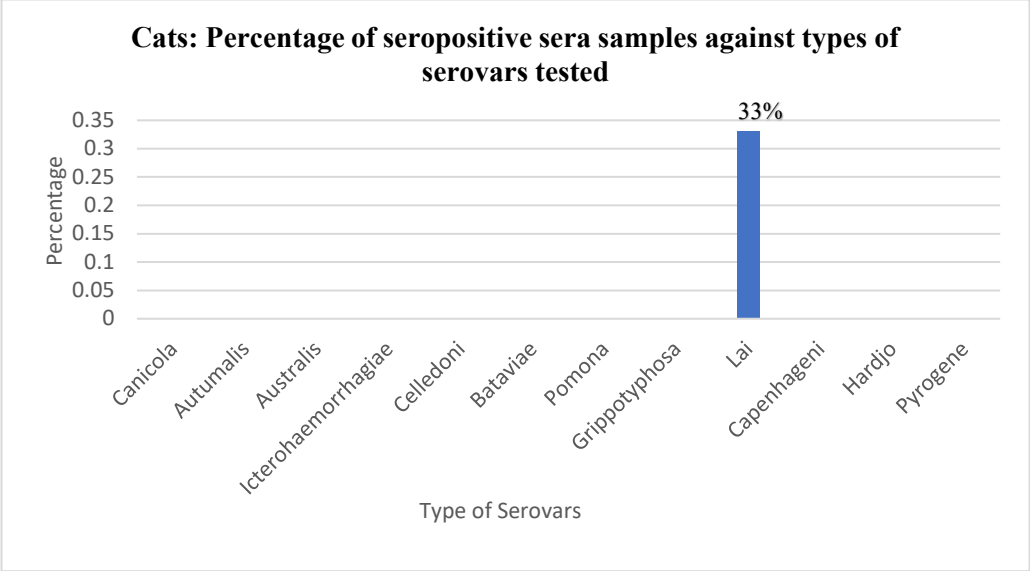

Figure 7 : Seroprevalence of Leptospirosis of cat's serum samples collected

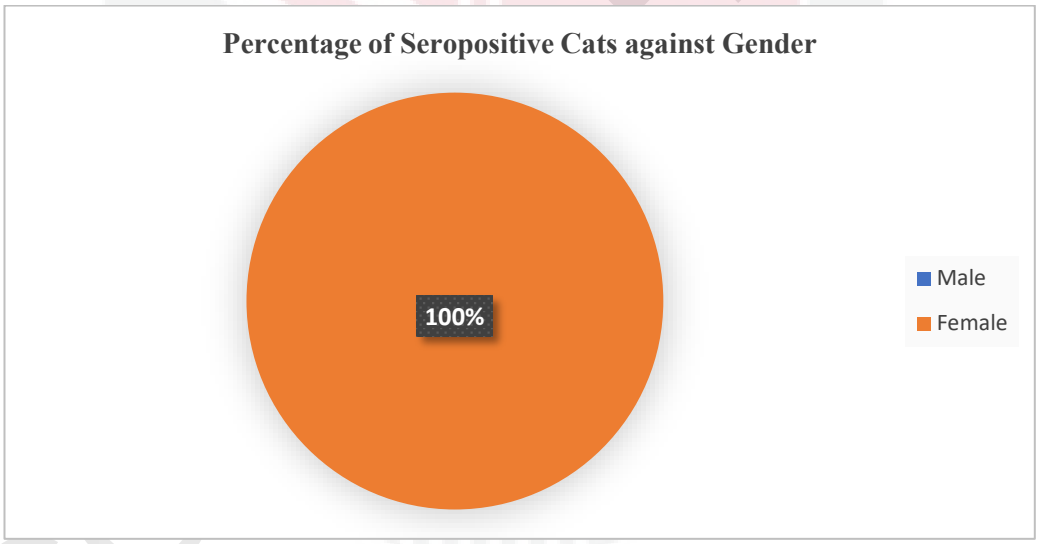

Figure 8 : Percentage of Seropositive Cats against Gender

5.0 DISCUSSION

In the study, the seroprevalence of the isolated population of dogs in Belum village was 8.1%. The positive cut-off point was set at 1:100 by following the standard set by OIE. The predominant serovars detected include *Cellodoni* (5.4%), *Lai* (2.7%) and *Australis* (2.7%). Out of the 3 seropositive samples, one of the sample showed seropositive toward 2 serovars which are *Cellodoni* and *Lai*. There were also low titer (1:50) reported in the study which include serovars *Icterohaemorrhagiae*, *Celledoni* and *Copenhageni* in 3 other samples. The finding shared a common serovar of *Australis* that also found in other canine leptospirosis studies done in Malaysia (Lau *et al*., 2016; Wong, 2016; Bo, 2017). Majority of the seropositive samples showed titers of 1:100 or 1:200 which may indicate the early stage of leptospirosis, previous infection or vaccination (Picardeau, 2013). But vaccination is not applicable in this group of isolated population. The low titer detected in 3 other samples cannot be neglected in the study as it can indicate an early infection or case of recovery from previous infection. One of the positive samples recorded a high titer of 1:400 against serovar *Celledoni* and this might indicate active infection of leptospirosis. The dog was in poor body condition score but apparently healthy without any clinical presentation of leptospirosis. All the dogs were scattered around the Belum village with majority living at the lakeside and known to be feral as none of the dogs was claimed by any of the indigenous people. Generally, the dogs were emaciated probably due to unbalanced diets. They are fed on scraps and sometimes fish offered by the villagers. The dogs were not known of their origin, immune and health status due to the isolated location in Belum. The temperament of the dogs was hardly approachable and fierce probably due to less domesticated.

