A SERO-EPIDEMIIOLOGICAL STUDY OF LEPTOSPIROSIS IN SARAWAK, MALAYSIA.

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This thesis is presented for the degree of Doctor in Philosophy
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DECLARATION

I declare this thesis is my own account of my research and contains as its main content work, which has not been submitted for a degree at any tertiary education institution.

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Sivapiragasam Thayaparan
ABSTRACT

Several recent outbreaks of leptospirosis involving human deaths have alarmed health professionals in Malaysia. The study outlined in this thesis was conducted to increase the understanding of the involvement of wildlife in the disease in Malaysia.

A strain of *Leptospira* (designated Lepto 175 Sarawak) was isolated from water in Sarawak, Malaysia. This strain did not produce any titres towards other known *Leptospira* sera, and thus represents a novel serovar. This serovar had 99.1% 16S rRNA gene sequence similarity with *Leptospira wolffii* and was the dominant strain present in the region.

In this study eight of the 12 non-human primates sampled (66.6%; 95% CI 34.9-90.1) and 73 of 155 wild small mammals (47.1%: 95% CI 39.0-55.3) were seropositive to leptospires. The seroprevalence was slightly higher in rats than in squirrels or bats. Seropositive animals were detected in all localities sampled, with the highest prevalence at Mount Singai (64.7%; 95%CI 38.3-85.8). Antibodies were detected to two different serovars in non-human primates, eight serovars were detected in rats, six serovars in bats and five in squirrels. Of 155 kidney samples from individuals, 17 were positive for *Leptospira* on PCR analysis (11%; 95% CI 6.5-17).

A cross-sectional serological survey of 198 humans was conducted in four villages around Kuching, Sarawak with 35.9% (95%CI 29.2-43.0) testing positive on the MAT. Antibodies to serovar Lepto 175 Sarawak were most commonly detected (31.3%; 95%CI 24.9-38.3) and were detected in individuals at all four locations. The presence of skin wounds (OR 3.1), farm animals (OR 2.5) and rats (OR 11.2) were
all significantly associated with seropositivity in a multivariable logistic regression model.

The results of the current study are important as wildlife may act as reservoirs of leptospires for humans. Health authorities should expand disease control measures to minimise the spill-over from wildlife to humans visiting, living or working in the sampled locations. The pathogenic status of serovar Lepto 175 Sarawak also requires further investigation.
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PUBLICATIONS

In the course of the undertaken research work, the following publication and posters were done:

A) PEER – REVIEWED JOURNALS


**MANUSCRIPT UNDER REVIEW**

B) CONFERENCE PAPERS

Thayaparan S., Amaran F., Robertson ID., Abdullah ID, Preliminary results on noval strain of Leptospira, Lepto 175 (Sarawak), in Sarawak Malaysia, 8th Scientific Meeting of International Leptospirosis Society, Fukuoka, Japan, During 8-11 October 2013.


THE POSTER PRESENTATION:


CHAPTER 1: GENERAL INTRODUCTION

Leptospirosis in Malaysia can be defined as an emerging or re-emerging endemic disease. The disease can affect both humans and other animals throughout Malaysia, and can result in serious socio-economic and public health consequences (Arief, 2013). Due to the abundance of wildlife and the tropical climate, infection in wildlife can lead to economic loss and pose a potential source of infection for communities in the country. This disease is caused by pathogenic or intermediate Leptospira, which belong to the family Leptospiraceae (Adler and de la Peña Moctezuma, 2010; Zakeri et al., 2010). Humans contract the disease through direct contact with infected blood, tissues, organs or urine of infected hosts. Transmission can also occur by direct penetration of the leptospiral organisms through the conjunctiva or surface epithelium (Faine, 1982; Russ et al., 2003). Indirect contact with the environment, including soil, mud, fresh water, vegetation, foodstuffs and workplaces infested with rodents, can also result in infection of humans (Turner, 1973). Tsai and Fresh (1975) reported the first human case of leptospirosis in Asia through contact or exposure to wild animals in Vietnam. In recent years outbreaks of human leptospirosis in Malaysia have been documented around wildlife reserves and parks (Lim et al., 2011; Thayaparan et al., 2013a) resulting in a high number of confirmed cases and associated mortalities. Wildlife tourism is an important source of revenue in Malaysia, particularly in the state of Sarawak (Yasak, 2000), and leptospirosis has the potential to impact on this important source of income.

The proposed study was carried out to determine the extent of exposure to leptospirosis in human communities on the periphery of wildlife reserves and in an urban area. In addition, small wildlife (bats, rodents, squirrels, treeshrew and non
human primates) were trapped in the forest habitats around the reserves and in the urban area and blood samples screened for the presence of leptospiral antibodies. This provided information on the possible zoonotic importance of wild mammals in maintaining this disease in communities in Sarawak. Serum samples were also collected from villagers who were at risk of having contact with small wildlife. The results will be used to develop suitable preventive and control measures for leptospirosis in the future. The results of this study will give a clear indication of the disease prevalence, reservoir host and the mode of transmission in the region and will also further understanding of the potential relationship between wildlife and humans in rural areas.

1.1 Background

Leptospirosis is a zoonosis of ubiquitous distribution, caused by the spirochaete *Leptospira*, of which there are two forms: the pathogenic species; and the non-pathogenic species (Bharti et al., 2003; Morey et al., 2006; Treml et al., 2002). Leptospirosis in humans was first reported 100 years ago by Adolf Weil in Heidelberg (Levett, 2001). The importance of occupation as a risk factor for infection was recognized early in the history of the disease (Turner, 1973). The role of rats as a source of human infection was discovered in 1917 (Levett, 2001) and subsequently some researchers have identified that flying foxes can carry pathogenic *Leptospira* in Australia (Cox et al., 2005; Smythe et al., 2002b; Tulsiani et al., 2011). In humans, leptospirosis can cause headaches, fever, chills and sweating (Edwards, 1960; Levett, 2001; Magill, 1998; Turner, 1973), with some highly pathogenic serovars causing pulmonary haemorrhage and even death. Leptospirosis
is increasing in importance as an occupational disease as intensive farming practices
and adventure tourism activities become more widely adopted in Malaysia. Diagnosis of leptospirosis by the presenting clinical signs is not definitive and the
disease can be confused with several other diseases including influenza, hepatitis
and various haemorrhagic fevers (Dutta and Christopher, 2005; Enria and Pinheiro,
2000). Early diagnosis in humans can be confirmed by culture of blood samples
during the initial fever and culture of urine samples during recovery from the fever.
One week after the onset of symptoms has passed, serological assays, such as the
Microscopic Agglutination Test (MAT) or Enzyme Linked Immune Sorbent Assay
(ELISA), may be used to detect antibodies in the blood. Only the pathogenic and
intermediate pathogenic *Leptospira* were investigated in the research reported in this
thesis.

In Malaysia isolation of leptospires from black rats (*Rattus rattus*) was first reported
in 1928 by Fletcher (Russ et al., 2003). From 1953 to 1955, 30 pathogenic serovars
were identified by researchers (El Jalii et al., 2002) from both military personnel and
civilians. Kennedy and Robinson, (1956) reported 31 cases of leptospirosis among
British army personnel in Malaysia. In a study highlighting the endemic nature of
leptospirosis in Malaysia, a high prevalence of antibodies to leptospires was
demonstrated in humans and animals throughout Malaysia (Lim et al., 2011;
Thayaparan et al., 2013a). The highest seroprevalence was reported in labourers
working in rubber estates and those working in the sewage, drainage, forestry and
cleaning industries (Bahaman and Ibrahim, 1988). Many more investigations on
human leptospirosis in Malaysia have revealed a high prevalence of infection
(Bahaman and Ibrahim, 1988; El Jalii and Bahaman, 2004; El Jalii et al., 2002).
However since 1986 no major investigations have been undertaken on human
leptospirosis in Peninsular Malaysia.

Studies carried out in Sabah in Malaysian Borneo, have highlighted the possible zoonotic importance of wild mammals in the maintenance of the disease within the community. One study (Russ et al., 2003) showed that a significant percentage (25.75%) of people living within the periphery of a wildlife park had been exposed to leptospiral antigens. Leptospirosis has been reported among British cavers in Sarawak (Self et al., 1987) and another incident occurred in an American caver returning from Sarawak, Malaysia in 1994 (Mortimer, 2005). Most recently in 2000 there was an outbreak of leptospirosis in international participants in an “Eco-Challenge” adventure race in Sabah, Malaysia (Haake et al., 2002; Sejvar et al., 2003).

1.2 Study hypothesis

For the purpose of this study, it is hypothesised that local communities (people) living around forested habitats that are in potential contact with wildlife (bats, rodents, tree shrews and non-human primates) will be more significant to infection with *Leptospira*.

1.3 Objectives

The research described in this thesis was designed to collect detailed information about the prevalence and pattern of *Leptospira* in wildlife and in humans adjacent to wildlife reserves in Kuching, Sarawak, Malaysia. This information will help in identifying risk factors for human infection and allow development of suitable
measures for disease control. The outcome of this study will give a clear indication of the carrier status of pathogenic *Leptospira* by small wildlife, the disease prevalence, possible reservoir hosts, and provide potential information on the mode of transmission within the region. The specific objectives were:

a. To determine the cause of recent human outbreaks around forested areas.

b. To determine the role of wild small mammals in the lifecycle of *Leptospira*.

c. To document animal hosts, and their seroprevalence around wildlife reserves and human settlements.

d. To investigate the epidemiology of human leptospirosis, including risk factors for infection.

### 1.4 Anticipated outcomes

The anticipated outcomes of this study are:

1. To identify the leptosomal serovars in wildlife and humans.

2. To provide information about the epidemiology of human leptospirosis, including the prevalence of the disease and potential risk factors for disease.

3. To provide information upon which prevention measures could be developed and applied to reduce the potential for disease.

4. To provide data on the distribution of infection in wildlife to medical authorities to enable prediction of future outbreaks of disease.

5. To publish results in high impact journals.
1.5 Study constraints and limitations

The Malaysian government has classified all species of flying foxes as protected, especially in Sarawak, and all studies involving these mammals have been prohibited. However, it was possible to obtain results from other species including small bats, rodents and squirrels, as well as non-human primates. As the role played by flying foxes in the spread of leptospirosis needs to be investigated further, this places challenges on undertaking research on these species in Malaysia.

With leptospirosis being a major health concern in Malaysia, it would be desirable to undertake a study on wildlife and at-risk humans across the country, however, due to funding constraints; this study focused on Sarawak. This state was selected, as it appeared to be having the most serious problem in keeping the disease in check. The success of this particular research would possibly facilitate similar studies being conducted in other areas of Malaysia. This study was possible because of the collaboration with the Zoology Department of the University Malaysia Sarawak (UNIMAS), the medical faculty of UNIMAS and the Institute of Medical Research (IMR), Kuala Lumpur. Such relationships will certainly be helpful in future endeavours to investigate leptospirosis in Malaysia.
1.6 Thesis structure

In order to achieve these aims, this thesis is presented in seven chapters.

- **Chapter 1 General introduction.** This provides a brief introduction on the status and the reason for initiation of research into leptospirosis in wildlife and humans in Sarawak, Malaysia. It also provides the aims and structure of the thesis.

- **Chapter 2 Literature review.** In this chapter the history and nature of leptospirosis worldwide and in Malaysia is reviewed. The epidemiology, clinical features, diagnostic facilities in Malaysia, treatment, control and prevention of leptospirosis are also discussed in this chapter.

- **Chapter 3 Leptospira, Lepto 175 Sarawak Nov., isolated from an environmental sample in Sarawak.** This chapter describes the isolation of the organism, including the molecular and serological methods used to identify the strain and to confirm it as a new strain of *Leptospira*.

- **Chapter 4 Serological study on leptospirosis in captive and free ranging non-human primates in Sarawak, Malaysia.** In this chapter the prevalence of leptospirosis is estimated in non-human primates using a MAT.

- **Chapter 5 Serological and molecular study of *Leptospira* in Rats, Squirrels, Treeshrews and Bats around wildlife reserves and disturbed forest in Kuching, Sarawak, Malaysia.** In this chapter the results of testing of samples collected from small wild mammals is reported. The seroprevalence of *Leptospira* is estimated using the MAT, the pathogen is cultured and some organisms isolated.

- **Chapter 6 Serological study and analysis of risk factors that may be involved in the introduction of leptospirosis to a human community located around wildlife reserves in Kuching, Sarawak, Malaysia.** In this chapter the seroprevalence to *Leptospira* in humans is reported through examining the results of
the MAT and ELISA tests. Also a risk assessment on the likelihood of *Leptospira* entering a community from wild small mammals is reported. Data are collected using questionnaires applied during the blood collection.

➢ **Chapter 7 General discussion and conclusions.** In this chapter the results of the thesis are discussed. The prevalence in animals and humans is discussed and the possible risk offered by wild animals to humans evaluated.
CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Leptospirosis is a tropical disease of major concern to countries such as Malaysia and has been linked to exposure to tropical jungles (MacKay-Dick, 1971). Many species of wildlife can be found in jungle environments, and some of these species may be vectors of pathogenic leptospires. In 1917 rats were identified as carriers of leptospires (Levett, 2001; Monahan et al., 2009), and subsequently flying foxes have been found to also carry pathogenic leptospires in Australia and South America (Cox et al., 2005; Matthias et al., 2005; Smythe et al., 2002a; Tulsiani et al., 2011). Although the role of flying foxes as carriers of pathogenic \textit{Leptospira} is not fully understood, it is known that several other species of wildlife can also act as potential carriers (Cox et al., 2005; Hartskeerl and Terpstra, 1996). The bacteria can cause polymorphic disease conditions in wild and domestic animals, as well as in humans (Cox et al., 2005). However, to date there has been little research undertaken on the role of wildlife in outbreaks. Due to the current significant level of deforestation occurring and the involvement of humans in tropical jungles, there is the potential for exposure of humans to new serovars of \textit{Leptospira}. Leptospirosis in wildlife can have negative consequences for biodiversity, livestock health, animal welfare and the economy of a country, as well as human health (Caley and Ramsey, 2002; Russ et al., 2003). In this thesis, the focus was on the disturbed jungle habitat surrounding the capital city of Sarawak, Kuching, where pockets of human inhabitation can be found.

Zoonotic diseases represent key biological threats to human health. Members of the
genus *Leptospira* can infect a broad range of animal species, including humans. Wildlife can also play a major role in the organism’s transmission and understanding the role of wildlife in the disease’s epidemiology is important when addressing disease in domestic animals and humans. Leptospirosis in wildlife is also important in its own right, with impacts on biodiversity as well as animal welfare. The proximity of wildlife to potential livestock carriers, the amount of infective agents in the environment, geographical, climatic, and socio-cultural factors affecting animal husbandry and agricultural practices may result in cross-over of infection from domesticated animals to wildlife species.

Dramatic societal and environmental changes, particularly involving deforestation and development of large scale agricultural industries (Colchester, 1994; Persoon, 2004), have caused the spectrum of infectious disease to change rapidly (McMichael et al., 1996). Across the world, explosive growth in the human population and the associated expanding poverty and urban migration, along with increased international travel and commerce, increase the risk of humans to exposure to infectious agents such as *Leptospira*. In this chapter the impacts of leptospires on wildlife, and the current status of surveillance and the options to strengthen control policies for leptospirosis in Malaysia are reviewed.

### 2.2 History of leptospirosis

#### 2.1.1 World wide

Leptospirosis has been documented worldwide, but formal reporting systems vary widely between countries (Lau et al., 2010a; Lau et al., 2010b; Pappas et al., 2008).
Frequently the disease gains public attention when outbreaks occur in association with natural disasters, such as flooding in Nicaragua in 1995 or among foreign travellers and extreme athletes (Mortimer, 2005). Southeast Asia is an endemic area for leptospirosis, and infection in humans has been reported throughout the region with 70% of the major pathogenic serovars having been isolated from Asia (Laras et al., 2002). Although leptospirosis is likely to have been present for millions of years (Levett, 2001), it is only in relatively recent times that the bacterial cause of the disease was confirmed. In 1886 Adolf Weil published an account detailing the icteric form of leptospirosis, and the disease was subsequently named after this discoverer. However, his observations had already been postulated by others, including Hippocrates (Levett, 2001). It has been hypothesised that leptospirosis was responsible for an epidemic among the natives of the Massachusetts coastal region, USA just before the arrival of the Pilgrims in 1620 (Marr and Cathey, 2010). What Weil described was the icteric form of leptospirosis with jaundice as the key clinical sign. In contrast the milder forms were harder to diagnose at that time due to a lack of advanced bacteriological techniques. Spirochaetes continued to cause problems for the later part of the 19th century, and it was only in 1907 that Stimson managed to isolate a leptospire from a patient (Heath et al., 1965). His subsequent research highlighted that the bacteria were concentrated in the renal tubules and were shaped like question marks. This gave rise to the name “*Spirocheta interrogans*”, and this name has been retained (Heath et al., 1965).

### 2.1.2 Southeast Asia

Leptospirosis is emerging as a serious concern to public health in Southeast Asia (Bengis et al., 2004; Bhatia and Narain, 2010; Flynn, 1999). The disease has been recognised in patients from Indonesia with clinical jaundice and non-malarial fever,
patients with clinical jaundice from Laos and Vietnam, and patients with haemorrhagic fever in Cambodia (Laras et al., 2002; Vijayachari et al., 2008). In addition, a cross-sectional community-based study was conducted in Laos to obtain estimates of the background seroprevalence. These findings suggested that leptospirosis was under-reported in Southeast Asia due to the wide range of clinical symptoms associated with acute leptospiral infection (Vijayachari et al., 2008).

During the Indochina War, the French experienced three epidemics of leptospirosis in 1950, 1951 and 1952, as well as having one quarter of their men incapacitated during one operation (MacKay-Dick, 1971). Easton, (1999) reported a spike in infection in Manila following flash floods during the monsoon season. The bacteria would most likely have entered the patients’ bodies through cuts on the skin when they waded through the floodwaters. Residents in low-lying areas and rice farmers were identified as a high-risk group for infection. One possible source of the problem was the increasing rat population with farmers from one province culling approximately 800 rats per night in their rice fields during the dry season (Easton, 1999). Studies in various locations in Thailand found that, after rats, dogs were the most important reservoir for leptospirosis with a seroprevalence of 11%, with the Bataviae serogroup being the most prevalent (5.2%) (Meeyam et al., 2006). Contact with sewage, staying outdoors more than half the time and consuming raw meat increased the likelihood of seropositivity in the sampled dogs. It was possible that the dogs acquired their infection from rats, which were responsible for an outbreak in north-eastern Thailand between 1999 and 2003 (Meeyam et al., 2006; Thaipadungpanit et al., 2007). During that period Thaipadungpanit et al., (2007) developed a scheme of multi-locus sequence typing of pathogenic Leptospira and
they confirmed that serovar Autumnalis was responsible for the outbreak and was being maintained by bandicoot rats (*Bandicota bengalensis*).

Besides infecting local residents, *Leptospira* can also cause illness in visitors to tropical regions, particularly those associated with eco-tourism and adventure travel (van Crevel et al., 1994). van Crevel et al., (1994) investigated leptospirosis in 32 Dutch travellers between 1987 and 1991 and found 28 of them had returned from Southeast Asia with the majority visiting Thailand and 21 of these had taken a rafting tour. From this research it was recommended that doctors should consider leptospirosis in their differential diagnosis whenever a patient with fever returned from the tropics. Due to the increasing number of reported cases of leptospirosis in the western world, it has been recommended that travellers try to avoid high-risk aquatic activity in the tropics and undertake chemoprophylaxis if they do take part in water-sports in Southeast Asia (Haake et al., 2002; Monahan et al., 2009). Saunders, (1979) examined 78 cases of leptospirosis in Pahang and reported the lack of specificity of the clinical syndrome, highlighting the challenges in diagnosing the disease purely on presenting clinical symptoms.

In Thailand, researchers conducted a serological survey of workers who had been involved in cleaning a pond in Khumuang, Buriram Province and analysis by multivariable logistic regression indicated that wearing long pants or skirts was protective against leptospiral infection, while the presence of more than two wounds on the body was associated with infection (Phraisuwan et al., 2002).

### 2.1.3 Malaysia

In Malaya, Fletcher isolated *Leptospira* from black rats (*Rattus rattus*) in 1928 (Bahaman and Ibrahim, 1988; Russ et al., 2003). Subsequently, many investigations
have demonstrated a high prevalence of infection in humans (El Jalii and Bahaman, 2004) with Kennedy and Robinson, (1956) reporting 31 cases among British army personnel in Malaysia. Also, 35% of febrile illness among British forces in Malaysia was attributed to leptospirosis (MacKay-Dick, 1971). Between 1953 and 1955, 30 pathogenic leptospiral serovars were identified by Alexander and his colleagues (Bahaman et al., 1987) from both military personnel and civilians. Their studies demonstrated a high seroprevalence in humans throughout Malaysia. The highest distribution was found in labourers working in rubber estates and those working with the sewage, drainage, forestry and town cleaning industries (Bahaman et al., 1987). In the 1950s and 1960’s a comprehensive study of leptospirosis in Malaysia was undertaken that included testing various mammals from a range of environments, as well as assessing occupational risks to humans. The results suggested that rats were the main maintenance hosts for leptospirosis, despite the presence of infection across many animal species. Over one hundred (104) strains were isolated and identified (Smith and Turner, 1961). Bahaman et al., (1987) conducted a cross-sectional serological survey of domestic animals in West Malaysia and found that approximately one quarter of the animals examined had agglutinating antibodies to *L. interrogans*. Cattle, buffaloes and pigs were all observed to have a high seroprevalence with temperate cattle breeds appearing more susceptible to infection than local breeds. A subsequent study found that the seroprevalence in cattle and buffaloes was 14.4% (Bahaman and Ibrahim, 1988). A new serovar was isolated from a bovine kidney, while six other serovars were isolated for the first time from Malaysian cattle. Serovar Hardjo was shown to be maintained in Malaysian cattle (Bahaman et al., 1987). Recently leptospirosis has been reported in detention centres for refugees in Malaysia (Jones, 2011; Wright,
Health authorities managed to contain an outbreak at the Juru Detention Centre, but the death of six Burmese at an undisclosed detention centre in September 2009 raised fresh concerns for several Malaysian NGOs. In July 2010, cases of leptospirosis were reported nation-wide and eight people who took part in a search and rescue operation in Lubok Yu, Maran, Pahang died from the disease, causing the picnic spot to be closed to the public (Hin et al., 2012; Sapian et al., 2012). Other cases were reported in Kedah during July 2011, with one fatality at Lata Bayu (Lim et al., 2011). As recent as March 2012, a national service trainee died of suspected leptospirosis at Sungai Siput in Perak, Malaysia (Thayaparan et al., 2013b). This resulted in a suspension of water-based activities at all National Service Training army camps.

According to data from the Ministry of Health the number of leptospirosis cases increased dramatically from 2004 to 2009 (Table 2.1), however the number of deaths from leptospirosis did not change from 2004 to 2007, although it increased markedly to 47 and 62 deaths in 2008 and 2009, respectively. The highest numbers of cases were reported in the state of Perak during 2005, 2006, 2008 and 2009 with 71, 93, 289 and 280 cases, respectively (Table 2.1). In 2004, more cases (32) were reported in Sarawak than in other years and in 2007 the highest numbers of cases (184) were reported in Pahang. The states of Sarawak, Selangor and Terengganu showed a gradual increase in the number of cases over this period. No cases were reported in WP Labuan owing to its small geographical area with no forest habitat and the fact that it is surrounded by seawater.

Case fatality rates (CFR) over the period from 2004 to 2009 varied from 1.8% to 7.6% (Figure 2.1) with an average of 4.44%. During this period a total of 5,267 cases of leptospirosis were reported nationwide with 234 known fatalities. Perak had
the highest CFR for this period (6.81%), followed by Sarawak (6.42%) and Perlis (6.25%). Approximately one-fifth of all cases of leptospirosis for this period were documented in Perak, of which 71 proved fatal (Figure 2.2). The almost threefold increase in the number of cases since 2007 may be due to improved diagnostic techniques or a greater awareness of the disease. This may also have contributed to reducing the CFR from 2004. It is also possible that climate change in the last four years of this period played a part in influencing the pattern of infection, as outbreaks of leptospirosis usually follow after a major flood during monsoon seasons (Lau et al., 2010).
Figure 2.1: Case fatality rate (CFR) of leptospirosis in Malaysia from 2004 to 2010 Source: (Hakim, 2011).

Figure 2.2: The CFR of leptospirosis in different Malaysian states (2004 to 2009) Source: (Hakim, 2011).
Table 2.1: Status of leptospirosis in humans in Malaysia from 2004 to 2009.

<table>
<thead>
<tr>
<th>State</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Deaths</td>
<td>Cases</td>
<td>Deaths</td>
<td>Cases</td>
<td>Deaths</td>
<td>Cases</td>
</tr>
<tr>
<td>Sarawak</td>
<td>32</td>
<td>2</td>
<td>71</td>
<td>2</td>
<td>37</td>
<td>3</td>
<td>70</td>
</tr>
<tr>
<td>Sabah</td>
<td>13</td>
<td>1</td>
<td>12</td>
<td>1</td>
<td>19</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>Perak</td>
<td>29</td>
<td>4</td>
<td>71</td>
<td>4</td>
<td>93</td>
<td>9</td>
<td>280</td>
</tr>
<tr>
<td>Selangor</td>
<td>16</td>
<td>5</td>
<td>20</td>
<td>3</td>
<td>37</td>
<td>0</td>
<td>208</td>
</tr>
<tr>
<td>Pahang</td>
<td>29</td>
<td>1</td>
<td>24</td>
<td>0</td>
<td>51</td>
<td>3</td>
<td>184</td>
</tr>
<tr>
<td>Kelantan</td>
<td>15</td>
<td>1</td>
<td>38</td>
<td>1</td>
<td>17</td>
<td>1</td>
<td>138</td>
</tr>
<tr>
<td>Terengganu</td>
<td>7</td>
<td>1</td>
<td>17</td>
<td>0</td>
<td>42</td>
<td>1</td>
<td>126</td>
</tr>
<tr>
<td>Kedah</td>
<td>15</td>
<td>1</td>
<td>27</td>
<td>0</td>
<td>31</td>
<td>0</td>
<td>106</td>
</tr>
<tr>
<td>Negri-Sembilan</td>
<td>27</td>
<td>1</td>
<td>41</td>
<td>0</td>
<td>24</td>
<td>0</td>
<td>91</td>
</tr>
<tr>
<td>Johor</td>
<td>30</td>
<td>1</td>
<td>29</td>
<td>6</td>
<td>31</td>
<td>2</td>
<td>59</td>
</tr>
<tr>
<td>Kuala Lumpur</td>
<td>31</td>
<td>0</td>
<td>20</td>
<td>1</td>
<td>27</td>
<td>1</td>
<td>54</td>
</tr>
<tr>
<td>Penang</td>
<td>9</td>
<td>0</td>
<td>25</td>
<td>1</td>
<td>28</td>
<td>0</td>
<td>32</td>
</tr>
<tr>
<td>Melaka</td>
<td>7</td>
<td>1</td>
<td>9</td>
<td>1</td>
<td>79</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>WP Putrajaya</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Perlis</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>WP Labuan</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>263</td>
<td>20</td>
<td>378</td>
<td>20</td>
<td>527</td>
<td>22</td>
<td>1418</td>
</tr>
</tbody>
</table>

Source: (Hakim, 2011)
2.1.4 Sabah and Sarawak

As well as Peninsular Malaysia, scientific research has also focused on Malaysian Borneo. There have been reports of leptospirosis in humans in Sabah and Sarawak, notably an outbreak following the 2000 Eco-challenge in Sabah (Lam and Kan, 2005; Sejvar et al., 2003). This highlighted the risks of eco-tourism and adventure travel, as most of the victims were athletes from western countries. In the Eco-challenge event, participants competed in jungle trekking, swimming and kayaking in freshwater and caving. A team of researchers from the CDC Atlanta contacted 189 athletes from 27 countries following the outbreak (Sejvar et al., 2003). Eighty athletes met their case definition, with 29 cases being hospitalised, although no fatalities were reported. Another study in Sabah revealed that swimming in the Segama River identified as a risk factor for infection and 20 athletes reported taking doxycycline prophylactically and this seemed to have a protective effect, with a preventive efficacy of 55% (Haake et al., 2002; Ricaldi and Vinetz, 2006).

Studies carried out in Sabah reported a high seroprevalence (25.75%) in people living within the periphery of a national park, presumably due to exposure/contact with wild mammals (Russ et al., 2003). Leptospirosis has also been reported in a caver from the USA after he returned from Gunung Mulu National Park in Sarawak (Mortimer, 2005). Recently researchers from Universiti Malaysia Sarawak (UNIMAS) and the Sarawak Health Department have conducted research in the Rejang Basin area, Sarawak finding that 31% of humans sampled were seropositive to leptospires and antibodies were associated with farming and/or water activities (Stoye, 2012; Suut et al., 2011). In Bakun in Sarawak, a Chinese national working on the hydro-electric dam project site was hospitalised with a serious condition suspected to be leptospirosis, and the subsequent death of 10 workers involved in
the translocation of animals from the Bakun Dam water catchment area to higher ground were speculated to be due to leptospirosis or melioidosis (Baer, 2009; Sibon, 2011).

In 2011, 186 cases of leptospirosis were reported in humans in Sarawak including 13 deaths, compared with 49 cases in the previous year (Koh, 2011). The CFR associated with this outbreak (6.9%) was higher than that in previous years. On the 12th December 2011, an outbreak occurred at RSAT Army camp at Penrissen Batu 8, Kuching, Sarawak with five army recruits showing clinical signs of leptospirosis and the disease was subsequently confirmed serologically. The source of infection was identified as drinking and bathing activities in the small river near the camp (Safii, Downloaded from jknsarawak.moh.gov.my/en/ on 18th March 2012a). The Kuching Divisional Health Office was notified of another suspected leptospiral outbreak on the 30th December 2011 by the Sarawak General Hospital. This incident involved two army recruits from Blok G10, Kem Semenggok, Kuching and both were serologically positive for leptospirosis (Safii, Downloaded from jknsarawak.moh.gov.my/en/ on 7th Dec 2012). The health authority of Sarawak confirmed an outbreak of leptospirosis in residents of Tiong Hua Road and leptospires were isolated from drain water in Sibu during June 2012 (Safii, Downloaded from jknsarawak.moh.gov.my/en/ on 18th March 2012b). In the state of Sarawak, the number of cases of leptospirosis was similar from 2004 to 2010, however in 2011 and 2012 there was a large increase in the number (Figure 2.3).
Figure 2.3: Number of confirmed cases of leptospirosis in humans in Sarawak
Source: (Hakim, 2011).

The four-fold spike in the number of cases in Sarawak is a valid cause for concern for health authorities in both Kuching and Kuala Lumpur. For 2011 alone, the CFR was 6.9%. The number of cases (186) reported in 2011 alone was already equivalent to 60% of the combined total from 2004 to 2009. The record high of 271 documented cases the following year may be attributed to the implementation of mandatory reporting procedures or increased awareness of the symptoms of leptospirosis by medical practitioners. This epidemic may also reflect the living conditions of residents in various areas of Sarawak.

2.3 Taxonomy and classification

The genus *Leptospira* is characterized by Gram-negative flexuous, helical organisms. The classification of leptospires has been unclear and problematic. Currently there are two methods that can be used for classification of the genus *Leptospira*: serological and molecular biological based methods.
2.3.1 Serological classification

Leptospires are spirochaetes in the order Spirochaetales, family of Leptospiraceae and include two genera, *Leptospira* and *Leptonema* (Adler and de la Pena Moctezuma, 2010). *Leptospira* are obligate aerobes with an optimum growth temperature ranging from 28°C to 30°C (Chang, 1947; Johnson et al., 1969). The genus *Leptospira* was divided into two species based on serological classification: *Leptospira interrogans*, which comprises all the pathogenic strains and *Leptospira biflexa*, the saprophytic strains, which are present in soil, fresh water or marine environments (Bharti et al., 2003; Cerqueira and Picardeau, 2009; Dikken and Kmety, 1978). Each species is divided into serogroups on the basis of serological cross reactivity and these are further divided into serovars. According to this classification, leptospires are classified into over 250 serovars by the Microscopic Agglutination Test (MAT), that uses specific antisera to identify the distinct serovars (Dikken and Kmety, 1978; Faine, 1994). Serovars that show 10% cross-agglutination are amalgamated into a serogroup (Faine, 1994). Classification and identification of leptospires using serology is a difficult and time-consuming procedure. The serogroups of *L. interrogans* and their common serovars are shown in Table 2.2.
Table 2.2: Serogroups and serovars of clinical importance in *L. interrogans*.

<table>
<thead>
<tr>
<th>Serogroups</th>
<th>Serovars</th>
</tr>
</thead>
<tbody>
<tr>
<td>Icterohaemorrhagiae</td>
<td>Icterohaemorrhagiae, Copenhageni, Lai</td>
</tr>
<tr>
<td>Hebdomadis</td>
<td>Kremastos, Hebdomadis, Jules</td>
</tr>
<tr>
<td>Autumnalis</td>
<td>Autumnalis, Fort-bragg, Bim</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>Pyrogenes, Zanoni</td>
</tr>
<tr>
<td>Bataviae</td>
<td>Bataviae</td>
</tr>
<tr>
<td>Sejroe</td>
<td>Hardjo, Sejroe, Saxkoebing</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>Grippotyphosa</td>
</tr>
<tr>
<td>Pomona</td>
<td>Pomona</td>
</tr>
<tr>
<td>Canicola</td>
<td>Canicola, Portlandvere</td>
</tr>
<tr>
<td>Tarassovi</td>
<td>Tarassovi</td>
</tr>
<tr>
<td>Australis</td>
<td>Australis, Bratislava</td>
</tr>
<tr>
<td>Javanica</td>
<td>Javanica</td>
</tr>
<tr>
<td>Ballum</td>
<td>Ballum, Arborea</td>
</tr>
<tr>
<td>Djasiman</td>
<td>Djasiman</td>
</tr>
</tbody>
</table>

Source: (Wai'in, 2007)

2.3.2 Genotypic classification

With the development of tools in molecular biology, classification and taxonomy of leptospires has changed. Genetic taxonomy involves DNA/DNA hybridization and guanine-plus-cytosine mol percentage (G+C mol%) content of the bacteria’s DNA (Wai'in, 2007). This has given rise to a number of genomo-species, which include serovars of both *L. interrogans* and *L. biflexa*. However unfortunately, genomo-species of *Leptospira* do not correspond to the previous two species and pathogenic and non-pathogenic serovars can be classified within the same species (Table 2.3).
<table>
<thead>
<tr>
<th>Genomo-species</th>
<th>Serogroup</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. interrogans</em></td>
<td>Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, Pyrogenes, Grippotyphosa, Djasiman, Hebdomadis, Sejroe, Bataviae, Ranarum, Louisiana, Mini, Sarmin</td>
</tr>
<tr>
<td><em>L. noguchii</em></td>
<td>Panama, Autumnalis, Pyrogenes, Louisiana, Bataviae, Tarassovi, Australis, Shermani, Djasiman, Pomona</td>
</tr>
<tr>
<td><em>L. santarosai</em></td>
<td>Shermani, Hebdomadis, Tarassovi, Pyrogenes, Autumnalis, Bataviae, Mini, Grippotyphosa, Sejroe, Pomona, Javanica, Sarmin, Cynopteri</td>
</tr>
<tr>
<td><em>L. meyeri</em></td>
<td>Ranarum, Semaranga, Sejroe, Mini, Javanica</td>
</tr>
<tr>
<td><em>L. fainei</em></td>
<td>Hurstbridge</td>
</tr>
<tr>
<td><em>L. biflexa</em></td>
<td>Semaranga, Andamana</td>
</tr>
<tr>
<td><em>L. borgpetersenii</em></td>
<td>Javanica, Ballum, Hebdomadis, Sejroe, Tarassovi, Mini, Celledoni, Pyrogenes, Bataviae, Australis, Autumnalis</td>
</tr>
<tr>
<td><em>L. kirschneri</em></td>
<td>Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Australis, Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Bataviae</td>
</tr>
<tr>
<td><em>L. weilii</em></td>
<td>Celledoni, Icterohaemorrhagiae, Sarmin, Javanica, Mini, Tarassovi, Hebdomadis, Pyrogenes, Manhao, Sejroe</td>
</tr>
<tr>
<td><em>L. inadai</em></td>
<td>Lyme, Shermani, Icterohaemorrhagiae, Tarassovi, Manhao, Canicola, Panama, Javanica</td>
</tr>
<tr>
<td><em>L. alexanderi</em></td>
<td>Manhao, Hebdomadis, Javanica, Mini</td>
</tr>
</tbody>
</table>

Source: (Wai'in, 2007)
2.4 Biology of leptospires

2.4.1 Morphology

Leptospires are tightly-coiled spirochaetes, typically measuring 10 to 20 µm in length, although cultures occasionally contain longer cells. Their helical amplitude is about 0.1 to 0.15 µm, with a wavelength in the region of 0.5 µm (Faine et al., 1999). The cells have pointed ends, at least one which is bent like a question mark, hence the name “Interrogans”. In the periplasmic space are two axial filaments with polar insertions (Swain, 1957). Leptospires move in two distinct ways, translational and rotational (Faine et al., 1999). Although all leptospires are morphologically indistinguishable, the morphology of individual isolates may vary with subculture in vitro, although this can be restored with passage through hamsters (Ellis et al., 1983).

Like other spirochaetes, leptospires have a distinctive double membrane structure. The cytoplasm and peptidoglycan cell wall are closely associated and overlain by an outer membrane (Haake et al., 2000). The outer membrane appears fluid and is porous, allowing the exchange of solutes between the periplasmic space and the environment. Salt water and desiccation can cause disorganisation of the envelope.

The composition of leptospiral lipopolysaccharide is similar to that of other Gram-negative bacteria, albeit with lower endotoxic activity (Vinh et al., 1986).

Leptospires are obligate aerobes that thrive at temperatures between 28°C to 30°C. They lack the ability to synthesise fatty acids, only reproducing in nature within animal hosts (Plank and Dean, 2000). They can grow in simple media enriched with vitamins, notably Vitamins B2 and B12, long-chain fatty acids and ammonium.
Long-chain fatty acids are utilised as the sole source of carbon and are metabolised by beta oxidation salts (Levett, 2001).

2.4.2 Genomic organization

Leptospires have complex genomes and it was only recently that the entire sequence for the serovar Lai was established (Ren et al., 2003). In comparison to the genomes of other spirochaetes, the leptospiral genome is large. Hence it is capable of surviving in various habitats including living freely in the environment, as well as in animals (Bharti et al., 2003). Both pathogenic and saprophytic leptospires have genomes of about 5000kb in size (Baril and Girons, 1990), although smaller genomes of 2000kb have been reported (Taylor et al., 1997). The genome consists of two sections: a 4400kb chromosome; and a smaller 350kb chromosome (Wai’in, 2007). In the *L. biflexa* genome has 3,590 protein-coding genes distributed across three circular replicons: a major one; a smaller one, and a third 74-kb replicon (Adler and de la Peña Moctezuma, 2010; Tulsiani, 2010). Pathogenic leptospires possess two sets of 16S and 23S ribosomal rRNA genes but only one 5S rRNA gene, with each rRNA gene located far from the others on the genome (Baril et al., 1992; Fukunaga and Mifuchi, 1989). In pathogenic serovars, but not in saprophytic species, copies of several insertion-sequence-like elements coding for transposases have been identified (Boursaux-Eude et al., 1995; Kalambaheti et al., 1999).
2.5 Pathogenesis

The most adverse clinical and pathological signs usually occur in young animals, especially when their maternally-derived immunity is waning (Boulanger et al., 1958). However, the common signs of infection with *Leptospira* in farm animals are abortions, stillbirths, decreased milk production and a failure to thrive (Alston et al., 1958; LaGrange et al., 1953; Rocha, 1998). In all species congenital infection and its sequelae are well reported. The most important difference between infection of animals and humans is the presence of chronic carriers in animals through reservoirs in the kidneys and genital tract (Acha and Szyfres, 2001; Alston et al., 1958; Wai'in, 2007; Sullivan, 1974).