On the other hand, the seroprevalence of cats reported in the study was 33% and the predominant serovar detected was *Lai*. The only seropositive cat in the study was pregnant. To date, there is no feline leptospirosis prevalence study published in Malaysia and hence the result is incomparable. But according to Jamshidi *et al* (2009), the prevalence of feline leptospirosis was 27% with predominant serovars of *Canicola*, *Hardjo* and *Icterohaemorrhagiae* in Iran. The cats were living closer to the villagers and mainly staying underneath their raised-floor houses. The seropositive cat recorded

a surprisingly high titer of 1:400 against serovar *Lai*. This can be due to previous exposure to leptospiral antigens or active infection. The cat was apparently healthy and show no clinical signs of leptospirosis. Cats are known to be infected with leptospirosis but rarely presented with clinical signs (Dickeson & Love, 1993; Agunloye & Nash, 1996). The pathogenesis of feline leptospirosis is similar to canine infection, but even with that, clinical signs are rarely developed in cats despite the development of histological lesions in the kidneys and liver (Jamshidi, 2009). However, the infected cats are still able to shed the *leptospires* through their urine up to 3 months based on an experiment (Willoughby, 2004). This possessed a zoonotic risk of spreading *leptospires* to the indigenous people.

In the study, it highlighted that uncommon serovars such as *Celledoni* and *Lai* can be found in dogs. This finding is contrast with other canine leptospirosis studies reported in Malaysia where *Canicola*, *Icterohaemorrhagiae*, *Bataviae*, *Australis*, *Pomona* and *Pyrogenes* are detected.

The sample size of the study may be inadequate which possibly lead to overestimate or underestimate the seroprevalence. As mentioned, the feral dogs were unapproachable and fierce, and due to lack of manpower and restraining tools, sampling was unrewarding during the study. Feral cat was limited and scattered around the village which lead to the least number of samples. Seroprevalence can also be underestimated due to false negative results of MAT. This happened if the infecting serovars are not included in the testing panel and highly possible in our study due to unknown origin of the feral dogs and cats.