2.5.1 Entry

Leptospires are capable of entering the lymphatics or bloodstream via a number of sites. In some animals, they enter through the eyes, genital tract and mucous membranes. In many human cases, the bacterium has also been found to have entered the body through abraded skin. Evidence has also pointed to transplacental infection during pregnancy (Wai'in, 2007). Transmission of disease to accidental hosts occurs through indirect contact with a maintenance host (Richardson and Gauthier, 2003).

2.5.2 Spread and growth

The ability of leptospires to thrive in tissues is a major factor for their virulence. They gain immediate exposure to the non-specific factors like pH, redox potential,
electrolytes, fatty acids and other organic compounds, some of which may be nutrients affecting the ability of them to survive and grow (Faine et al., 1999). Their resistance to innate (non specific) immunoglobulins in tissue fluids mediates their survival in the host body. When present in tissues, leptospires do not cause acute inflammatory responses (Arimitsu et al., 1989). They can spread rapidly following entry via lymphatics to the bloodstream, circulating to all tissues of the body.

Leptospires are first found in the lungs and subsequently in the liver and spleen (Faine, 1964). In the renal tubules, leptospires move through the interstitial space and attach to the renal epithelial cells. The time taken for lesions to develop is dependent on the size of the infecting dose, the rate of organism growth in the host, their level of toxicity and the opsonic immunity development rate. Toxicity is usually a function of the serovars of leptospires in a given host (Faine et al., 1999).

2.5.3 Persistence and carrier sites

Following clearance from the bloodstream, leptospires may persist and multiply in certain tissues in immunologically privileged sites. These sites include the proximal renal tubules, brain, anterior chamber of the eye and genital tract (Faine et al., 1999). Growth of leptospires in the kidneys continues exponentially and reaches a maximum concentration about 21 to 28 days after infection (Faine, 1962b).

2.5.4 Toxin production and virulence factors

Several leptospiral serovars have been reported to produce endotoxins (Levett, 2001). In biological assays, leptospiral lipopolysaccharide preparations exhibit
endotoxic activity, but at much lower potencies than in the host (Masuzawa et al.,
1990). In strains of Pomona and Copenhageni, a protein cytotoxin has been
demonstrated (Miller et al., 1970) and cytotoxic activity has been found in the
plasma of infected animals (Knight et al., 1973). Leptospites virulence factors such
as hemolysins, lipopolysaccharide, glycolipoprotein, peptidoglycan, heat shock
proteins, flagellin, KatE and Htp6 may contribute to this (Picardeau et al., 2008).
This toxin has been shown to induce a histopathological effect with infiltration of
macrophages, polymorphonuclear cells, also virulent factors can activate apoptosis
in macrophages (Yam et al., 1970).

2.6 Clinical features of leptospirosis

2.6.1 Domestic animals

In domestic species clinical signs are influenced by several factors including the
species affected, the inoculation dose, the immune status and the age of the animal.
Clinical signs may vary from high fever, pulmonary congestion, jaundice, weight
loss, abnormal milk secretion, haemoglobinuria, abortion, stillbirths or persistent
infection in newborns, and death. Leptospirosis can cause serious economic loss to
the livestock industries (Bennett, 1993; Lewis, 2003) and is a major cause of
abortions in cattle (Prescott et al., 1988; Shapiro et al., 1999). Chronic infection can
also lead to infertility and reproductive failure in cattle, goats and horses (Hanson,
1982; Ngbede et al., 2013; Prescott et al., 1988; Shapiro et al., 1999). As the disease
is common in domestic animals, humans and wildlife, control in domestic animals
can have a positive impact on the level of disease in wildlife and humans.
2.6.2  Wild animals

Knowledge on naturally occurring leptospiral infections in wildlife is limited, although experimental challenge of captive wild animals has resulted in clinical signs ranging from an inapparent infection to a febrile response, abortion, and death in *Odocolileus virginianus* and *Eumetopias jubatus* (white tailed deer and Steller sea lion, respectively) (Gulland et al., 1996; Twigg et al., 1969). The full impact of leptospirosis in wildlife is not known and studies on the impact of the disease on wildlife are required. Data on the seroprevalence in wildlife around the world is presented in Table 2.4.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Location</th>
<th>Seroprevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small rodents, Shrews</td>
<td>Zurich, Switzerland</td>
<td>12.6%</td>
<td>(Adler et al., 2002)</td>
</tr>
<tr>
<td>Mammals</td>
<td>Peruvian Amazon</td>
<td>20%</td>
<td>(Bunnell et al., 2000)</td>
</tr>
<tr>
<td>Rodents</td>
<td>Peruvian Amazon</td>
<td>20%</td>
<td>(Bunnell et al., 2000)</td>
</tr>
<tr>
<td>Marsupials</td>
<td>Peruvian Amazon</td>
<td>35%</td>
<td>(Bunnell et al., 2000)</td>
</tr>
<tr>
<td>Chiropteran (Bats)</td>
<td>Peruvian Amazon</td>
<td>35%</td>
<td>(Bunnell et al., 2000)</td>
</tr>
<tr>
<td>Carnivores</td>
<td>Peruvian Amazon</td>
<td>89%</td>
<td>(Cirone et al., 1978)</td>
</tr>
<tr>
<td>Rodents</td>
<td>Peruvian Amazon</td>
<td>60%</td>
<td>(Cirone et al., 1978)</td>
</tr>
<tr>
<td>Flying fox</td>
<td>Australia</td>
<td>11-39%</td>
<td>(Cox et al., 2005)</td>
</tr>
<tr>
<td>Possums</td>
<td>Australia</td>
<td>35%</td>
<td>(Durfee and Presidente, 1979)</td>
</tr>
<tr>
<td>Californian sea lion</td>
<td>California</td>
<td>33-71%</td>
<td>(Gulland et al., 1996)</td>
</tr>
<tr>
<td>Black rats</td>
<td>New Zealand</td>
<td>34%</td>
<td>(Hathaway and Blackmore, 1981)</td>
</tr>
<tr>
<td>Animal</td>
<td>Location</td>
<td>Seroprevalence</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------</td>
<td>----------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Brown rats</td>
<td>New Zealand</td>
<td>26%</td>
<td>(Hathaway and Blackmore, 1981)</td>
</tr>
<tr>
<td>Lynx</td>
<td>Spain</td>
<td>32%</td>
<td>(Millán et al., 2009)</td>
</tr>
<tr>
<td>Fox</td>
<td>Spain</td>
<td>47%</td>
<td>(Millán et al., 2009)</td>
</tr>
<tr>
<td>Mongoose</td>
<td>Spain</td>
<td>20%</td>
<td>(Millán et al., 2009)</td>
</tr>
<tr>
<td>Genet</td>
<td>Spain</td>
<td>12%</td>
<td>(Millán et al., 2009)</td>
</tr>
<tr>
<td>Badger</td>
<td>Spain</td>
<td>50%</td>
<td>(Millán et al., 2009)</td>
</tr>
<tr>
<td>Cat</td>
<td>Spain</td>
<td>20%</td>
<td>(Millán et al., 2009)</td>
</tr>
<tr>
<td>Dog</td>
<td>Spain</td>
<td>36%</td>
<td>(Millán et al., 2009)</td>
</tr>
<tr>
<td>Feral Pigs</td>
<td>NSW Australia</td>
<td>20%</td>
<td>(Mason et al., 1998)</td>
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<td>Caribou</td>
<td>Alaska</td>
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<td>(Zarnke, 1983)</td>
</tr>
<tr>
<td>Moose</td>
<td>Alaska</td>
<td>3%</td>
<td>(Zarnke, 1983)</td>
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<tr>
<td>Grizzly bears</td>
<td>Alaska</td>
<td>5%</td>
<td>(Zarnke, 1983)</td>
</tr>
<tr>
<td>Black bears</td>
<td>Alaska</td>
<td>4%</td>
<td>(Zarnke, 1983)</td>
</tr>
</tbody>
</table>

### 2.6.3 Humans

More than 184 distinct serovars of *L. interrogans* belonging to 20 serogroups have been identified across the world (Nascimento et al., 2004). In Southeast Asia, the most common serovars associated with disease of domestic animals and humans are *Icterohaemorrhagiae, Autumnalis, Canicola, Pomona, Patoc, Grippotyphosa, Australis* and *Poi* (Srivastava, 2006; Victoriano et al., 2009). The first three are the most important serovars with respect to veterinary and public health perspectives. Fever is an important and common presentation of tropical diseases and may sometimes be the only manifestation of a serious illness. Diagnosis in humans is often difficult because of the many diagnostic possibilities, symptoms which may often be non-specific, and because many medical doctors are unfamiliar with the spectrum of tropical diseases. Treating the disease appropriately will reduce the risk to public health, and this can only come following a correct diagnosis. With the
recent popularity of adventure travel and triathlete competitions, there is an increased awareness of the potential for leptospiral infection amongst those who partake in such activities (Cachay and Vinetz, 2005; Morgan et al., 2002).

2.7 Pathology

Symptoms can vary from a mild non-specific influenza-like infection to Weil’s disease, where serious complications can occur (Esen et al., 2004). The primary histological lesion observed in clinical leptospirosis is the damage to the endothelial membrane of the capillaries, caused by leptospiral toxin (Wai’in, 2007). This has the effect of loosening intercellular junctions, allowing both fluid and leptospires to migrate into extravascular spaces, followed by red blood cells, wherever the damage is severe. Secondary effects of ischaemic change, anoxia and increased tissue pressure reinforce damage, resulting in cellular functional disintegration and death of the cell (Ellis, 1994). In the kidneys, the main finding is interstitial nephritis. This is accompanied by intense cellular infiltration composed of neutrophils and monocytes (Penna et al., 1963), but renal disease is uncommon.

2.8 Epidemiology of leptospirosis

Leptospires are widespread and their abundance is due to their ability to infect a range of animal species, including humans, as well as the ability to survive outside the host, if environmental conditions are favourable (Duncan et al., 2011; Hathaway and Blackmore, 1981; Smith et al., 1961). The main sources of infection are urine of infected or carrier animals and contaminated surface water, mud and soil (Monahan
et al., 2009). Transmission can happen as a result of direct or indirect contact with infected animals or their secretions (Monahan et al., 2009). The major route of infection is via mucous membranes, however carnivores can be infected through the ingestion of leptospiral-infected carcasses (Murray et al., 2006). Usually leptospires appear in the blood four to 10 hours after infection and can be detectable in the blood for a few hours up to seven to 10 days (Plank and Dean, 2000; Smith et al., 1994; Tsai and Fresh, 1975). Clinical signs may not occur in every case but severe fever is an important sign of acute leptospirosis. Animals that have recovered from leptospirosis may become carriers, with the organism present in the renal tubules for periods of days to years (Babudieri, 1958; Bahaman et al., 1987) with subsequent shedding of leptospires via urine into the environment (Babudieri, 1958).

Infectious diseases are transmitted globally through animal and human movements due to eco-tourism, wildlife research, reintroduction, rehabilitation, hunting, pet trade, laboratory and food industry demands and farming (Beatty et al., 2008; Osofsky, 2005). These movements and activities are major contributing factors for the transfer of leptospirosis to animals and humans and the spread of the disease to new areas.

Research on leptospirosis has highlighted that rodents (20%), marsupials (35%) and bats (35%) are most likely to spread the disease (Bunnell et al., 2000). Marsupials and chiropterans have recently been implicated as more significant reservoir hosts of leptospires pathogenic to humans than previously was recognized (Bunnell et al., 2000). Studies on leptospirosis involving bats have produced equivocal results (Cox et al., 2005), however the fact that leptospires can potentially be spread through bats is a major concern due to their abundance, their ability to travel long distances and their potential to expose humans to infected urine. Ground-dwelling species, such as
rodents foraging under bat roosts, could also encounter *Leptospira* contaminated urine and potentially spread the organism to urban areas (Tulsiani et al., 2011). From the limited research on wildlife, several pathogenic serovars have been isolated with the most prominent serovars including Grippotyphosa and Pomona from white-tailed deer (*Odocoileus virginianus*) in North America (Cantu et al., 2008).

Surveillance of leptospirosis is important to determine the emergence of new strains, which have the potential to cause an outbreak. Although it is not economically viable to survey all wildlife species, it is important to regularly screen particular species which live on the periphery of forests and have the potential to interact with humans (eg: wild rats, carnivores and bats). Screening these ‘sentinels’ provides useful cost-effective information on the health status of a broader range of species.

### 2.8.1 Geographical distribution of leptospirosis worldwide and in Southeast Asia

Leptospirosis is a major health concern globally, more so in tropical regions such as South America, South Asia and Southeast Asia. Major outbreaks have been reported recently in India, Indonesia, Sri Lanka and Thailand (Ciuchi-Nicolau, 2010). In Sarawak, East Malaysia, leptospirosis has been reported to have occurred at numerous locations, sometimes involving Western tourists. Given the regional peculiarities of climate, population density and the degree of contact between humans (accidental hosts) and animals (maintenance hosts), Southeast Asia is conducive for maintaining a high level of transmission of leptospirosis (Ciuchi-Nicolau, 2010). Numerous pathogenic serovars circulate and there are abundant
reservoir host species including rodents, farm animals and dogs present in this region.

Transmission is not only occupational as is observed in temperate climates, but is the consequence of exposure to a contaminated environment, in particular during the wet season when outbreaks can occur configuring an epidemic-endemic pattern of disease (Levett, 2001). According to Pappas et al., (2008), the Caribbean and Latin America, the Indian subcontinent, Southeast Asia, Oceania and to a lesser extent Eastern Europe, are the most significant foci of the disease, including areas that are popular travel destinations.

Between 1987 and 1991, researchers surveyed 32 Dutch travellers with leptospirosis who had mainly been to Thailand and other Southeast Asian countries. Their studies found that the main infective agents belonged to the serogroups Australis and Sejroe (van Crevel et al., 1994).

2.8.2 Geographical distribution of leptospirosis in Malaysia

At least 37 serovars have been isolated from humans and animals in Malaysia (El Jalii and Bahaman, 2004) and serovars from the Pyrogenes, Canicola, Autumnalis and Pomona serogroups have been isolated from human patients (Tan, 1964). During the 1950’s a group of researchers successfully isolated leptospiral serovars from serogroups Hebdomadis, Grippotyphosa and Canicola from three species of Malaysian rodents and a serogroup of Icterohaemorrhagiae from *Rattus whiteheadi* from Sabah (Wisseman et al., 1955). In 2004, researchers discovered a new pathogenic serovar, Lai, from a patient who had returned from Langkawi Island in West Malaysia (Wagenaar et al., 2004). As late as 2009, a new serovar, *L. Kmetyi*
Bejo-Iso 9, was isolated from a soil sample in the southern state of Johor (Slack et al., 2009). In the next chapter, the discovery of a novel serovar in Sarawak Leptospira, Lepto 175 Sarawak is discussed.

2.8.3 Sources and mode of transmission of leptospirosis

Domestic and wild mammals, reptiles and amphibians are maintenance hosts for different leptospiral serovars (Wai'in, 2007), however rodents and cattle are considered the main source of human infection (Levett, 2001; Smythe et al., 2000), although dogs may also be an important source (Meeyam et al., 2006). In addition, bats have recently also been suggested as a possible source of exposure to Leptospira for humans (Cox et al., 2005; Matthias et al., 2005), particularly because of their size, abundance, spatial distribution, and interrelationship with domestic animals (Kunz, 1982; Matthias et al., 2005; Vashi et al., 2010). Leptospires colonise the kidneys of carrier animals and are shed in the urine, which is the primary source of environmental contamination. It is estimated that between 10,000 and 1,000,000 leptospires are shed by carriers in every ml of urine passed (Faine et al., 1999). Humans and other animals are usually infected by exposure to this urine from the infected animals (Ellis and Michna, 1976).

Transmission modes can be direct or indirect. Direct transmission happens when animals come into contact with the urine of chronically infected animals (Ellis et al., 1986; Ellis and Thiermann, 1986). However in cattle and pigs, evidence exists of leptospires crossing directly from the genital tract to the foetus (Goldenberg and Thompson, 2003). Indirect transmission happens when animals or humans acquire infection from the environment via contamination of the conjunctivae, oral mucosa,
and respiratory tract mucous membrane or via skin cuts (Levett, 2001). Also human-to-human transmission has been postulated through sexual transmission (Harrison and Fitzgerald, 1988) and breastfeeding (Bolin and Koellner, 1988).

2.8.4 Cycle of host infection

The epidemiology of human leptospirosis reflects the ecological relationship between humans and chronically-infected mammalian hosts (Wai'in, 2007). Humans are regarded as incidental dead-end hosts from which further transmission has not been demonstrated. Two natural cycles of leptospiral infection have been identified: a sylvatic cycle and a domestic cycle (Faine et al., 1999). In the former case, leptospirosis is accidentally transmitted to livestock and humans from numerous species of rodents and chiropterans. The main mode of spread and continuity of infection in rodents is by direct transmission from mother to the young (Wai'in, 2007). Humans can pick up infection through contact with an environment contaminated with urine, particularly that of rodents or bats. Bats forage in fruit orchards and forest clearings created by humans, and roost in buildings, water cisterns, culverts, abandoned structures and bridges (Kunz, 1982). In contributing to a sylvatic cycle of leptospiral transmission, ground-dwelling species, such as rodents or marsupials, that reside or forage under bat roosts could be exposed to Leptospira-contaminated urine (Matthias et al., 2005).

Animals that do not exhibit signs of clinical infection but shed leptospires for long periods of time are regarded as maintenance (or reservoir) hosts. Different animal species may be reservoirs for distinct serovars. The domestic cycle of leptospiral
transmission involves cattle, swine, sheep, buffalo, goats and dogs. These animals are known to be maintenance hosts of specific serovars (Voronina et al., 2014).

2.8.5 Survival of leptospirosis in the environment

The extent to which leptospires are transmitted depends on the survival of them in the environment which is influenced by temperature, climate, soil pH and soil moisture (Ringen and Okazaki, 1956). The optimal survival condition for leptospires is a warm, wet environment with neutral to slightly alkaline water (Farrar, 1995). Like other spirochaetes, leptospires are well-adapted to viscous environments, where they show greater translational motility than other bacteria (Kaiser and Doetsch, 1975). They can survive for approximately two weeks in soil contaminated with urine from an infected host (Karaseva et al., 1977).

2.8.6 Major leptospirosis outbreaks in Malaysia

Records of leptospiral human infections in Malaysia date back to 1925 (El Jalii and Bahaman, 2004; Lim et al., 2011; Thayaparan et al., 2013b). Cases of febrile illness among both military and civilian personnel were frequent during the 1950’s, drawing attention to leptospirosis as an emerging disease in both Peninsula and East Malaysia (Broom, 1953). According to Lam and Kan, (2005), there have been two known outbreaks in recent years. The first involved a group of schoolboys who had been swimming in a river. The patients reported fever, headache, giddiness, vomiting, body ache, cough, chest pain and conjunctivitis. Tragically, one of them succumbed to the disease, which was believed to be leptospirosis. The second instance involved the widely-publicised Eco-Challenge in Sabah in 2000, in which
many participants, most from Western countries, became ill when they returned home. Serological studies carried out in Sabah on people who lived near national parks in the early 2000s reported a high seroprevalence to leptospirosis, possibly from exposure to infected wildlife (Russ et al., 2003). Leptospiral infection of inmates at various immigration detention centres all over the Malay Peninsula have also been reported, with six confirmed fatalities, causing major concern among human rights groups (Jones, 2011; Wright, 2011). In Terengganu, there were outbreaks reported in Hulu Terengganu and Sungai Berua, as well as in an Orang Asli settlement (Lim et al., 2011). Leptospirosis has also been implicated in the death of a Universiti Malaysia Terengganu staff member in June 2013 (Thayaparan et al., 2013b). Patterns of infection in Malaysia had appeared to be sporadic up until 2010, with the disease concentrated mostly in one or a few regions at any time (Lim et al., 2011) and occurring during the monsoon seasons. However, with climate changes, disease has been reported throughout the year, as has been demonstrated in Sarawak during the last two years (Thayaparan et al., 2013b). Although human-to-human transmission has not been reported, the severity of the disease raises significant concerns when outbreaks occur. MacKay-Dick, (1971) suggested that the severity of leptospirosis within a community was co-related to the length of time its members spent in the jungle. With leptospirosis being prevalent in wildlife, containment of the disease is difficult due to a lack of a suitable and acceptable approach for wildlife management (Thayaparan et al., 2013a).

2.8.7 Leptospirosis and ecotourism

Leptospirosis is a zoonosis with a wide geographical distribution, primarily because the pathogen can be harboured by a wide range of wild and domestic animals.
Although leptospires cannot survive in dry environmental conditions, the presence of moist soil, standing water or surface waters allows them to maintain their virulence and persistence outside their animal hosts (Faine, 1982). Contact with these contaminated surfaces poses an occupational risk to humans, although recently more people are exposed to the spirochaete during recreational activities. One such activity would be adventure travel. Southeast Asia is a popular destination for such travellers, many of whom hail from the Western world. The region’s tropical climate provides a suitable environment for leptospires to thrive, which puts such tourists at risk of disease when they visit. However, leptospirosis is often misdiagnosed, even though it is endemic, because of the non-specific symptoms associated with infection and the difficulty in confirming a diagnosis (Sejvar et al., 2003).

Eco-tourism comprises a range of adventure activities including four-wheel driving, flying microlight planes and white water rafting and in Malaysia there are large numbers of tour operators involved in this sector of the tourism industry. It is believed that 7% of tourist activity could be nature-related with over 500,000 international eco-tourists visiting Malaysia each year (Yasak, 2000). Furthermore it is estimated that 7 to 10% of all overseas tourists undertake some form of ecotourism activities, and a further 14% are interested in walking, hiking or trekking. This amounts to potentially over one million international tourists each year (Yasak, 2000). Statistics from Tourism Malaysia (2011) (http://corporate.tourism.gov.my/) reveal that 24.6 million tourists visit Malaysia each year, contributing to a major part of the country’s income. Most of these tourists also want to see Malaysia’s native wildlife (Thayaparan et al., 2013a). Although ecotourism is a major industry in Malaysia and other Southeast Asian countries, its potential impact on wildlife and the people raises significant concerns.
Furthermore the threat of diseases such as leptospirosis could result in bad publicity and loss of an important source of foreign revenue for the country.

2.8.8  Deforestation and wildlife-human conflict

Urbanisation inevitably causes deforestation, which can result in displacement of wildlife and extinction of specific species. The loss of habitat can disrupt animal’s food supply, and many species take shelter near human settlements where there exists a potential food source. Some species, such as bats, have spatial and temporal dynamics that are particularly sensitive to anthropogenic activity (Matthias et al., 2005). In the Iquitos region in Peru, bats are believed to be a major player in the sylvatic cycle of leptospiral infection. Deforestation not only results in a loss of habitat and food for wildlife but also results in increased contact with humans, increasing the risk of transmitting zoonotic diseases.

2.8.9  Ecotourism and wildlife contact

Although little physical contact with wildlife has been documented by eco-tourists, it is virtually impossible to avoid contact with contaminated water and soil. The majority of infections would appear to result from exposure to contaminated freshwater rather than exposure directly to animal urine. Most tourists come from more temperate climates and do not wear suitable protective clothing due to the high heat and humidity of the tropics (Mortimer, 2005), putting themselves at risk of exposure and subsequent infection. The Malaysian jungle is home to numerous wildlife species, including bats; whose numbers populate many cave formations in Borneo. Other species that may be potential reservoirs of *Leptospira* are primates,
and the role these play in leptospirosis will be discussed in Chapter 4. With forest areas shrinking it is likely there will be even greater interaction between humans and wildlife in the future and the potential for diseases, such as leptospirosis, to reach epidemic proportions (Thayaparan et al., 2013a).

2.9 Economic impact of leptospirosis

The economic impact of leptospirosis is usually felt by those who are involved in the livestock industries. Primarily, it has had a significant impact on the cattle industry through abortions, stillbirths and mastitis. However with the disease becoming endemic and the zoonotic aspect becoming more important, the consequences are now greater for the wider community and general economy.

As with other zoonotic pandemics, fear and anxiety can grip the communities most vulnerable to infection. The economic impact of the disease includes loss of income to the affected individuals, loss of export products from decreased productivity of diseased livestock, loss of income from reduced tourism over concerns about the disease, costs associated with the investigation and control of an outbreak and compensation to farmers for the loss of their animals (Arief, 2013).

2.10 Laboratory Diagnostic Methods

Diagnosis of leptospirosis requires use of laboratory methods as the clinical presentation is not specific. The diagnostic method selected depends on the available samples and the purpose of testing. Identification of the infecting serovar is
important both epidemiologically and clinically, since this may assist in determining the source and likely outcome of infection. Different assays have been developed in an attempt to diagnose leptospirosis accurately, however the majority are unsuitable for use in developing countries due to their requirement for the maintenance of multiple strains and the need for expensive equipment. Several methods exist for the diagnosis of leptospirosis. These include simple microscopic demonstration of the organism to more advanced tests such as the Polymerase Chain Reaction (PCR).

2.10.1 **Microscopic demonstration of Leptospira**

Leptospires may be visualized in clinical material through dark-field microscopy, immunofluorescence or light microscopy after appropriate staining (Wai’in, 2007). Where laboratory resources are limited, dark-field microscopic examination of body fluids has been used to rapidly detect the presence of leptospires. However the technique lacks sensitivity (Faine et al., 1999) with a detection limit of $10^4$ leptospires/ml (Turner, 1970). Microscopic examination of blood samples needs to take place within the first few days of acute illness during which time leptospiromaemia is present (Levett, 2001). Serum protein and fibrin strands and other cell debris in the blood can resemble leptospires, while the concentration of organisms in the urine of humans and animals is frequently too low to be detectable by this method (World Health Organization, 2003). This method calls for high operator skill and no information on the infecting serovar can be gained (Smith et al., 1994).

For post-mortem diagnosis and to increase the sensitivity of direct microscopic examination, specific staining methods have been deployed. Standard stains for leptospires have been silver impregnation techniques, strong carbol fuchsin and
methylene blue, or Gram stain using a carbol fuchsin counterstain. However, like dark-field microscopy, staining methods also present a high risk of false-positive or false-negative results (World Health Organization, 2003). Immunofluorescent staining is also used for demonstrating leptospires in clinical and environmental specimens, such as urine, body fluids, frozen kidney samples, soil and water, because of the ease of identifying leptospires and the serovars can be presumptively determined (Ellis et al., 1982; Faine et al., 1999). Recently the more sensitive immune-histochemical methods have been applied, notably the immunogold silver staining method. This method produces a permanent image that does not fade with time. Furthermore a fluorescent microscope is not required.

2.10.2 Cultural methods
Leptospires can be cultured from the blood, body fluids and tissues of infected subjects. These bacteria grow in culture media containing dilute animal (usually rabbit) serum or bovine serum albumin (Faine et al., 1999; World Health Organization, 2003). The Ellinghausen-McCullough-Johnson-Harris (EMJH) formula is currently most commonly used. Its base is a serum-free oleic acid-albumin medium with derivatives containing Tween 80 as the source of fatty acids and BSA as a detoxifier (Ellinghausen and McCullough, 1965a; Johnson and Harris, 1967). The anti-microbial agent, 5-fluorouracil, is sometimes added to media to inhibit the growth of contaminants present in clinical specimens (Johnson and Rogers, 1964). To make EMJH media semi-solid or solid, sugar at respective concentrations of 0.1-0.2% and 0.8-1.5% is added (Faine et al., 1999).
Unfortunately, culture is a slow process requiring several weeks of incubation, and has low sensitivity (Bharti et al., 2003). Palmer and Zochowski, (2000) recommended that samples be inoculated into media within 24 hours of collection. Growth may occasionally be detectable after one week culture, however it often takes longer. The culture medium should therefore be checked for growth of leptospires at regular intervals for a period up to 4 months (World Health Organization, 2003). In semi-solid media, growth reaches maximum density beneath the surface of the medium, which becomes increasingly turbid as incubation proceeds (Palmer and Zochowski, 2000; Wai’in, 2007).

Leptospiral cultures are maintained by repeated subculture, though this repeated process makes the organisms less virulent (Adler and de la Pena Moctezuma, 2010). Isolates can be grown to reasonable density in semisolid EMJH medium at 30°C, and can subsequently be stored in the dark at room temperature for up to three years. Long-term storage at -70°C in glycerol is also used (Palit et al., 1986).

During acute and chronic infections, isolation of leptospires is frequently attempted from various clinical specimens. Suitable specimens, including blood (whole and clotted), serum, urine, cerebrospinal fluid and tissue (particularly kidney) samples, can be inoculated into EMJH medium containing 5-fluorouracil (Wai’in, 2007). Cultures should be incubated at 29 ± 1°C for at least 16 weeks, and preferably for 26 weeks and examined by dark-field microscopy every 1 to 2 weeks (OIE, 2008). Identifying isolates to the serovar level is usually performed at reference laboratories and involves time-consuming cross-absorption agglutination procedures with panels of monoclonal antibodies (Levett, 2004; Smith et al., 1994).
2.10.3  **Microscopic Agglutination Test (MAT)**

The MAT relies on a panel of live cultures as diagnostic antigens, making the quality assurance of this test particularly difficult (Chappel et al., 2004). This is the standard diagnostic test for leptospirosis but requires sophisticated equipment and is time consuming.

2.10.4  **ELISA**

Although the ELISA can detect different classes of antibody, it may be subject to false positive reactions and requires confirmation of these results by the MAT (Smythe et al., 2002b). In contrast to the MAT, ELISAs can be performed rapidly and without the need for very sophisticated equipment. Two commercially available ELISA kits – the INDX Dip-S-Ticks and the PanBio ELISA were evaluated against the MAT, using sera from the University Hospital, Kuala Lumpur, between 1991 and 1997 (Sekhar et al., 2000). Results from this research suggested that the INDX Dip-S-Ticks test was a practical alternative to the MAT.

2.10.5  **Polymerase Chain Reaction (PCR)**

The PCR has been evaluated by several groups for its usefulness in detecting leptospiral DNA from both humans and animals. Although PCR technology is now widely used for the diagnosis of many diseases, its general value for the rapid diagnosis of leptospirosis has not been evaluated worldwide and it is not yet widely used in tropical and subtropical countries (World Health Organization, 2003). Over the years, the PCR has evolved from a gel-based method to a real-time one, incorporating specific probes and primers and can be very sensitive, detecting as few
as 2 to 3 organisms per sample. However, the test requires selection of specific primers to allow for amplification of the DNA. A number of primer pairs have been described based on specific gene targets (Renesto et al., 2000), including the 16S and 23S ribosomal RNA genes found in all pathogenic leptospires (Merien et al., 1992). Other primers have also been constructed from genomic libraries (Gravekamp et al., 1993).

There is evidence that PCR assays display greater sensitivity than conventional diagnostic methods, such as culture or dark-field microscopy, although the sensitivity of culture may vary between laboratories (Brown et al., 1995; Heinemann et al., 2000; Merien et al., 1992). The PCR is very useful for obtaining an early diagnosis of leptospirosis, when bacteria may be present but before antibody titres are at detectable levels (World Health Organization, 2003). The ability of PCR assays to identify specific serovars is limited, with authors often describing genotypic groupings of serovars instead of serovar-specific groupings (O'Keefe, 2002).

2.10.6 Multi-Locus Sequence Typing (MLST)

The Multi-Locus Sequence Typing (MLST) database has information on numerous bacterial species, including leptospires. It currently has information on over 200 isolates, most of which have been isolated from Thailand between 2000 and 2005 (Thaipadungpanit et al., 2007; Boonslip et al 2013). Ahmed et al., (2006) developed a robust MLST method for genotyping *Leptospira*. This method of typing is comparably more reproducible, robust, consistent and portable than other methods. The MLST approach allows a better study of the genetic relatedness of leptospires.
and can be used for molecular epidemiological studies and population genetics (Ahmed et al., 2006). It is a simple PCR-based technique using DNA sequences to assign and characterise alleles present in different target genes (Romero et al., 2011a). However, Romero et al., (2011a) reported that a major drawback of the MLST was its lack of discriminatory power when applied on uncommonly occurring isolates.

2.10.7 Modern rapid tests

A more recent method of detecting leptospires is the Lepto Dipstick test (Sehgal et al., 2000). This uses a broadly reactive antigen for detecting IgM antibodies. This method was evaluated on 867 sera collected from patients confirmed with leptospirosis and controls from the Andaman Islands between 1993 and 1997. Using these samples the Lepto Dipstick had a sensitivity of 78.7%, a specificity of 88.3% and a positive predictive value of 91.0%. The test had a good level of agreement with the standard criteria for diagnosis using paired MAT results (K = 0.64). The sensitivity of the Lepto Dipstick was high between the second and fourth weeks of the disease, however lower, but still acceptable, sensitivities were recorded before and after that period. This test is easy to perform as it does not involve sophisticated equipment. The reagents and dipsticks can also be stored for long periods, even at room temperature, and therefore it is suitable for use as a rapid screening test for the diagnosis of leptospirosis.

Sehgal et al., (2003) evaluated the Lepto lateral flow assay on samples from the Andaman Islands between October 1999 and December 2000. Like the Lepto Dipstick, it was developed at the Dutch Royal Tropical Institute. This test can be
performed at the patient’s bedside, as whole blood can be used. One hundred and seventeen patients suspected to have leptospirosis were included in the study with acute serum samples collected from all of them and convalescent samples from 104. The results of the lateral flow test were compared with the standard criteria for diagnosing leptospirosis, and the test was found to have a sensitivity of 52.9% (37/70) in the first week of illness and 86% (49/57) during the second to fourth weeks of illness. Corresponding specificities were 93.6% (44/47) and 89.4% (42/47), respectively. Agreement of the results with the standard criteria was low during the first week, but high during weeks 2 to 4. All indices of validity and utility of the lateral flow test were similar to those of the IgM ELISA and Lepto dipstick (Sehgal et al., 2003).

Samples from inhabitants of the Andamans were again selected for the Lepto Dri Dot card agglutination test (Vijayachari et al., 2002). The results of this test were compared with blood culture and MAT tests on paired serum samples. Based on these criteria, 74 of 124 patients were diagnosed with leptospirosis. The Lepto Dri Dot had a sensitivity of 67.6% (50/74) and a specificity of 66.0% (33/50) during the first week. During weeks 2 to 4 the values increased to 85.5% (47/55) and 80% (40/50), respectively. An IgM ELISA was also performed on the serum samples for comparison and this was marginally less sensitive, but more specific, during the first week of illness. The Lepto Dri Dot test does not require special storage or sophisticated equipment and can be performed by relatively low skilled personnel.

In Hawaii, eight different IgM detection methods were compared using 379 serum samples obtained in 1998 and 1999 from 236 patients, of which 33 were confirmed infected (Effler et al., 2002). The median time between the onset of illness and the collection of samples was 9 days. This is the most comprehensive field evaluation of
screening tests for leptospirosis reported to date. The sensitivity of the tests ranged from 29 to 86% and specificity from 85 to 100% for seven of the eight tests evaluated. The sensitivity was particularly low (<25%) on samples collected during the first week of illness for seven of the tests. The data indicated that IgM detection tests have limited use for diagnosing leptospirosis during the initial stage of disease, a time when important therapeutic decisions are usually made (Effler et al., 2002).

2.11 Diagnostic techniques used in Malaysia

Several methods are used in the diagnosis of leptospirosis in Malaysia. These include the simple MAT, PCR and ELISA’s. The MAT is inexpensive, but time consuming and laborious, and is only undertaken at the Institute for Medical Research (IMR) Kuala Lumpur, Makmal Kesihatan Awam Kebangsaan (MKAK) Sungai Buloh Malaysia for community outbreaks and at universities undertaking research on leptospirosis (Hakim, 2011). ELISA’s are performed at hospitals with the necessary facilities as well as the IMR and MKAK. Currently culture of Leptospira is only performed at the IMR (Hakim, 2011). Diagnosis of animal leptospirosis is only undertaken at the Veterinary Research Institute, Ipoh and the Wildlife Department, Kuala Lumpur (PERHILITAN). Environmental samples can be tested at the MKAK, Sungai Buloh, Malaysia (Hakim, 2011).

Diagnosis of leptospirosis in wildlife requires evaluation of presenting clinical signs and serological test results. There are three methods used to diagnose leptospirosis in mammals: serological, demonstration and histological methods. The serological methods consist of fluorescent antibody and microscopic and macroscopic agglutination tests, which detect antibodies to leptospires. Depending on the severity or stage of infection, an animal may react serologically to one or all antigens used to
determine the serological profile. The demonstration methods involve examining body fluids and tissues under dark field microscopy, and histological examination involves examination of biopsy material with specific silver stains (Warthin-Starry) or a Giemsa stain.

2.12 Treatment, control and prevention of leptospirosis

A range of antibiotics are used to treat hosts with leptospirosis, with intravenous C-penicillin (2M units 6 hourly for 5-7 days) being commonly used in severe adult human cases (Ganoza et al., 2006; Hakim, 2011). Less severe cases are usually treated with oral antibiotics such as doxycycline, tetracycline, ampicillin or amoxicillin (Hakim, 2011). A trial on the penicillin derivative, doxycycline, on the prevention of infection and clinical disease was conducted in the North Andaman Islands in 1999 (Sehgal et al., 2000). Although the findings indicated that doxycycline prophylaxis did not prevent leptospiral infection in an endemic area, such treatment did have a significant protective effect in reducing the morbidity and mortality during outbreaks.

It is important to investigate the nature of leptospirosis, and identifying the endemic strain in an area will largely dictate the direction of any studies on the organism and the disease in wildlife and humans. This will determine the resources required to be invested into control and prevention strategies. After several outbreaks of leptospirosis around wildlife reserves, the IMR, Malaysia undertook an investigation to determine the cause of the disease in Sarawak. During the initial surveillance project, Leptospira-like organisms were collected around the study area for the research described in this thesis. In the next chapter a novel strain of Leptospira collected in the study area near Kuching, Sarawak is described.
CHAPTER 3: *LEPTOSPIRA*, LEPTO 175 SARAWAK SP. NOV., ISOLATED FROM AN ENVIRONMENTAL SAMPLE IN SARAWAK, MALAYSIA.

3.1 Introduction

Leptospirosis is emerging as a grave public health concern in Malaysia and other Southeast Asian countries (Arief, 2013; Bhatia and Narain, 2010; Coker et al., 2011; Victoriano et al., 2009). There are many animal hosts for the bacteria, and these hosts can shed leptospires in their urine for many years (Monahan et al., 2009). Soil and water contaminated by this urine can result in severe illness when humans and other animals come into contact with it. Leptospirosis is a widespread zoonotic disease resulting in morbidity and mortality of humans and animals, and the disease is endemic in Malaysia (Antony, 1996). Outbreaks of leptospirosis have recently been reported in and around wildlife reserves/parks and mortalities have previously been reported in humans in Malaysia as reported in the previous chapter (Koay et al., 2004; Lim et al., 2011; Thayaparan et al., 2013b).

Although leptospires survive best in moist conditions, flourishing in pools of stagnant water for up to several days, they cannot thrive in salt water (Dutta and Christopher, 2005) surviving for only a few hours in this medium. Ecuadorian researchers have shown that leptospires can remain motile in pure water for up to 110 days without nutrients (Trueba et al., 2004). However they are easily destroyed by desiccation, exposure to disinfectants and detergents, and heating to 50°C for five
minutes (Ferguson, 1991). Although reports on the isolation of leptospires from environmental samples are numerous, little direct information is available on their actual distribution in various bodies of water or in the soil in Malaysia.

In 2009 a novel strain, *L. kmetyi* Bejo-Iso 9, was identified from a soil sample obtained from the southern Malaysian state of Johor (Slack et al., 2009). More recently in June 2012, the Borneo Post reported that contamination from rat urine with stagnant water was responsible for the widespread distribution of leptospires around Sibu City in Sarawak (Thayaparan et al., 2013b). Health authorities warned that children should stay away from stagnant ponds to avoid “untoward incidents such as coming into contact with leptospirosis or other water-borne diseases” (Boon, 2012).