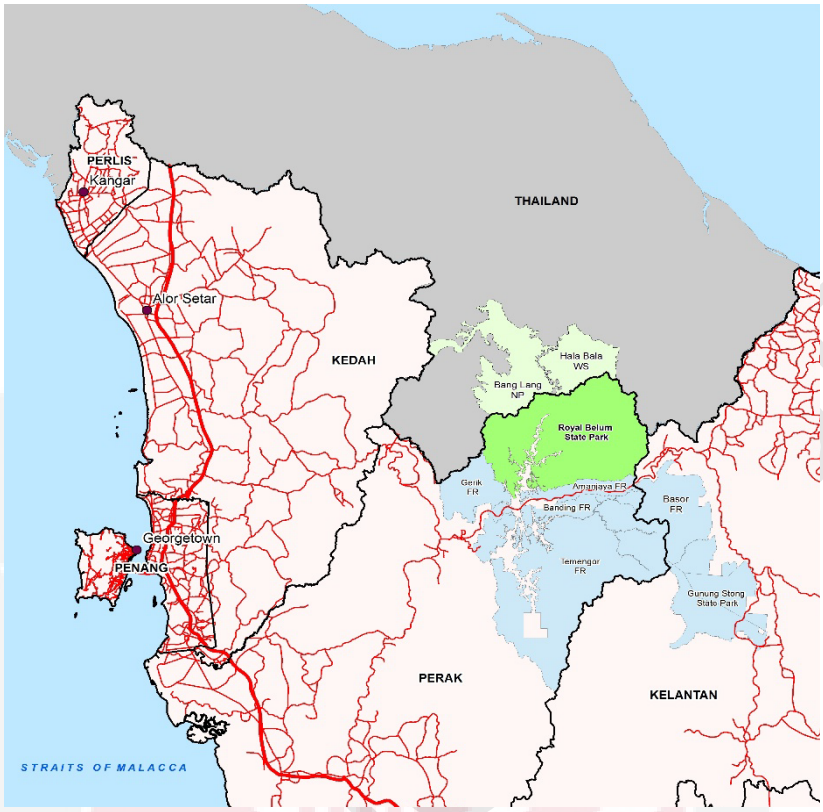

Figure 9 : Map of Royal Belum State Perak (source from WWF Malaysia)

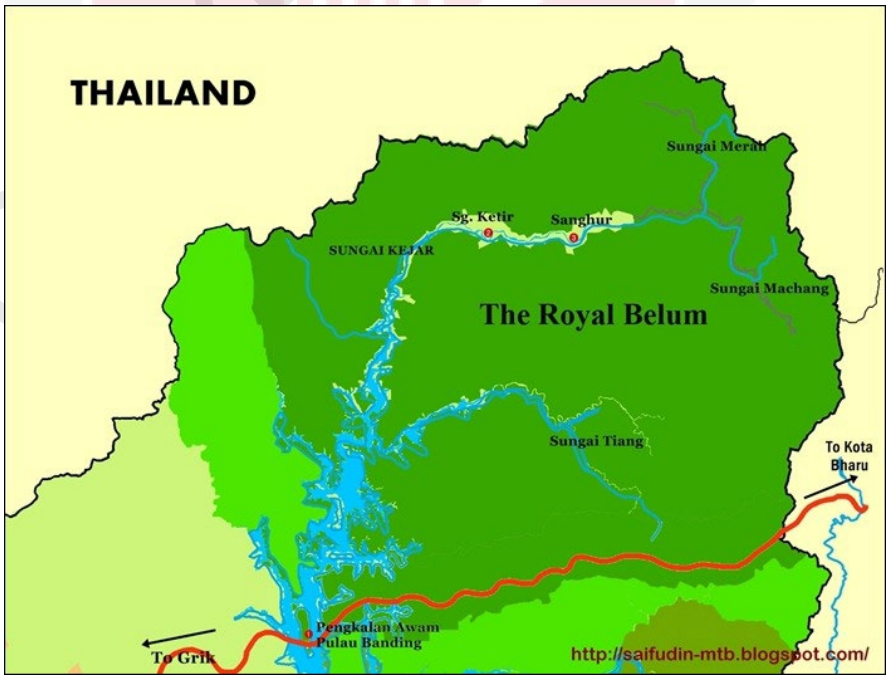

Figure 10 : Sungai Tiang, Royal Belum (source from http://saifudin-mtb.blospot.com)