The genus *Leptospira* is divided into pathogenic, intermediate and non-pathogenic leptospires. Members of the pathogenic group are associated with disease outbreaks and some intermediate strains have also been isolated from humans and other animal species displaying signs of disease (Slack et al., 2008; Zakeri et al., 2010). The isolation of members of pathogenic leptospires from water and soil has been well documented (Ganoza et al., 2006; Henry and Johnson, 1978; Henry et al., 1971).

One of the problems with the diagnosis of leptospirosis in humans in Malaysia has been the confusing nature of the symptoms associated with the disease. Since it is a febrile illness and several tropical diseases also manifest with fever in the early stages, the disease is easily misdiagnosed (Saunders, 1979), thus delaying the commencement of effective treatment. However, recent improvements in environmental sampling and isolation and identification of *Leptospira* strains have been reported (Hakim, 2011). To gain additional information on the extent of the
distribution of leptospires in wildlife and humans around wildlife reserves/park in Sarawak, environmental samples were collected and the results are described in this chapter. Initially, strain Lepto 175 Sarawak was isolated from a sample of water collected from Sarawak by researchers from the IMR who were undertaking a survey around a suspected leptospirosis outbreak area. The morphological, serological and molecular characterization of a novel species of the genus *Leptospira* is reported in this Chapter.

### 3.2 Materials and Methods

Several 50 mL of surface stagnant water was collected from different spots in sterile polypropylene centrifuge tubes and filtered through Whatman no. 1 filter paper to eliminate large particles prior to filtering through a 0.45 μm nitrocellulose filter membrane, then a 0.22 μm filter was used to filter only leptospires (Ganoza et al., 2006; Henry and Johnson, 1978). 1 mL of filtered water was inoculated, in duplicate, into EMJH (Difco) media and Fletcher media, which were incubated at 30°C. The media were examined weekly using dark-field microscopy for the presence of leptospires. If leptospires were detected under dark field microscopy, 1 mL of the media was inoculated into EMJH and Fletcher media for subculture at 2 weekly intervals.

#### 3.2.1 Morphological identification of *Leptospira*, Lepto 175 Sarawak

Dark field microscopy was used to demonstrate leptospires in the media. Microscopy was undertaken at 200 and 400x after four weeks of culture in the EMJH media.
3.2.2 **Serological identification of Leptospira, Lepto 175 Sarawak**

Strain Lepto 175 Sarawak was isolated and forwarded to the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, Australia, for further identification. The strain was maintained in EMJH and Fletcher medium at 30°C. Serological identification of the isolate was performed with the Cross Agglutination Absorption Test (CAAT) carried out with the recommended techniques (Babudieri, 1971) using hyper-immune serum from rabbits against the known serotypes in the reference laboratory (Table 3.1).

3.2.3 **Analysis of PCR products**

Cultures were prepared for isolation of DNA by centrifugation as described previously (Thaipadungpanit et al., 2007), followed by genomic DNA extraction using a High Pure Viral Nucleic Acid kit (Roche, Germany). Amplification of the 16S rRNA was performed as described by others (Slack et al., 2008; Slack et al., 2006; Thaipadungpanit et al., 2007) with the following modifications: PCR amplification was performed in 25 µL volumes containing 1x Taq PCR buffer, 2.0 mM MgCl₂, 200 uM dNTPs, 10.0 pmol Forward primer (5’- GTT TGA TCC TGC TGG CTC AG 3’) and 10.0 pmol Reverse primer (5’- CCG CAC CTT CCG ATAC-3’) (Thaipadungpanit et al., 2007), 1U Taq Polymerase (Fermatas, Cat#-EP0405), 2.5 µL template DNA and nuclease free water to make up the final volume of 25 µL.
Table 3.1: Serological groups tested against Lepto175.

<table>
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<tr>
<th>Serogroup</th>
<th>Serovar</th>
<th>Strain</th>
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<td>Salinem</td>
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<td>-</td>
<td>Licerasiae</td>
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3.2.4 Polymerase chain reaction using conventional PCR

The DNA was amplified by using the following thermal-cycling profile: 95°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute, and a final extension for 7 minutes at 72°C. DNA sequencing was performed using the Big Dye Terminator (BDT) sequencing kit version 3.1 (Applied Biosystems) using the original primer as described previously (Thaipadungpanit et al., 2007). The PCR products were confirmed by agarose gel electrophoresis (1.5%w/v) of 5µL PCR products for 45 minutes at 116V.

3.2.5 DNA sequencing

The cycle-sequencing products were purified by using sodium acetate/alcohol precipitation as per the manufacturer’s instructions (Applied Biosystems) and the purified products were forwarded to the First Base Laboratories Sdn Bhd, DNA sequencing facility, Selangor, Malaysia, for capillary electrophoresis using an ABI 3130XL instrument.

3.2.6 Analysis of sequence results

The sequences were assembled and trimmed to a minimum of two contiguous sequences using Bio Edit software (Invitrogen). Sequences from strain Lepto 175 Sarawak and representative 16S rRNA (1331 bp) gene sequences from members of the genus Leptospira were aligned with CLUSTAL W (Thompson et al., 1997). By using MEGA 5 (Tamura et al., 2011), distances of aligned 16S rRNA gene sequences were estimated by the Jukes–Cantor method (Jukes and Cantor, 1969),
bootstrapped 1000 times and the tree topology was determined by the neighbour-joining method (Slack et al., 2008; Slack et al., 2009; Slack et al., 2006). The final phylogenetic tree was rooted by using *Turneriella parva* serovar Parva strain H as an out-group and bootstrap values (1000) were displayed as percentages (Thaipadungpanit et al., 2007).

### 3.3 Results

#### 3.3.1 Morphological Features of *Leptospira Lepto 175 Sarawak*

The bacterial cells were thin, finely coiled and actively motile. The size of the cells ranged from 10–13 µm in length and 0.2 µm in diameter. Under low power magnification (x200) cells appeared as a small piece of thread with hooked ends and were visible as dense, dot like structures (Figure 3.1).

![Image of Leptospira Lepto 175 Sarawak under dark field microscopy (x200).](image)

**Figure 3.1:** *Leptospira, Lepto 175 Sarawak* under dark field microscopy (x200).

#### 3.3.2 Serological identification of *Leptospira Lepto 175 Sarawak*

When strain Lepto 175 Sarawak was tested against hyper-immune antiserum representing the major Leptospiral serogroups, there was no significant titre to any
leptospiral group (Table 3.2). This suggests that strain Lepto 175 Sarawak is serologically distinctive to other leptospires maintained by the reference laboratory.

Table 3.2: Results of serological groups tested against Lepto 175 Sarawak.

<table>
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<tr>
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<tr>
<td>Sejroe</td>
<td>Saxkoebing</td>
<td>Mus 24</td>
<td>6400</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Semarang</td>
<td>Patoc</td>
<td>Patoc 1</td>
<td>800</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Wolffii</td>
<td>Khorat</td>
<td>Khorat-H2</td>
<td>800</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Inadai spp.</td>
<td></td>
<td></td>
<td>1600</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Serogroup</td>
<td>Serovar</td>
<td>Strain</td>
<td>A/S titre</td>
<td>Lepto 175</td>
</tr>
<tr>
<td>-----------</td>
<td>---------</td>
<td>------------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Ranarum</td>
<td>Ranarum</td>
<td>ICF</td>
<td>1600</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Louisiana</td>
<td>Louisiana</td>
<td>LSU 1945</td>
<td>3200</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Manhao</td>
<td>Lichuan</td>
<td>Manhao 4</td>
<td>3200</td>
<td>&lt;50</td>
</tr>
<tr>
<td></td>
<td>Fanei</td>
<td>Hurstbridge</td>
<td>1600</td>
<td>&lt;50</td>
</tr>
<tr>
<td></td>
<td>Bejo</td>
<td>ISO 9</td>
<td>1600</td>
<td>&lt;50</td>
</tr>
<tr>
<td></td>
<td>Licerasiae</td>
<td>VAR 10</td>
<td>800</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

3.3.3 Molecular characterization of *Leptospira*, Lepto 175 Sarawak

The 16S rRNA gene sequence similarity between strain Lepto 175 Sarawak and the 21 previously described species in the genus *Leptospira* was in the range of 87.19-99.10% (Figure 3.2; Table 3.3). *Leptospira licerasiae*, *Leptospira fainei* and *Leptospira wolffii* sv. Khorat strain Khorat-H2 (99.1 %) showed the highest levels of 16S rRNA gene sequence similarity to strain Lepto 175 Sarawak. Phylogenetic analysis using 16S rRNA gene sequences showed that strain Lepto 175 Sarawak was placed within the radiation of the genus *Leptospira* and was found to cluster with the intermediate *Leptospira* species.
Figure 3.2: The phylogenetic tree of Lepto 175 Sarawak with other strain 16s rRNA partial sequences.

A - Pathogenic strains; B - Intermediate strains; C- Non-Pathogenic strains; D - Outgroup

3.4 Discussion

Strain Lepto 175 Sarawak was identified as an intermediate strain of *Leptospira*. This means that it is considered to be generally non-lethal, however it could be potentially fatal under suitable environmental and host situations. Thus it could be placed in the same group as *Leptospira wolffii*, a strain recently isolated from the urine of a patient from Nakhon Rachasima, Thailand (Slack et al., 2008) and from humans, sheep, buffalo, cattle, rodents and dogs from Iran and India (Balamurugan et al., 2013; Zakeri et al., 2010). Intermediate strains of leptospires present a new dimension in the fight against this disease. They have the possibility of becoming
pandemic and causing mortality in victims if not properly treated. Since the majority of patients infected with intermediate strains are likely to make a full recovery, scientists may overlook the public health significance of these strains (Balamurugan et al., 2013; Zakeri et al., 2010). However, any new strains of *Leptospira* that may potentially result in fatalities should be taken seriously and efforts made to minimize the risk of exposure and improve the level of medical treatment. By studying intermediate leptospiral strains in detail, the scientific community may gain more insight into how they should tackle these bacterial strains. It is imperative that Lepto 175 Sarawak and similar strains be studied in greater detail so as to combat this potentially new threat.

So far, several known serovars of *Leptospira*, notably *L. interrogans* Icterohaemorrhagiae have been shown to be highly lethal (Viriyakosol et al., 2006). A handful of others are intermediate and saprophytic and fatalities attributed to them are uncommon. Treatments for the different categories of leptospires already exist and these are administered according to the magnitude of infection (Sehgal et al., 2000). Newly discovered intermediate strains of *Leptospira* thus pose a challenge in the treatment of this illness. As fatalities may arise in patients infected with intermediate strains, administering the correct treatment is important.
**Table 3.3: Pair wise distance and sequence similarities of *Leptospira* strains against Lepto 175 Sarawak.**

<table>
<thead>
<tr>
<th>Leptospiral strains</th>
<th>Pair wise distance</th>
<th>Sequence similarities (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. wolffii</em> sv Khorat Khorat-H2</td>
<td>0.009</td>
<td>99.10</td>
</tr>
<tr>
<td><em>L. lecanasiae</em> sv Varillal VAR010</td>
<td>0.013</td>
<td>98.74</td>
</tr>
<tr>
<td><em>L. broumii</em> 5399</td>
<td>0.021</td>
<td>97.85</td>
</tr>
<tr>
<td><em>L. fainei</em> sv Hurstbridge BUT6</td>
<td>0.022</td>
<td>97.76</td>
</tr>
<tr>
<td><em>L. inadai</em></td>
<td>0.036</td>
<td>96.41</td>
</tr>
<tr>
<td><em>L. kmetyi</em> sv Malaysia.</td>
<td>0.046</td>
<td>95.43</td>
</tr>
<tr>
<td><em>L. borgpetersenii</em> str Veldrat</td>
<td>0.047</td>
<td>95.34</td>
</tr>
<tr>
<td><em>L. santarosai</em> sv Shermani LT</td>
<td>0.047</td>
<td>95.34</td>
</tr>
<tr>
<td><em>L. noguchii</em> sv Panama CZ 214</td>
<td>0.047</td>
<td>95.25</td>
</tr>
<tr>
<td><em>L. alexanderi</em> sv Nanding_str M</td>
<td>0.048</td>
<td>95.16</td>
</tr>
<tr>
<td><em>L. interrogans</em> str RGA</td>
<td>0.048</td>
<td>95.16</td>
</tr>
<tr>
<td><em>L. kirschneri</em></td>
<td>0.048</td>
<td>95.16</td>
</tr>
<tr>
<td><em>L. santarosai</em></td>
<td>0.048</td>
<td>95.16</td>
</tr>
<tr>
<td><em>L. alexanderi</em> sv Manha3L60</td>
<td>0.048</td>
<td>95.16</td>
</tr>
<tr>
<td><em>L. interrogans</em></td>
<td>0.048</td>
<td>95.16</td>
</tr>
<tr>
<td><em>L. sv. Sichuan</em> str 79601</td>
<td>0.050</td>
<td>94.98</td>
</tr>
<tr>
<td><em>L. noguchii</em> Fort Bragg</td>
<td>0.050</td>
<td>94.98</td>
</tr>
<tr>
<td><em>L. weilii</em> Sarmin</td>
<td>0.052</td>
<td>94.80</td>
</tr>
<tr>
<td><em>L. biflexa</em> Patoc I</td>
<td>0.126</td>
<td>87.37</td>
</tr>
<tr>
<td><em>L. wolbachii</em> sv Codice CDC</td>
<td>0.126</td>
<td>87.37</td>
</tr>
<tr>
<td><em>L. meyeri</em> sv Ranarum Iowa City</td>
<td>0.128</td>
<td>87.19</td>
</tr>
<tr>
<td><em>T. parva</em> sv Parva H</td>
<td>0.195</td>
<td>80.48</td>
</tr>
</tbody>
</table>

Medical practitioners should be more alert if a patient presents with febrile symptoms. Probing questions should be asked, especially if the patient is a returned traveller from the tropics as early diagnosis of leptospirosis is important to prevent the disease becoming established in the patients home country (Ricaldi and Vinetz, 2006).
3.5 Description of *Leptospira*, Lepto 175 Sarawak sp. nov.

The morphology and motility under dark-field microscopy of the new strain were consistent with those for the genus *Leptospira*. Cells were 10–13 µm in length and 0.2 µm in diameter, with a wavelength of 0.5 um and amplitude of approximately 0.2 µm. No serologically titres were produced against the currently recognised members of *Leptospira*. Phylogenetically the isolate was placed within the radiation of the genus *Leptospira* (based on 16S rRNA gene sequence analysis).

Identifying host animals and reservoirs for intermediate leptospiral strains, such as *L. Lepto 175* Sarawak, will help in containing the disease it may induce. Understanding the animal reservoirs for serovars and strains is critical in controlling leptospirosis in both the human and animal (domestic and non-domestic) community (Nascimento et al., 2004). In the following chapter the results of a serological study in non-human primates from Sarawak are described.
CHAPTER 4: LEPTOSPIRAL AGGLUTININS IN CAPTIVE AND FREE RANGING NON-HUMAN PRIMATES IN SARAWAK, MALAYSIA

4.1 Introduction

The role of wildlife as reservoirs of leptospirosis has been widely documented from many countries; however it is still poorly understood (Matthias et al., 2005; Smythe et al., 2002a; Tulsiani, 2010; Tulsiani et al., 2011). The disease can result in economic losses in domesticated animals and has the potential to be an important zoonotic disease of humans (Russ et al., 2003), as well as an infectious agent for non-human primate species, which represent potential reservoir hosts. Leptospires were first isolated from rats in 1917 in Malaysia and it is widely acknowledged that rats are a key source of infection for humans (Levett, 2001). However, recently Australian and Peruvian researchers have reported that bats can also carry pathogenic *Leptospira* (Cox et al., 2005; Matthias et al., 2005; Smythe et al., 2002a), although their role as carriers is not fully understood. Other wildlife, such as primates, wild boar, and deer, also can act as potential carriers of these pathogens (Bengis et al., 2004; Cantu et al., 2008; Cole and Landres, 1995; Diesch et al., 1970; Hathaway et al., 1981; Kilbourn et al., 2003). However to date there has been little research conducted on free ranging wildlife. Due to heavy deforestation and human involvement in the jungles of Malaysia, there is the potential for exposure of humans to leptospires from wildlife and the opportunity to be exposed to yet to be recognised leptospiral serovars. This is particularly relevant to tourists in Malaysia with 24.7 million tourists visiting the country annually, many of whom are keen to
see native wildlife (Jayaraman et al., 2010). Leptospirosis in wildlife can have negative consequences on biodiversity, human and livestock health, animal welfare and the national economy (Russ et al., 2003). At present Malaysian surveillance of disease in wildlife is poorly coordinated and emerging zoonotic infectious diseases represent a growing threat. Ecotourism is a major industry in Malaysia, especially in the state of Sarawak (Chin et al., 2000; Musa, 2000; Yasak, 2000; Yea, 2002); however its impact on wildlife and the local people is controversial. Ecotourism can have a direct effect on wildlife species, communities and populations by influencing their feeding, and reproductive and social behaviours, as well as indirect effects through loss of vegetation, pollution, intra/inter-specific competition, close contact with humans, and introduction of disease (Cole and Landres, 1995). Knowledge on the leptospires naturally infecting wildlife is limited, although experimental infections in captive wild animals have resulted in clinical signs ranging from inapparent infections to febrile responses, abortions and deaths (Gulland et al., 1996; Twigg et al., 1969).

The research reported in this study arose from the need to investigate the role of wildlife in outbreaks of leptospirosis in humans (Thayaparan et al., 2013b) in Sarawak. Previous studies have mainly focused on rodents and bats as carrier of leptospires, as well as the effects on domestic animals and livestock. Few detailed investigations on primates have been performed and few have concentrated on animals from south-east Asia (Lilenbaum et al., 2002; McClure et al., 1986; Minette, 1966; Perolat et al., 1992; Pinna et al., 2012; Stasilevich et al., 2000). The study outlined in this chapter was designed to evaluate the seroprevalence to *Leptospira* in non-human primates in Sarawak.
4.2 Materials and methods

4.2.1 Study Area

Trapping of non-human primates was carried out around the Bako National Park and the Matang Wildlife Centre.

4.2.1.1 Bako National Park

Bako National Park is located 37 km from Kuching, Sarawak, East Malaysia (Figure 4.1). It is Sarawak’s oldest national park, covering an area of 2,727 hectares and is located at the tip of the Muara Tebas Peninsula (Chin et al., 2000; Ngui, 1991). It is one of the smallest national parks in Sarawak, yet one of the most interesting, as it contains almost every type of vegetation found in Borneo. The well-maintained network of nature trails, from easy forest strolls to full-day jungle treks, allows visitors to get the most out of this unique environment. Long-tailed macaques (*Macaca fascicularis*), silvered langur (*Trachypithecus cristatus/Presbytis cristata*), proboscis monkey (*Nasalis larvatus*), common water monitors lizards (*Varanus salvator*), plantain squirrels (*Collosciurus notatus*), wild boar (*Sus scrofa*) and mouse deer (*Tragulus kanchil*) are commonly found around the reserve. The major attraction of this place is the proboscis monkey and many visitors visit this park solely to view this species in their natural setting (Chin et al., 2000).

4.2.1.2 Matang Wildlife Centre

The Matang Wildlife Centre is situated at the western corner of the Kubah National Park in Sarawak (Ngui, 1991), East Malaysia, and covers 180 hectares of lowland forest (Figure 4.2). It is dedicated to education, research, conservation and
recreational activities (Ngui, 1991). In this park there are several species of confiscated wildlife for public viewing and research is also conducted by local and international scientists. There are long tailed macaques, pig/short-tailed macaques (Macaca nemestrina), Bornean gibbons (Hylobates muelleri), orangutans (Pongo pygmaeus) and sun bears (Helarctos malayanus) housed for various purposes.
Figure 4.1: Location of Bako National Park.
Figure 4.2: Location of Matang Wildlife Centre
4.2.2 Sampling procedure

In this study eight captive primates (three pig/short-tailed macaques, two Bornean Gibbons, a long-tailed macaque and two orangutans) and four free ranging primates (one silvered langur and three proboscis monkeys) were tested for the presence of leptospiral antibodies. The use of wildlife was approved by the Murdoch University Animal Ethics Committee (Animal ethics No: W2376/10) and Sarawak Forestry Department, Sarawak Malaysia (Permit No: NCCD.907.4.4 (V)-235). Free-range animals were first tracked down and tranquillised with Zoletil (5 mg/kg; 100 mg/mL) whilst captive animals were sedated with Zoletil (5 mg/kg). Five ml of blood was collected from the saphenous vein in a plain tube from each animal. The tubes were maintained at room temperature for 15 minutes and then centrifuged at 15,000 RPM for 5 minutes. The serum was then separated and stored at -20°C until it was analysed.

Figure 4.3: Silvered langur and proboscis monkey being prepared for blood collection.
4.2.3 Microscopic Agglutination Test (MAT)

The MAT was performed at the IMR according to the methods of Faine and Organization, (1982) to check for *Leptospira*-specific antibodies to 17 serovars endemic in Malaysia (Table 4.1).

4.2.3.1 Preparation of leptospiral antigens

Subcultures of the 17 serovars were prepared by inoculating 200-300 µL of well-grown culture from previous subcultures maintained by the IMR into fresh EMJH media. The serovars were subcultured in triplicate and incubated at 30°C for 5 days prior to testing. During incubation the cultures were checked daily for growth and for the presence of contamination. Once there was confluent growth, the cultures were used in the antigen panel.

4.2.3.2 Dilution of sample sera

64 µL of serum from sampled animals was mixed with 1536 µL of phosphate buffered saline (PBS) to make a 1:25 dilution.

4.2.3.3 MAT screening procedure

Two micro-titre plates were used to screen 11 serum samples. These plates were labelled with the respective antigens in the columns and the serum reference number in the rows. The details of the screening procedure were:
1. 50 µL of PBS was added to wells in column 1.

2. 50 µL of the 1:25 diluted serum of each animal sample was added to the respective column.

3. 50µL of leptospiral cultures were then added to the wells in the respective rows as labelled.

4. The plate was then shaken for 1 minute.

5. The plate was covered and incubated at 30°C for 2 hours.

6. After the incubation a loopful of the suspension was transferred via a wire loop to each well of a slide and the density of the leptospires observed under dark field microscopy at x100 magnification. The agglutination in wells containing the animal’s sera were compared with the controls in column 1.

7. Positive agglutination was considered when the approximate number of free leptospires was ≤ 50% of the number in the control wells.

8. Results were recorded on the result sheet.

9. For positive sera, full titration of the sera was performed and agglutination was observed with the 17 specific serovars common to Malaysia as reported below.

4.2.3.4 MAT full titration

1. The microtitre plates were labelled.

2. 50 µL of PBS were added to wells in columns 1, and 3 until 12 for the relevant rows.

3. 50 µL of the 1:25 diluted sera were added to wells 2 and 3 according to their respective rows.
4. The sera from wells 3 to 12 were titrated using a multi-channelled pipette. The sera were mixed with PBS in well 3 and then 50 µL of this mixture was transferred into well 4 and the procedure repeated until well 12.

5. 50 µL of the antigen was added into all of the wells according to the respective rows.

6. The plate was incubated at 30°C for 2 hours.

7. Agglutination was detected by observing free leptospires in each well and this was compared with the control wells. Positive agglutination was considered when the approximate number of free leptospires was <50% of the number in control wells.

8. The titre for each sample was recorded for each serovar as the last dilution that showed <50% of free leptospires compared to the control wells. Sera were considered to be positive if the titre was ≥ 1:100 by MAT.
Table 4.1: Serovars of Leptospira commonly found in Malaysia and used as antigens in the MAT panel.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Serovar</th>
<th>Reference Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australis</td>
<td>Australis</td>
<td>Ballico</td>
</tr>
<tr>
<td>Autumnalis</td>
<td>Autumnalis</td>
<td>Akiyami A</td>
</tr>
<tr>
<td>Bataviae</td>
<td>Bataviae</td>
<td>Swart</td>
</tr>
<tr>
<td>Canicola</td>
<td>Canicola</td>
<td>Hond Utrecht IV</td>
</tr>
<tr>
<td></td>
<td>Icterohaemorrhagiae</td>
<td></td>
</tr>
<tr>
<td>Celledoni</td>
<td>Celledoni</td>
<td>Celledoni</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>Grippotyphosa</td>
<td>Moskva V</td>
</tr>
<tr>
<td>Javanica</td>
<td>Javanica</td>
<td>Veldrat batavia 46</td>
</tr>
<tr>
<td>Pomona</td>
<td>Pomona</td>
<td>Pomona</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>Pyrogenes</td>
<td>Salinem</td>
</tr>
<tr>
<td>Sejroe</td>
<td>Hardjo</td>
<td>Hardjoprajitno</td>
</tr>
<tr>
<td>Semaranga</td>
<td>Patoc</td>
<td>Patoc 1</td>
</tr>
<tr>
<td>Djasiman</td>
<td>Djasiman</td>
<td>Djasiman</td>
</tr>
<tr>
<td>-</td>
<td>Lai</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>Copenhageni</td>
<td>Hebdomadis</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>Lepto175 Sarawak</td>
</tr>
</tbody>
</table>

4.3 Results

Antibodies to leptospires were detected in 8 (66.6%; 95% CI 34.9-90.1) of the 12 animals sampled (Table 4.2). All tested primates were negative to all serovars tested except Lepto 175 Sarawak and L. interrogans Lai. Of the eight captive non-human primates six (three short-tailed macaque, two Müller's Bornean gibbon and one orangutan) were seropositive (75%; 95% CI 34.9-96.8). Two of the four free ranging non-human primates (one proboscis monkey and one silvered langur) also were seropositive (50%, 95% CI 6.8-93.2).
Table 4.2: MAT results from the 12 positive non-human primates for *L. Lepto* 175 Sarawak and *L. interrogans* Lai.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Species</th>
<th>Location</th>
<th><em>L. Lepto 175</em> Sarawak</th>
<th><em>L. interrogans</em> Lai</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-tailed macaque</td>
<td><em>Macaca nemestrina</em></td>
<td>Matang (C)</td>
<td>800</td>
<td>400</td>
</tr>
<tr>
<td>Short-tailed macaque</td>
<td><em>Macaca nemestrina</em></td>
<td>Matang (C)</td>
<td>400</td>
<td>200</td>
</tr>
<tr>
<td>Short-tailed macaque</td>
<td><em>Macaca nemestrina</em></td>
<td>Matang (C)</td>
<td>400</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Proboscis monkey</td>
<td><em>Nasalis larvatus</em></td>
<td>Bako (F)</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Proboscis monkey</td>
<td><em>Nasalis larvatus</em></td>
<td>Bako (F)</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Proboscis monkey</td>
<td><em>Nasalis larvatus</em></td>
<td>Bako (F)</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>Müller's Bornean gibbon</td>
<td><em>Hylobates muelleri</em></td>
<td>Matang (C)</td>
<td>400</td>
<td>200</td>
</tr>
<tr>
<td>Müller's Bornean gibbon</td>
<td><em>Hylobates muelleri</em></td>
<td>Matang (C)</td>
<td>200</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Orangutan</td>
<td><em>Pongo pygmaeus</em></td>
<td>Matang (C)</td>
<td>200</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Orangutan</td>
<td><em>Pongo pygmaeus</em></td>
<td>Matang (C)</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Long-tailed macaque</td>
<td><em>Macaca fascicularis</em></td>
<td>Matang (C)</td>
<td>&lt;50</td>
<td>&lt;50</td>
</tr>
<tr>
<td>Silvered langur</td>
<td><em>Trachypithecus cristatus</em></td>
<td>Bako (F)</td>
<td>400</td>
<td>&lt;50</td>
</tr>
</tbody>
</table>

(C) – Captive animal; (F) – Free ranging animals
The antibody titres for seropositive animals varied from 1:100 to 1:800. One of the sera from a captive short-tailed macaque had a titre of 1:800 to serovar *Leptospira* Lepto 175 Sarawak (Table 4.2). Two thirds of the primates were positive (n = 8) for serovar Lepto 175 Sarawak and one third (n = 4) for Lai (Table 4.3). Four primates were positive for both serovars Lai and Lepto 175 Sarawak (Table 4.2).

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Number positive (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lai</td>
<td>4 (33.3%; 9.9-65.1)</td>
<td></td>
</tr>
<tr>
<td>Lepto 175 Sarawak</td>
<td>8 (67.6%; 34.9-90.1)</td>
<td></td>
</tr>
</tbody>
</table>

**4.4 Discussion**

Antibodies to leptospires were detected in captive primates from the Matang Wildlife Centre. These infections may have arisen from poor sanitation or inadequate rodent control and adopting a program to improve these may result in reducing the spread of leptospires to other animals, including humans. In this study the highest antibody titre (1:800) was detected against Lepto 175 Sarawak in a short-tailed macaque. This single high antibody titre may indicate active infection (Kilbourn et al., 2003), but assessment of its significance requires testing of serial samples which was not possible in the current study.

Szonyi et al., (2011) described an outbreak of leptospirosis in Capuchin (*Cebus capucinus*) monkeys and advised of the risk of disease from these animals. According to the EAZWV transmissible Disease Fact Sheet (Hartskeerl and Ellis,
2003) the Macaca spp., Cercopithecus aethiops (Green monkey), Pongo pygmaeus, Saimiri sciureus (common squirrel monkey), Saguinus oedipus (Tamarins) and Galago senegalensis (Bush babies) are susceptible to infection with serovars of *L. interrogans*. In primates leptospirosis is mostly asymptomatic; however in fatal cases anaemia, facial oedema, vomiting, jaundice, weakness, lethargy and fever may occur (Hartskeerl and Ellis, 2003; Kessler and Everard, 1988). Palmer et al., (1987) experimentally challenged Grivet/Green monkeys with the Hardjo serovar and found these animals to be a suitable model for infection in humans.

In this study a positive titre in a proboscis monkey was possibly first published report in the world. Information on Lepto 175 Sarawak is deficient, although it would appear to be endemic in Sarawak. As reported from the results of the previous chapter, Lepto 175 Sarawak has close genetic similarities with *Leptospira wolffii*. This latter species has been isolated from humans and wildlife species, including non-human primates, from Thailand, India, Iran and Sabah (Balamurugan et al., 2013; Kilbourn et al., 2003; Slack et al., 2008; Zakeri et al., 2010). Although the pathogenicity status of Lepto 175 Sarawak is not yet confirmed, it is important that the public health authorities are aware of these preliminary seroprevalence results and the potential for an outbreak in humans visiting Bako National Park should be evaluated and monitored. In contrast it is known that the serovar Lai is pathogenic and is a major cause of zoonotic spread to humans involved in adventure activities (Sejvar et al., 2003). Studies by Kilbourn et al., (2003) on free-ranging orangutans in Sabah found a high prevalence of antibodies against *L. interrogans* serovars, notably Grippotyphosa and Autumnalis, as well as the novel species *Leptospira wolffii*. Experimental infection of marmoset monkeys (*Callithrix jacchus*) with serovar Copenhageni resulted in histological tissue reactions including intra-alveolar
haemorrhage (Pereira et al., 2005). Most animals had microscopic changes to the kidneys when they were euthanased 6 and 12 days after challenge.

According to the results of previous researchers, natural infection of monkeys by leptospires is an unusual event, however it has been observed in serological surveys or under exceptional conditions in captivity (Pereira et al., 2005). Baulu et al., (1987) studied Vervet monkeys (*Chlorocebus pygerythrus*) in Barbados and found that they retained naturally acquired antibodies to *Leptospira* for at least 2.5 years. The primates were transmitting the organism between themselves independently of other animals and were not a major source of infection for humans. Kessler and Everard, (1988) surveyed free-ranging Rhesus monkeys (*Macaca mulatta*) in Puerto Rico and found evidence of seropositivity. However these primates were apparently healthy, despite their contact with rats and ingestion of stagnant water. Lilenbaum et al., (2002) found anti-*Leptospira* agglutinins in lion tamarins (*Leontopithecus rosalia*) in Rio de Janeiro, Brazil, even though these animals showed no clinical signs or prior history of disease. Pinna et al., (2012) suggested that captive New World monkeys should be screened for leptospirosis before they are released into the wild, as there is a danger they may have been exposed to serovars originating from urban areas prior to release. Following the death of an endangered Colobus monkey (*Colobus guereza*) at the National Zoo in Washington DC (Hutchins, 2006), William Foster of the American Zoo and Aquarium Association reported that leptospirosis was not often diagnosed in zoo animals. He advocated tougher pest-control policies to tackle the rodents that carry the bacterium. Romero et al., (2011b) also reported on a potential risk of transmission of leptospires from zoo animals to staff in Zoo Colombia.
This study has found evidence of exposure of langur leaf and proboscis monkeys to leptospires and there is an urgent need to undertake more investigations on free ranging non-human primates in Malaysia. Macaques are scavengers that search for food in garbage heaps, which mean they may ingest food possibly contaminated by rodent urine or have direct contact with it through damaged skin. This results in leptospires entering their bodies and infecting them, potentially resulting in them becoming maintenance hosts for the bacteria. When the macaques urinate onto the ground, there is danger that leptospires shed could be transferred to silvered leaf langur and proboscis monkeys through contact with the organisms in the environment. Besides contact with rats, ingestion of stagnant water contaminated with leptospires could be a potential source of infection for these non-human primates. The latter possibility was highlighted by Famatiga, (1973) who observed silvered langur and proboscis monkeys drinking from freshwater streams and rivers. These water bodies are known to be suitable habitats for leptospires and when these two predominantly arboreal primate species drink from such places, they may become infected. Proboscis monkeys are also competent swimmers (Jalil, 2009) and are known to have the most aquatic lifestyle of primates. They usually live near water bodies, rarely ranging more than one kilometre from it. Such activities would increase the opportunity for exposure to leptospires.

In Bako National Park several short and long-tailed macaques and wild boars roam freely eating leftover food and garbage (Personal observation). Based on the previous discussions it is possible that these two species of macaque may transmit leptospires to proboscis and silvered langur, as well as to the many tourists who visit this national park. Humans are also at risk through contact with contaminated soil or water. It is important to educate visitors to national parks not to leave food out
which may attract rodents or other scavengers. Additionally, animals in rehabilitation centres should be thoroughly screened for leptospirosis prior to their release to the natural environment to prevent them from spreading the pathogen on release. The results of this study are important as they indicate that macaques possibly form a key node in the chain of leptospiral transmission.

4.5 Conclusions

Although leptospires are endemic in Sarawak this is the first reported evidence of the organisms in non-human primates with the proboscis monkey having the highest seroprevalence. As mentioned previously, rodent control is a vital step in containing and controlling leptospirosis. Litter prevention in and around national parks is an important component of this to reduce the likelihood of macaques getting infected and passing on the leptospires to humans or other animals. The possibility of non-human primates acting as reservoir hosts for leptospires requires further investigation.

Other mammals, including bats, play a key role in the distribution of Leptospira. Infection of native mammals is potentially deleterious to the animals themselves but also to the fledgling nature tourism industry in Sarawak, as these animals may inadvertently result in infection of foreign or domestic visitors. In the following chapter the results of a serological survey of other wildlife (small mammals) in Sarawak are reported.
CHAPTER 5: SEROLOGICAL AND MOLECULAR STUDY OF *LEPTOSPIRA* IN SMALL WILDLIFE AROUND WILDLIFE RESERVES AND DISTURBED FOREST REGIONS IN KUCHING, SARAWAK, MALAYSIA.

5.1 Introduction

Leptospirosis is endemic to tropical regions of the world and is re-emerging as a new danger to public health in Southeast Asia, including Malaysia. Although studies have been conducted in Malaysia for more than 70 years (Hanson, 1982), leptospirosis and the threat it poses is still not well understood. Malaysian jungles are home to numerous species of wildlife and peri-domestic animals. These include bats, squirrels and rats, as well as primates. Bats and rats are known to harbour leptospiral serovars and pass them to other species including humans (Faine et al., 1999; Matthias et al., 2005; Richardson and Gauthier, 2003; Roth, 1964; Vashi et al., 2010).

With deforestation becoming more commonplace in many tropical environments, including Malaysia, there is increased likelihood of contact between humans and wildlife due to the disturbance of natural habitats. Bats are known to respond to habitat modification, loss and fragmentation at the population level, so their spatial and temporal dynamics are particularly sensitive to anthropogenic activities. They
forage in fruit orchards and forest clearings, and roost in buildings, water cisterns, culverts, abandoned structures and bridges (Matthias et al., 2005). This often results in humans coming into contact with bat urine or surfaces contaminated with it. Also, ground-dwelling species, such as rodents or marsupials, that reside or forage under bat roosts, could encounter *Leptospira*-contaminated urine (Zarnke, 1983).

Sarawak has seen an increase in human cases of leptospirosis in recent years. As summarised in Chapter 2, leptospirosis is suspected to have made a Chinese national working at Bakun Dam seriously ill, and contamination by rat urine was believed to have been the source of infection in Sibu city in June 2012. The increasing popularity of eco-tourism raises another risk for the disease. Such tourism brings visitors to Malaysia close to nature and native wildlife, some of which may be reservoir hosts for leptospirosis, as well as potentially interfering with an already-fragile ecosystem.

### 5.1.1 Objectives

The objectives of the study reported in this chapter were to:

a. Determine the role of bats, rats, squirrels and treeshrews in the lifecycle of *Leptospira* in and around wildlife reserves and disturbed forest areas in Sarawak.

b. Determine the seroprevalence to *Leptospira* in these species

c. Determine the carrier stage of animals by performing culture and molecular studies.
5.1.2 Rats and leptospirosis

Rats have been shown to be carriers of leptospires throughout the world and are important reservoirs of infection for animals and humans (Priya et al., 2007; Roth, 1964). Sarawak, East Malaysia is a tropical region with diverse animal species including 61 different species of rats and mice (Santa Rosa et al., 1975). The Müller’s rat (*Sundamys muelleri*), the rice field rat (*Rattus argentiventer*) and the brown spiny rat (*Maxomys rajah*) have a broad-based distribution between the border of forested areas and human settlements and many different leptospiral serovars have been isolated from these species in Sarawak and other countries (Smith et al., 1961). There has been a higher seroprevalence reported in rice field rats compared to other species of rats (Smith et al., 1961). The brown spiny rat is generally regarded as the maintenance host for leptospires of the *Icterohaemorrhagiae* serogroup and the species can be an occasional carrier of other serovars.

Researchers have indicated a striped field mouse (*Apodemus agrarius*), a forest vole (*Clethrionomys glareolus*), *Apodemus* spp. and common voles (*Microtus arvalis*) as the prevailing species infected by leptospires (Slavica et al., 2008; Treml et al., 2002). Treml et al., (2002) found striped field mice and forest voles in the Czech Republic and reported a seroprevalence of 20.6% to sv. *Grippotyphosa*. Similar findings in voles were described in the Netherlands by other researchers (Kuiken et al., 1991). Rodents have long been associated with leptospirosis as reservoir or maintenance hosts and verminous rodents are considered a key source for the distribution of leptospires in an urban setting (Emanuel et al., 1964; Faine et al., 1999; Hathaway et al., 1981; Lau et al., 2010a; Lau et al., 2010b; Mohan et al., 2009; Slack et al., 2006; Turner, 1970). The bacteria colonise the renal tubules of
rodents, who may then shed leptospires continuously or intermittently throughout their life (Farrar, 1995). Although leptospires are endemic in rodents, clinical disease is not reported and serovar-specificity is observable (Levett, 2001). Rodents may also present low titres to serovars of *Leptospira* that are not endemic to their species (Webster et al., 2009). The higher seroprevalence in rodents is also influenced by seasonal effects with a higher population of rats in the wet than in the dry season (Kasso et al., 2010). This population growth also coincides with outbreaks of leptospirosis in humans (Pappas et al., 2008).

5.1.3 Bats and leptospirosis

Bats have been implicated in the spread of leptospirosis to humans (Mortimer, 2005; Vashi et al., 2010). Mortimer, (2005) reported the case of an American caver who may have had contact with bat (and possibly rat) urine-contaminated water while carrying out a reconnaissance project at Gunung Buda, Sarawak.