The indigenous village of our study located at Tiang River, Belum. The only human inhabitant in the village is Jahai community. Under the Perak State Parks Corporation Enactment 2001, Royal Belum was gazetted as a protected area on 3 May 2007 and the area encompasses a total of 117 500 hectare in the most northern part of Perak, Malaysia (coloured in green on the map). Royal Belum state is bordered with Thailand on the north, Kelantan state on the east and Gadong River on the west. The landscape of Royal Belum consists of forest, small areas of grassland, abandoned agricultural plots, and a large man-made lake, Temenggor Lake. The Lake was built to generate electricity and water supply to the town. Due to the presence of lake, flood is a common incidence in the area especially during rainy season. A flood incidence had hit the Royal Belum Park in early year of 2015 and caused damage to the wooden houses of the indigenous people (New Straits Times, 2015). According to Centres for Disease Control and Prevention (CDC) 2017, exposure of flood water carries the potential of leptospirosis infection and outbreak are usually followed by flood. Other than that, the water from the lake was shared among the indigenous people and the feral dogs and cats in the area. The indigenous people stated that they prefer to stay near to river as it ease them from getting domestic usage and catching fish (New Straits Times, 2016). Domestic usage such as washing clothes, bathing and cleaning by the indigenous people were observed during our study in the area. Besides, the water also acts as a medium of transportation for the villagers to get to the other location by boat. The feral dogs and cats use the water as a source of drinking water. Another risk of transmission of leptospirosis between the people and the feral are proposed due the reason.

According to Suut *et al* (2016), a local study done in rural communities in Sarawak reported that a seroprevalence of human leptospirosis was 37.4%. The seroprevalence is higher than other studies done in Malaysia which only ranged from 8.4% to 25.75% (Rafizah *et al.,* 2013; Shafei *et al.*, 2012; Karim *et al.,* 2003). The proposed reason of the high prevalence is due to the demographics of the rural communities live in settlement built along the river and engaged in economic activities such as fishing, collecting jungle produce and farming. Other than that, poor sanitation, low awareness against zoonotic diseases and low educational level are known to the

contributing factors to the high prevalence. The situation is akin to the Jahai community in Sungai Tiang, Belum.

On the other hands, wildlife can also act a potential carrier of *leptospire* (Bengis, 2004). Belum state is notable for its wildlife diversity and majority of the species are characteristic of the tropical rainforest whilst minority of the species are from seasonal tropical forest of Thailand. A study conducted locally by Thayaparan *et al* (2013) on seroprevalence of leptospiral infection in wildlife revealed high seropositive among monkey, rats, bats, squirrel and mongoose species. Rats from Royal Belum rainforest was tested positive for carrying pathogenic *leptospires* (Mohamed-Hassan *et al*., 2012). The Belum area provide high risk for transmitting *leptospires* from the reservoir animals. The risk of transmission of leptospirosis from the wildlife to the feral dogs and cats is highly possible in the Belum region and this further increase the zoonotic burden to the indigenous people.

Another risk factor of leptospirosis spreading in Belum state is the close bordering with Thailand where leptospirosis is endemic. Thailand had reported a total case of 10217, 6864, and 4958 cases of leptospirosis in the year 2001, 2002 and 2003 respectively (Tangkanakul *et al.*, 2005). Sharing the same rainforest between Belum state and Thailand facilitates the movement of wildlife in the area.

With all the potential risk factors mentioned, leptospirosis outbreak is not an astonishing event that could happen in near future. Hence, thorough prevalence study which include other species (human, rats and other wildlife) is necessary to have an insight of leptospirosis status in this area. Intervention by the government in preventive measures are needed to curb and prevent future outbreak of leptospirosis in this isolated population. Lack of access to medical facilities, balanced diets, clean and treated water and public education are the major problems to the Jahai population.

6.0 CONCLUSION

In conclusion, the overall seroprevalence of canine leptospirosis was 8.1% in total of 37 dogs whilst feline leptospirosis recorded 33% in total of 3 cats studied. The predominant serovars reported were *Celledoni*, *Lai* and *Australis* in dogs and *Lai* in cats. These findings showed that *leptospires* are existing in the environment of Belum state.

7.0 RECOMMENDATIONS

In future study, more area of the Belum state should be included to have a larger sample size and a more precise estimation of seroprevalence of leptospirosis. Sampling methods need to be revise as the animals in the area are less domesticated and require proper restraining tools, restraining techniques and well-trained personnel to reduce stress and injuries to both the handlers and animals.