Despite the negative reputation that bats pose to public health, these creatures play a major role in the earth’s fragile ecosystem. Bats are a critical component of Southeast Asia’s threatened fauna; they constitute approximately 30% of the region’s mammal species, and can comprise as many as half of all mammal species in tropical rainforest regions (Kingston et al., 2006). Moreover, the region is pivotal for international bat conservation as it supports nearly 30% of the world’s bat fauna with 330 species listed (Kingston, 2010; Simmons, 2005). Habitat loss is the main threat to the survival of bats, which provide essential ecosystem services as pollinators (Corlett, 2005; Whittaker and Jones, 1994) and seed dispersers to more than 300 paleotropical plant species (Kingston, 2010). It is believed that without
bats both the regeneration of the forest ecosystem (Cox et al., 1991; Gumal, 2001; Marshall, 1985; McConkey and Drake, 2006; Rainey et al., 1995) and the success of bat-dependent fruit crops will be severely compromised. Other bats feed on insects and are responsible for eliminating pests of rice crops, reducing the need for insecticides (Cleveland et al., 2006) and the consequent impacts on local biodiversity that result from their use. Large bat colonies are also a source of guano, which is widely used in Southeast Asia as an organic fertilizer (Kingston, 2010).

The purpose of this particular study was to determine the common leptospiral serovars present in small wild mammals living around wildlife reserves/disturbed forest habitats and human communities.

### 5.2 Materials and Methods

From January 2011 to March 2012 blood and kidneys were collected from bats, rodents and squirrels trapped in Sarawak and tested to determine exposure to leptospires.

#### 5.2.1 Study/sampling sites

The sampling of animals was undertaken in the wider range of Kuching, and Kota Samarahan area included Universiti Malaysia Sarawak (UNIMAS) area, Matang & Kuba National Park, Bako National Park, Wind and Fairy Cave National Reserves and Mount Singai Conservation Area (Figure 5.1). Localities were chosen in such a way that different environments were represented (disturbed jungle habitats around Kuching and secondary forest located around human settlements) to gain an accurate
picture of the possible transmission of leptospires in the particular localities. During the investigation 155 small mammals (rats, squirrels, treeshrews and bats) were sampled.

5.2.1.1 UNIMAS area (UNIMAS, Kampung Sebayor and Kampung Etingan)

Universiti Malaysia Sarawak (UNIMAS) is located in Kota Samarahan, approximately 30 kilometres east of the state capital Kuching (Figure 5.2). The university is surrounded by forested and swampy areas, providing an ideal environment for leptospires to thrive (www.unimas.my). The eastern (old) campus contains two residential colleges while the western (main) campus has five colleges. The campus grounds have a disturbed habitat or deforested area. Kampung (Village) Sebayor is located inside the campus and Kampung (Kg) Etingan is located close to UNIMAS campus.

The locations and descriptions of Bako National Park and Matang Wildlife Centre were outlined in Chapter 4.
Figure 5.1: Sampling sites of small wildlife.
Figure 5.2: UNIMAS and surroundings where samples were collected.
5.2.1.2 Wind and Fairy Caves

The Wind and Fairy caves are part of the Bau Formation, a narrow belt of limestone covering approximately 150 sq km in south-west Sarawak. Most caves in this network are remote and inaccessible; however Wind Cave is within easy reach of Kuching and is a popular day trip and picnic destination (www.sarawakforestry.com). The Wind Cave Nature Reserve covers 6.16 hectares and includes the cave itself and the surrounding forest. Squirrels, shrews and a variety of birds can be found along the river and the limestone hill (Ngui, 1991). Black nest swiftlets (*Aerodramus maximus*) can be seen and heard inside the cave, as well as 14 species of bat (Barapoi, 2004). Immediately to the south of Wind Cave lies Fairy Cave (Wong and Chung, 2001) (Figure 5.3).

5.2.1.3 Mount Singai Conservation Area

Mount Singai is located midway between the town of Bau and the Matang wildlife reserve (Figure 5.4). This 333.3 metre (1,000-ft) high mountain can be reached by a bitumen road via Batu Kawa, while its flat top can be accessed via the same jungle trail used by village people to travel to their lowland farms (Ngui, 1991; Wong and Chung, 2001). Once occupied by members of the Bisingai community, the mountain is now overgrown with secondary vegetation and in recent times has been occupied by the Catholic Memorial Pilgrimage Centre. This mountain holds significant socio-ecological value as well as attracting many devout Catholics. Twenty-two species of mammals have been recorded at Mount Singai, including 10 species of bats, seven species of rodents and four species of tree shrews (Ngui, 1991; Wong and Chung, 2001).
Figure 5.3: Location of Wind Cave and Fairy Cave.
Figure 5.4: Location of Mount Singai Conservation Area.
5.2.2 Animal capturing

The species sampled in this study included small mammals (rats, squirrels, tree shrew and bats) that were captured in the selected areas over a one-year period from January 2011 to March 2012. The small mammals were trapped around bat caves, forest areas of the national parks and disturbed habitats. Trapping of wildlife was approved by the Murdoch University Animal Ethics Committee (Animal ethics No: W2376/10) and the Sarawak Forestry Department, Sarawak Malaysia (Permit No: NCCD.907.4.4 (V)-235).

5.2.2.1 Rats, squirrels and treeshrews

In Malaysia wild rats are mainly nocturnal and occur in a variety of habitats including coastal forests (especially mangroves), secondary forests and grasslands and have also adapted to rubber and oil palm plantations. One hundred and two species of bats and 61 species of rodents have been identified in Borneo (Santa Rosa et al., 1975).

The rats, squirrels and treeshrews were trapped using cage traps (Figure 5.5) (Baeumler and Brunner, 1988). A maximum of 50 cage traps were set at each sampling time. A mixture of one-part raisins and two parts peanut butter with rolled oats, banana and dried fish was used as bait and placed in each trap (Hickie and Harrison, 1930; Wood, 1984). Traps were opened at 10 pm and checked the following morning. Any by-catch was immediately released at the site of trapping. If pregnant or lactating small mammals were captured these were also released immediately at the captured site. Any trapped rats, squirrels and tree shrews were collected and taken to the UNIMAS laboratory. The cage containing the trapped
mammal was covered with cloth and kept in the animal house until processing. Each small mammal was housed in individually ventilated cages. During transportation the animals were fed sunflower seeds, cereals, cooked corn kernels and cheese and provided with water ad libitum.

Figure 5.5: Wire mesh trap used for trapping rats, squirrels and treeshrews

5.2.2.2 Trapping and sampling of bats

All the bats were wild caught either using a mist or harp net. A mist net (Figures 5.6 and 5.7) is a lightweight net that was placed in the flight path of the bats; for example across trails, over streams, adjacent to fruiting trees and at the mouths of caves. Bats that were caught in the nets were carefully removed from the net to ensure their delicate wings were not injured. Harp traps (Figure 5.8) were also set across trails or over small streams. Harp traps are made of vertical sets of fine fishing line strung under tension in a frame. The bats are not able to see or detect
them, and consequently get caught between the sets of line and slide down into a bag beneath the frame. They then remained in the bag until they were removed. Three harp traps and five mist nets were placed along bat flight paths for a maximum of six hours between 5 pm and 11 pm. Nets were checked every 30 minutes and any bats caught were removed from the traps. After removal each bat was placed in an individual cloth bag, which was tied and placed in a wicker basket. The basket was covered with a cloth and placed in a safe quiet place to minimise disturbance. If any birds were trapped these were removed and released immediately.

Figure 5.6: A fruit bat caught in a mist net.

Figure 5.7: Illustrated diagram of the setup of a mist net.
(http://www.ilmb.gov.bc.ca/risc/pubs/tebiodiv/bats/batsml20-04.htm)
5.2.3 Collection and preservation of samples from animals

5.2.3.1 Rats, squirrels and treeshrews

The captured animals were anaesthetised using Ketamine + Xylazine (50–75 mg/kg + 10 mg/kg IP, respectively) (Karwacki et al., 2001). Once the animals were anaesthetised 1-3 ml of blood was collected via cardiac puncture using a 25 G needle and a 5 mL syringe. After blood collection, the rats were euthanased with barbiturate (sodium pentobarbitone at 150-200 mg/kg) via either an intracardiac or intravenous (IV) injection. After euthanasia the animals were necropsied and kidney samples collected (Figure 5.9). Blood samples were left at room temperature for 30 minutes to clot and were then centrifuged at 3,000 RPM for 1 minute. The serum was removed and stored at -20°C until testing in the laboratory.
Bats were anaesthetised to reduce the stress of handling and to minimise the risk of the handler being bitten (Figure 5.10). Each animal was anaesthetised with an intramuscular injection of ketamine (6–7 mg/Kg) and medetomidine (60–70 µg/Kg) (Plowright et al., 2008). A blood sample (1 to 3 mL) was collected by cardiac puncture from each bat using a 5 mL syringe and a 25-gauge needle. Prior to collection the hair was sterilized with cotton wool dipped in 70% alcohol. Data were collected on the age, gender, location and general health status of each sampled animal.

The bats were then placed in a fabric pouch and placed in a plastic airtight container. Cotton balls were thoroughly saturated with isoflurane in the barrel of a six ml syringe (after removing the plunger). The syringe barrel containing the isoflurane
saturated cotton balls were then placed into the container and the lid closed. Bats were usually anaesthetised within seconds and death was confirmed by auscultation with a stethoscope. Kidney samples were removed for culturing and molecular analysis. Carcasses were stored in 99% ethanol for future use by UNIMAS students. All personnel who handled bats had been vaccinated against rabies and all samples were treated as potentially infectious.

Figure 5.10: Examining a fruit bat prior to the collection of blood.

5.2.4 Culture and isolation of leptospirosis

Kidney samples from all animals were cultured for leptospires. A cross-section of the cortex of one kidney was removed using a sterile scalpel and this section was cut into small pieces and inoculated into two EMJH and two Fletcher media tubes for
culture. The remaining part was deposited in 100% pure PCR grade alcohol for future PCR work. The tubes with the inoculated material were incubated at 30°C and checked twice weekly under dark field microscopy for 12 weeks. If there was no growth after 12 weeks, or if any fungal growth was detected, the specimen was discarded.

5.2.5 Microscopic Agglutination Test (MAT)

All serum samples were tested against a panel of 17 live antigens in the Microscopic Agglutination Test (MAT). The protocol followed was that described in Chapter 4. In this study an agglutination titre $\geq 50$ was indicative of seropositivity to Leptospira.

5.2.6 Molecular analysis

5.2.6.1 Preparation of genomic DNA from kidney samples

Approximately 30 mg of kidney was placed into a sterile Petri dish. Using a sterile scalpel and a needle the tissue was dissected into small pieces. The tissue was then transferred into a micro centrifuge tube and mixed with 50 μL protein kinase K (Qiagen) and 200 μL lysis buffer and incubated at 55°C overnight or until the tissue was completely digested.

5.2.6.2 Analysis of PCR products

The methods for DNA isolation, sequencing and analysis of the PCR have been described in Chapter 3.
5.3 Results

5.3.1 Results of capturing small mammals (Rats, Squirrels, Tree shrew and Bats)

In total 155 animals were sampled including 70 bats, 57 rodents, 20 squirrels and eight tree shrew. The rats trapped were Müller’s rat (*Sundamys muelleri*) (n = 29), ricefield rat (*Rattus argentiventer*) (n = 20), brown spiny rat (*Maxomys rajah*) (n = 7), and Whitehead’s rat (*Maxomys whiteheadi*) (n = 1). All squirrels were either Low’s squirrels (*Sundasciurus lowii*) (n=1) or plantain squirrels (*Callosciurus notatus*) (n=19) and the tree shrew were *Tupaia tana* (n=8). The bats trapped were dusky fruit bat (*Penthetor lucasi*) (n = 20), short-nosed fruit bat (*Cynopterus brachyotis*) (n = 22), spotted-winged fruit bat (*Balionycteris maculata*) (n = 13), fawn roundleaf bat (*Hipposideros cervinus*) (n = 5), Bornean horseshoe bat (*Rhinolophus borneensis*) (n = 2), bicolored roundleaf bat (*Hipposideros bicolor*) (n = 2), Malaysian slit-faced bat (*Nycteris tragata*) (n = 1), intermediate horseshoe bat (*Rhinolophus affinis*) (n = 1), dusky roundleaf bat (*Hipposideros ater*) (n = 1), lesser woolly horseshoe bat (*Rhinolophus sedulous*) (n = 1), papillose woolly bat (*Kerivoula papillosa*) (n = 1) and dayak roundleaf bat (*Hipposideros dyacorum*) (n = 1). The largest number of animals was caught in the locality of UNIMAS (40) followed by Wind Cave and Fairy Cave areas (36), Bako National park (31), Matang and Kubah (30) and Mount Singai (18) (Tables 5.1 and 5.2).
Table 5.1: Species, location and MAT results for animals other than bats caught.

<table>
<thead>
<tr>
<th>Species</th>
<th>UNIMAS</th>
<th>Mt. Singai</th>
<th>Wind &amp; Fairy Caves</th>
<th>Matang &amp; Kubah</th>
<th>Bako</th>
<th>Total # trapped</th>
<th>%</th>
<th>MAT (+)</th>
<th>% Positive (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sundamys muelleri</td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>29</td>
<td>34.1</td>
<td>20</td>
<td>68.9 (42.9, 84.7)</td>
</tr>
<tr>
<td>Rattus argentiventer</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>20</td>
<td>23.5</td>
<td>9</td>
<td>45.0 (23.1, 68.5)</td>
</tr>
<tr>
<td>Maxomys rajah</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>7</td>
<td>8.2</td>
<td>4</td>
<td>57.1 (18.4, 90.1)</td>
</tr>
<tr>
<td>Maxomys whiteheadi</td>
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<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1.2</td>
<td>0</td>
<td>0.0 (0, 97.5)</td>
</tr>
<tr>
<td>Sundasciurus lowii</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1.2</td>
<td>1</td>
<td>100.0 (2.5, 100)</td>
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<tr>
<td>Callosciurus notatus</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>6</td>
<td>19</td>
<td>22.4</td>
<td>10</td>
<td>52.6 (28.9, 75.6)</td>
</tr>
<tr>
<td>Tupaia tana</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>8</td>
<td>9.4</td>
<td>1</td>
<td>12.5 (0.3, 52.7)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
<td><strong>13</strong></td>
<td><strong>17</strong></td>
<td><strong>16</strong></td>
<td><strong>19</strong></td>
<td><strong>85</strong></td>
<td><strong>100.0</strong></td>
<td><strong>45</strong></td>
<td><strong>52.9 (41.8, 63.9)</strong></td>
</tr>
<tr>
<td><strong>%</strong></td>
<td>23.5</td>
<td>15.3</td>
<td>20.0</td>
<td>18.8</td>
<td>22.4</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
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</table>
Table 5.2: Species, location and MAT results for bats caught.

<table>
<thead>
<tr>
<th>Species</th>
<th>UNIMAS</th>
<th>Mt. Singai</th>
<th>Wind &amp; Fairy Caves</th>
<th>Matang &amp; Kubah</th>
<th>Bako</th>
<th>Total trapped</th>
<th>%</th>
<th>MAT (+)</th>
<th>% Positive (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penthetor lucasi</em></td>
<td>5</td>
<td>2</td>
<td>11</td>
<td>0</td>
<td>2</td>
<td>20</td>
<td>28.5</td>
<td>12</td>
<td>60.0 (36.1, 80.9)</td>
</tr>
<tr>
<td><em>Cynopterus brachotis</em></td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>22</td>
<td>31.4</td>
<td>9</td>
<td>40.9 (20.7, 63.6)</td>
</tr>
<tr>
<td><em>Balionycteris maculate</em></td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>13</td>
<td>18.5</td>
<td>3</td>
<td>23.1 (5.0, 53.8)</td>
</tr>
<tr>
<td><em>Hipposideros cervinus</em></td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>5</td>
<td>7.1</td>
<td>2</td>
<td>40.0 (5.3, 85.3)</td>
</tr>
<tr>
<td>Other bat species</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>14.2</td>
<td>2</td>
<td>20.0 (2.5, 55.6)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
<td><strong>5</strong></td>
<td><strong>19</strong></td>
<td><strong>14</strong></td>
<td><strong>12</strong></td>
<td><strong>70</strong></td>
<td><strong>100.0</strong></td>
<td><strong>28</strong></td>
<td><strong>40.0 (28.5, 52.4)</strong></td>
</tr>
<tr>
<td>%</td>
<td>23.5</td>
<td>15.3</td>
<td>20.0</td>
<td>18.8</td>
<td>22.4</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3.2 Results of serological analysis of trapped rats, squirrels, treeshrews and bats

Antibodies to leptospires were detected in 73 of the 155 animals tested (seroprevalence of 47.0%; 95% CI 39.0-55.3%). The seroprevalence for rats (57.9%; 95% CI 44.1-70.9) was slightly higher than that for squirrels (42.9%; 95% CI 24.5-62.8) and bats (40%; 95% CI 28.5-52.4), however this difference was not significant ($\chi^2 = 4.28; df=2; P=0.117$).

The highest seroprevalence was found in Müller’s rats (68.9%; 95% CI 42.9-84.7) followed by the brown spiny rat (57.1%; 95% CI 18.4-90.1), plantain squirrels (52.6%; 95% CI 28.9-75.6) and ricefield rats (45%; 95% CI 23.1-68.5). The highest seroprevalence in bats was observed in the dusky fruit bat (60%; 95% CI 36.1-80.9), followed by the short-nosed fruit bat (40.9%; 95% CI 20.7-63.6) and spotted-winged fruit bat (23.1%; 95% CI 5.0-53.8) (Tables 5.1 and 5.2). There was no significant difference in the seroprevalence between the different animal species ($\chi^2 = 24.9; df=18; P=0.126$).

Some seropositive animals were detected in all of the localities sampled. The highest prevalence was found at Mount Singai (64.7%; 95% CI 38.3-85.8) and the lowest at the UNIMAS area (35%; 95% CI 20.6-51.7) (Table 5.3). There was no significant difference overall in the seroprevalence among the five sampling locations ($\chi^2 = 4.28; df=4; P=0.117$); however significantly more seropositive animals were detected at Mount Singai than at UNIMAS (OR 3.4; 95% CI 1.04, 11.17).

Antibodies to 10 different serovars were detected in rodents and six different serovars in bats. Antibodies to serovar Lepto 175 Sarawak were detected in 30 (24.7%) rats, 11 (9.0%) squirrels and 27 (52.9%) bats (Table 5.4). Antibodies to serovar Icterohaemorrhagiae were detected in 20 (16.5 %) rodents, squirrels and treeshrew;
serovar Australis in 13 (10.6 %) blood samples and serovar Autumnalis in 12 (9.6 %) blood samples (Tables 5.5 and 5.6). Serovars Australis and Lai were detected in one dusky fruit bat and one short-nosed fruit bat respectively. Antibodies to serovar Pyrogenes were detected in 10 (19.6%) samples from dusky fruit bats and short-nosed fruit bats.

Table 5.3: Seroprevalence to leptospires in small mammals trapped in different localities.

<table>
<thead>
<tr>
<th>Locality</th>
<th>No. of seropositive mammals</th>
<th>Seroprevalence (95% CI)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNIMAS</td>
<td>14</td>
<td>35.0 (20.6, 51.7)</td>
<td>1.0</td>
</tr>
<tr>
<td>Mt. Singai</td>
<td>11</td>
<td>64.7 (38.3, 85.8)</td>
<td>3.4 (1.04, 11.17)</td>
</tr>
<tr>
<td>Wind &amp; Fairy Caves</td>
<td>17</td>
<td>47.2 (30.4, 64.5)</td>
<td>1.7 (0.66, 4.18)</td>
</tr>
<tr>
<td>Matang &amp; Kubah</td>
<td>17</td>
<td>56.7 (37.4, 74.5)</td>
<td>2.4 (0.92, 6.42)</td>
</tr>
<tr>
<td>Bako NP</td>
<td>14</td>
<td>45.2 (27.3, 64.0)</td>
<td>1.5 (0.59, 4.0)</td>
</tr>
</tbody>
</table>

Table 5.4: Seroprevalence to leptospires in different orders of small mammals.

<table>
<thead>
<tr>
<th>Animals</th>
<th>MAT Positive</th>
<th>Seroprevalence (95% CI)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>33</td>
<td>57.9 (44.1, 70.9)</td>
<td>1.0</td>
</tr>
<tr>
<td>Squirrels &amp;Tree shrew</td>
<td>12</td>
<td>42.9 (24.5, 62.8)</td>
<td>0.55 (0.24, 0.99)</td>
</tr>
<tr>
<td>Bats</td>
<td>28</td>
<td>40.0 (28.5, 52.4)</td>
<td>0.48 (0.22, 1.36)</td>
</tr>
</tbody>
</table>
Table 5.5: Number of animals belonging to the rats, squirrels and treeshrews seropositive to different leptospiral serovars.