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9.0 APPENDICES

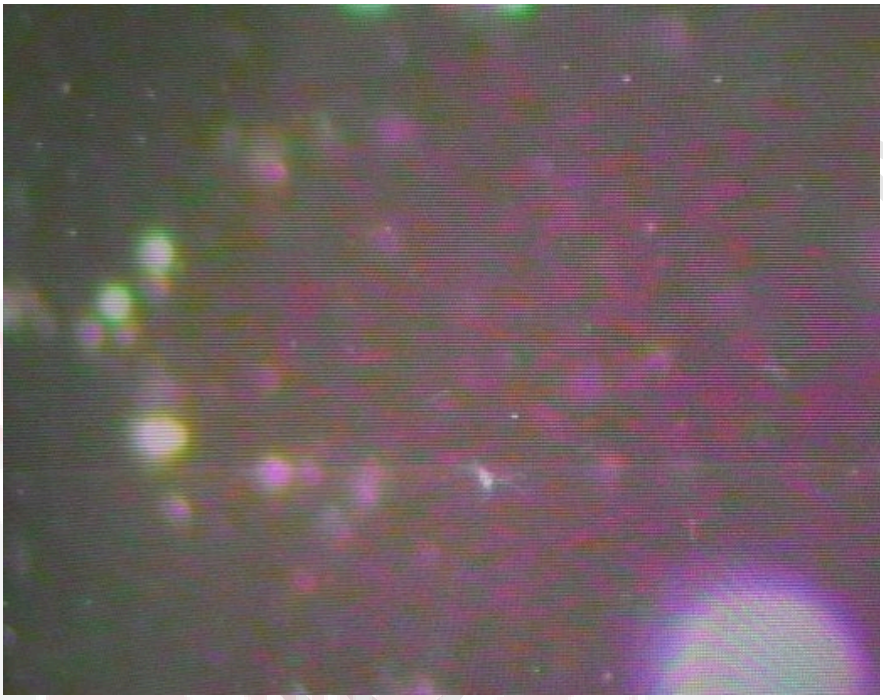

Figure 11 : MAT positive under 200x dark field microscopy

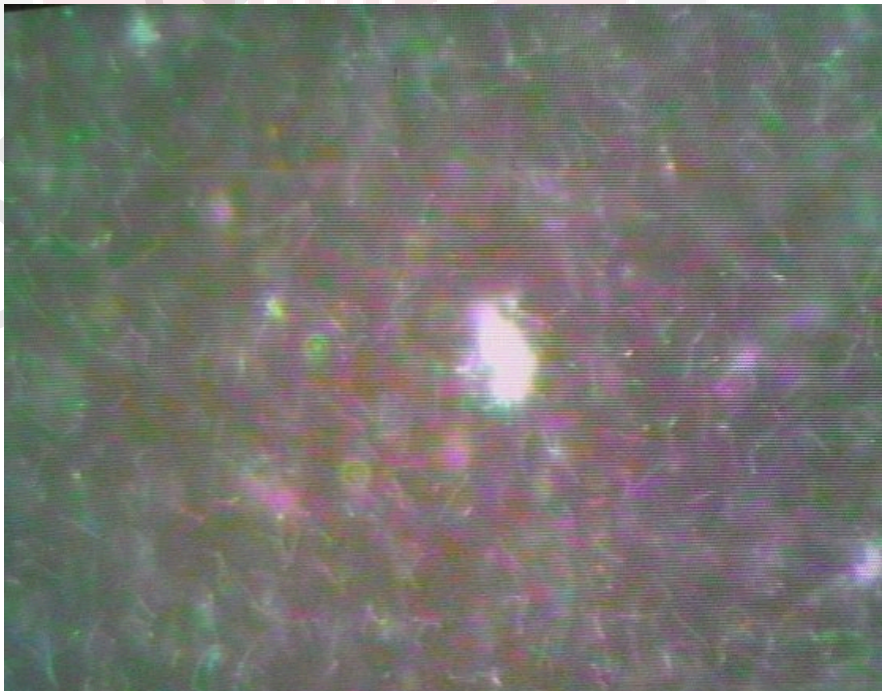

Figure 12 : MAT negative under 200x dark field microscopy