<table>
<thead>
<tr>
<th>Serovar</th>
<th>+ (%)</th>
<th>Lepto 175</th>
<th>Australis</th>
<th>Autumnalis</th>
<th>Ictero</th>
<th>Javanica</th>
<th>Patoc</th>
<th>Copenhageni</th>
<th>Pomona</th>
<th>Lai</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sundamys muelleri</em></td>
<td>66 (55.0)</td>
<td>17</td>
<td>13</td>
<td>9</td>
<td>14</td>
<td>0</td>
<td>8</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><em>Rattus argentiventer</em></td>
<td>23 (19.1)</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Maxomys rajah</em></td>
<td>9 (7.5)</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Sundasciurus lowii</em></td>
<td>1 (0.8)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Callosciurus notatus</em></td>
<td>19 (15.8)</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td><em>Tupaia tana</em></td>
<td>2 (1.7)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>120 (100)</td>
<td>41 (34.1)</td>
<td>13 (10.7)</td>
<td>12 (10)</td>
<td>20 (16)</td>
<td>5 (5)</td>
<td>15 (12.5)</td>
<td>6 (5.0)</td>
<td>5 (4.1)</td>
<td>3 (2.5)</td>
</tr>
</tbody>
</table>
Table 5.6: Number of bats seropositive to different leptospiral serovars.

<table>
<thead>
<tr>
<th>Species</th>
<th>+ (%)</th>
<th>Lepto 175</th>
<th>Australis</th>
<th>Icterohaemorrhagiae</th>
<th>Pyrogenes</th>
<th>Patoc</th>
<th>Lai</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Penthetor lucasi</em></td>
<td>21 (41.1)</td>
<td>12</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Cynopterus brachyotis</em></td>
<td>17 (33.3)</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td><em>Balionycteris maculate</em></td>
<td>7 (13.7)</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td><em>Hipposideros cervinus</em></td>
<td>3 (5.8)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Rhinolophus borneensis</em></td>
<td>2 (3.9)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><em>Nycteris tragata</em></td>
<td>1 (1.9)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>51 (100)</strong></td>
<td><strong>27 (52.9)</strong></td>
<td><strong>2 (3.9)</strong></td>
<td><strong>1 (1.9)</strong></td>
<td><strong>10 (19.6)</strong></td>
<td><strong>9 (17.6)</strong></td>
<td><strong>2 (3.9)</strong></td>
</tr>
</tbody>
</table>
The antibody titres for seropositive animals varied from 1:50 to 1:800. More animals had a serum titre of 1:100 (n = 65) than other titer (1:50 in 20 serum samples; 1:200 in 30 serum samples and 1:400 in 8 samples, while only one squirrel had a titer of 1:800) (Table 5.7).

Table 5.7: Titres of antibodies to different serovars found in small mammals.

<table>
<thead>
<tr>
<th>Serovar</th>
<th>+ ve</th>
<th>1:50</th>
<th>1:100</th>
<th>1:200</th>
<th>1:400</th>
<th>1:800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepto 175 Sarawak</td>
<td>68</td>
<td>23</td>
<td>27</td>
<td>12</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Australis</td>
<td>15</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Autumnalis</td>
<td>12</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td>21</td>
<td>5</td>
<td>13</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Javanica</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patoc</td>
<td>24</td>
<td>8</td>
<td>14</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Copenhageni</td>
<td>6</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pomona</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lai</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total/%</td>
<td>171/100</td>
<td>56/32.5</td>
<td>77/44.7</td>
<td>30/17.4</td>
<td>8/4.6</td>
<td>1/0.6</td>
</tr>
</tbody>
</table>

5.3.3 Influence of locality on seroprevalence.

Small mammals from Mount Singai were 3.4 times (95% CI 1.04-11.17) more likely to have leptospiral antibodies than animals captured from the UNIMAS area (Table 5.3). The prevalence in animals sampled from Matang and Kubah, Wind Cave and Fairy Caves and Bako National Park were similar to that of UNIMAS (all OR 95% CI
included the value 1.0) (Table 5.3). Representatives of rats and squirrels & treeshrew from Matang and Kubah were more likely to be seropositive than representatives from UNIMAS (OR 4.5; 95% CI 1.1, 19.1) (Table 5.8). All other locations had a similar seroprevalence to UNIMAS.

There was no significant difference among the seroprevalence in bats and the five different sampling sites (Table 5.9).

The squirrels and treeshrew were less likely to be seropositive than rats (OR 0.55: 95%CI 0.24, 0.99). Although the bats were less likely to be seropositive than animals from the order rats (0.48: 95% CI 0.22, 1.36), this difference was not significant.

Table 5.8: Seroprevalence to leptospires in mammals belonging to the order rats, squirrels and treeshrew from different localities.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Number Seropositive</th>
<th>Seroprevalence (95% CI)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNIMAS</td>
<td>8</td>
<td>40.0 (19.1, 63.9)</td>
<td>1.0</td>
</tr>
<tr>
<td>Mt. Singai</td>
<td>7</td>
<td>53.9 (25.1, 88.8)</td>
<td>2.1 (0.49, 9.0)</td>
</tr>
<tr>
<td>Wind &amp; Fairy Caves</td>
<td>10</td>
<td>58.8 (32.9, 81.6)</td>
<td>2.14 (0.57, 7.9)</td>
</tr>
<tr>
<td>Matang &amp; Kubah</td>
<td>12</td>
<td>75.0 (47.6, 92.7)</td>
<td>4.5 (1.06, 19.1)</td>
</tr>
<tr>
<td>Bako National Park</td>
<td>8</td>
<td>42.1 (20.3, 66.5)</td>
<td>1.1 (0.30, 3.9)</td>
</tr>
</tbody>
</table>
Table 5.9: Seroprevalence to leptospires in bats from different localities.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Number seropositive</th>
<th>Seroprevalence (95% CI)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNIMAS</td>
<td>6</td>
<td>30.0 (11.9, 54.3)</td>
<td>1.0</td>
</tr>
<tr>
<td>Mt. Singai</td>
<td>4</td>
<td>80.0 (28.4, 99.5)</td>
<td>9.3 (0.85, 10.9)</td>
</tr>
<tr>
<td>Wind &amp; Fairy Caves</td>
<td>7</td>
<td>36.9 (16.3, 61.6)</td>
<td>1.4 (0.36, 5.2)</td>
</tr>
<tr>
<td>Matang &amp; Kubah</td>
<td>5</td>
<td>35.7 (12.8, 64.9)</td>
<td>1.3 (0.30, 5.5)</td>
</tr>
<tr>
<td>Bako National Park</td>
<td>6</td>
<td>50.0 (21.1, 78.9)</td>
<td>2.3 (0.53, 10.3)</td>
</tr>
</tbody>
</table>

Table 5.10: *Leptospira* serovar PCR results from all positive kidneys of sampled small mammals.

<table>
<thead>
<tr>
<th>Place</th>
<th>Species</th>
<th>Serovar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt Singai</td>
<td>Callosciurus notatus</td>
<td>Pomona</td>
</tr>
<tr>
<td></td>
<td>Sundamys mueller</td>
<td>Icterohaemorrhagiae</td>
</tr>
<tr>
<td>UNIMAS</td>
<td>Sundamys mueller</td>
<td>Lai</td>
</tr>
<tr>
<td>Wind &amp; Fairy Caves</td>
<td>Callosciurus notatus</td>
<td>Pomona</td>
</tr>
<tr>
<td></td>
<td>Maxomys rajah</td>
<td>Icterohaemorrhagiae</td>
</tr>
<tr>
<td></td>
<td>Maxomys rajah</td>
<td>Icterohaemorrhagiae</td>
</tr>
<tr>
<td></td>
<td>Sundamys mueller</td>
<td>Lai</td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Pomona</td>
</tr>
<tr>
<td></td>
<td>Sundamys mueller</td>
<td>Icterohaemorrhagiae</td>
</tr>
<tr>
<td>Matang &amp; Kubah</td>
<td>Sundamys mueller</td>
<td>Icterohaemorrhagiae</td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Pomona</td>
</tr>
<tr>
<td>Bako National Park</td>
<td>Callosciurus notatus</td>
<td>Pomona</td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Pomona</td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Pomona</td>
</tr>
<tr>
<td></td>
<td>Sundamys mueller</td>
<td>Icterohaemorrhagiae</td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Pomona</td>
</tr>
</tbody>
</table>
Of 155 kidney samples from individual animals only 17 were positive for *Leptospira* on the molecular study (10.97%; 95% CI 6.5, 17) (Table 5.10). The majority of the positive results were from plantain squirrels (53%; 95%CI 27.8, 77), Müller’s rat (35%; 95%CI 14.2, 61.7) and brown spiny rats (12%; 95%CI 1.5, 36.4). No other animals were positive on the PCR. Nine samples were positive to Pomona, six to Icterohaemorrhagiae and two to serovar Lai (Table 5.10).

### 5.4 Discussion

All locations sampled in the current study were within 30 kilometres of Kuching, the capital city of Sarawak, East Malaysia. Two of these, Matang-Kubah and Bako, are situated within the confines of national parks while Mount Singai is a small mountain located between Matang and Bau. UNIMAS is an educational facility based in Kota Samarahan District. The Wind and Fairy Caves are located close to the town of Bau. A common trend in most of the human leptospirosis outbreaks in the Kuching region have been exposure to wildlife and contact with water contaminated with leptospires. Bako National Park, Matang-Kubah Wildlife Centre, Mount Singai and the Wind and Fairy Caves are known to contain several wildlife species that may be responsible for transmitting leptospires. UNIMAS is located in an area where the natural environment has been disturbed, similar to other areas of Sarawak. Certain parts of the UNIMAS campus are also known to be waterlogged and have patches of forest environment, increasing the possibility of exposure of students at the campus to leptospires.

Studies have shown that rodents and bats are reservoirs for leptospirosis (Matthias et al., 2005; Vashi et al., 2010), notably the pathogenic serovar Icterohaemorrhagiae
that causes fatalities in humans. These two wildlife groups are believed to be potential sources of infection for humans, and contact with them is highly likely when visiting the five sampled locations. The results indicate that small mammals from Mount Singai were 3.4 times more likely to have leptospiral antibodies than animals at UNIMAS. A greater proportion of rats were shown to be seropositive than bats, squirrels and tree shrews, highlighting the importance of these species as carriers of leptospires. These findings highlight potential risk for acquiring leptospirosis to villagers living around Mount Singai, as well as tourists visiting the mountain.

Deforestation, which is becoming commonplace across Malaysia (Aiken and Leigh, 1992; Jomo et al., 2004), can promote the emergence of infectious diseases, such as leptospirosis, by placing humans into contact with novel reservoirs or infectious agents (Arief, 2013; Matthias et al., 2005). Bats respond to the destruction of their habitat at the level of populations and communities, making their spatial and temporal dynamics particularly sensitive to anthropogenic activity (Calisher et al., 2006). Matthias et al., (2005) detected *Leptospira* serovar Icterohaemorrhagiae in one bat from the Peruvian Amazon region and proposed a rodent-bat cycle of infection (Vashi et al., 2010), adding further support to the growing awareness of the role played by bats in the transmission of this pathogen to humans and other animals.

Forests in Southeast Asia, including Malaysia, are characterised by flowering and fruit production, substantially contributing to the abundance of rodents (Nakagawa et al., 2007). A ricefield rat captured by Mohamed-Hassan et al., (2012) at a military training camp in West Malaysia was identified as a carrier of leptospires. Rats are also reservoirs of a number of other parasites and infectious pathogens, including the
agent of plague, *Yersinia pestis* (Zahedi et al., 1984), and are key sources of infection for humans.

Culture of leptospires or a positive PCR result from kidney samples was considered to be indicative of a carrier status of this pathogen. In contrast animals positive on the MAT are not necessarily carriers as *Leptospira*-specific antibodies can be detected in convalescent sera and positivity to an MAT indicates past or current infection but not necessarily renal shedding (Faine et al., 1999). In the current study, *Leptospira* serovar Pomona was most prevalent among the plantain squirrels, being detected in kidneys collected from animals from four out of the five regions sampled. Müller’s rats were identified as carriers of both serovars Lai and Icterohaemorrhagiae, with the latter also found in brown spiny rats. Many small mammals also displayed high levels of antibodies to the newly discovered strain serovar Lepto 175 Sarawak, with plantain squirrels and Müller’s rats being more commonly affected with this serovar. Lepto 175 Sarawak was also found in a large number of dusky fruit bats tested. At present Lepto 175 Sarawak is best considered an intermediate strain, as its lethal capacity is unknown. In Chapter 3 Lepto 175 Sarawak was shown to have a close similarity to *Leptospira wolffii*, which has been isolated from Thailand, Iran and India (Slack et al., 2008; Zakeri et al., 2010). Evidence of infection with Icterohaemorrhagiae, a well-known pathogen implicated in many fatal cases of leptospirosis in humans (Viriyakosol et al., 2006), was evident in this study being slightly less common than Australis and Autumnalis. Pyrogenes appeared to be more common in bats while Autumnalis was found mainly in rodents. Rats tested positive for at least two known pathogenic serovars (Icterohaemorrhagiae and Lai).
This particular study should generate concerns and lead to the health authorities expanding disease control measures as there are significant levels of human activity at all five locations where the animals were sampled. Visitors should be advised to protect themselves and avoid direct contact with contaminated soil or water. Wherever possible, pest control measures should be implemented to contain the rat population, particularly considering that these rodents appear to be the main source of infection in these locations.

Wildlife that is undergoing rehabilitation should be screened for *Leptospira* periodically before their eventual re-introduction to the natural environment. This helps prevent them from transmitting the disease to other species in the wild or to humans involved in their release (Birtles, 2012).

The pathogenesis of serovar Lepto 175 Sarawak also needs to be monitored closely considering its similarities to serogroup Wolffii. It remains to be seen if serovar Lepto 175 Sarawak will be as virulent as Wolffii and hence extra vigilance is required and the Malaysian health authorities need to implement disease control measures to prevent the occurrence of a potential epidemic.

In the following chapter risk factors for infection in humans are investigated so that risk factor modification can be implemented to reduce the number of cases of leptospirosis in humans.
CHAPTER 6 : PREVALENCE AND RISK FACTORS OF LEPTOSPIRA EXPOSURES IN HUMAN LIVING IN FOREST BORDER AREAS AND DISTURBED JUNGLE ENVIRONMENTS IN SARAWAK MALAYSIA.

6.1 Introduction

6.1.1 General Introduction
Determining the seroprevalence of *Leptospira* spp., especially the novel *Leptospira Lepto 175 Sarawak*, is an important step in understanding its role in infection and will largely influence the nature of future work on leptospirosis in Sarawak, Malaysia. Across the world researchers have focussed on the more pathogenic strains and their zoonotic significance, epidemiology, control and prevention; however future studies on intermediate pathogenic strains are important to prevent new disease outbreaks in communities. There are many technical challenges in confirming the virulence factors of intermediate strains, although genotyping and proteomic methods have been developed to investigate these factors (Ganoza et al., 2006; Plank and Dean, 2000). The pathogenicity of a Leptospire serovar for humans can only be established after it is isolated from a patient presenting with the clinical symptoms of leptospirosis.

Leptospirosis is known worldwide for its harmful effects on both humans and animals, resulting in morbidity and mortality and it is classed as a direct anthropozoonosis (Sanders et al., 1999; Schwabe, 1969). Infection in domestic animals can result in severe economic losses due to decreased milk production,
abortions, stillbirths and infertility, although fatal infection is rare (Sullivan, 1974). Pathogenic leptospires thrive in the kidneys of mostly mammals and are excreted through the urine into the environment, where they survive for up to several months if conditions are favourable. When humans come into direct contact with infected animals, their urine or urine-contaminated environments, there is the risk of infection. The bacteria enter the body via open wounds or through the mucous membranes. Symptoms of illness in humans are varied and easy to misdiagnose as other diseases. In extreme cases, a fatal condition characterised by organ failure and gross haemorrhage can result (Daher et al., 2003). In Malaysia, leptospirosis is a disease that is subject to mandatory reporting on human due to its severity and the difficulty in bringing it under control (Hakim, 2011). In Sarawak alone, the number of cases rose almost fourfold from 49 in 2010 to 186 in 2011, as discussed in Chapter 2. It is widely believed that bats and rodents may be responsible for the spread of leptospirosis in Sarawak. With increased urbanisation and rapid deforestation happening across the state, the possibility of people coming into contact more frequently with wildlife becomes all the more inevitable. This can put them at risk of contracting diseases such as leptospirosis. Furthermore, domestic animals can be exposed to a large number of leptospiral serovars and may act as vital maintenance hosts for the bacterium (Bahaman et al., 1988).

6.1.2 Risk factors for leptospirosis in humans

It is well established that leptospirosis is an occupational disease (Waitkins, 1986) through contact with infected animals or urine-contaminated surfaces, such as soil or water. Shafei et al., (2012) investigated municipal workers of Kota Bharu, the capital of Kelantan state in northern Malaysia, and found that garbage collectors and
street cleaners had a high seroprevalence of leptospirosis. Earlier, Russ et al., (2003) reported that a significant number of inhabitants of settlements surrounding Sabah’s Crocker Range had been exposed to leptospiral antigens if they were involved in agricultural activities or used water sourced from rivers for their household use. Unsanitary living conditions that allowed rats to thrive also increased the risk of leptospiral infection in humans, as did residing in flood-prone areas (Easton, 1999).

Poor knowledge of disease control can also be considered a potential risk factor for infection of humans (Wiwanitkit and Wiwanitkit, 2006). Rafizah et al., (2013) studied 999 febrile patients originating from 10 hospitals in north-eastern Malaysia and found that those who were seropositive for leptospires (n=84) had all been exposed to the organism through their occupation or through involvement in recreational activities.

The study described in the current chapter was conducted to assess the extent of exposure to leptospires in humans living on the fringes of forested areas and disturbed jungle environments. The major objective of this study was to determine which strains had led to antibody development in the sampled humans.

### 6.2 Materials and methods

A cross-sectional serological survey was conducted in four different locations around Kuching, Sarawak, between January 2011 and March 2012. A total of 198 individuals over the age of 18 were sampled from four villages. The villages were selected based on their location adjacent to areas where wildlife inhabited. A single venous blood sample (5 mL) was collected from each participant. At the same time
as the blood was collected, a questionnaire survey was administered to gather information about exposure, contact with animals, general knowledge of leptospirosis and socio-economic status (Appendix 1). Households were randomly selected for this study using the detailed information provided by the village chief of the respective village. If the selected household member was not interested in participating in the study the next household member was selected. A minimum of 10% of village people >18 years old were targeted for sampling from each village. This research was approved by the Murdoch University Human Ethics Committee (Permit number: 2010/240) and the Malaysian Research Ethics Committee (NMRR-10-879-7153). A case was defined as a person who participated in the survey and who was positive for anti-leptospiral antibodies by an IgG and IgM ELISA kit (CTK Biotech cat: E0331) indicating current or past leptospiral infection. At the same time the MAT was performed on all samples to identify the serovar responsible for the infection. A titre ≥1:100 on the MAT was considered as evidence of recent or past infection with *Leptospira*.

### 6.2.1 Study areas

Four locations were chosen for this particular study: Kampung (Kg) Sebayor, Kg Etingan, Mount Singai and Wind and Fairy Caves (described in Chapter 5).

### 6.2.2 Study design

#### 6.2.2.1 Blood collection

Blood samples (5 mL) were collected from the cephalic vein from apparently healthy participants of either gender over the age of 18 years. Disposable needles
and vacuum tubes were used to collect blood from each participant by a qualified Medical Laboratory Assistant (MLT) of the Universiti Malaysia Sarawak Medical Faculty. Samples were maintained at 20°C for 6 hours to allow coagulation and then were centrifuged at 3,000 RPM for 1 minute to separate the sera. The serum was then removed and transferred to vials and stored at -70°C until testing was performed.

6.2.2.2 Information about potential risk factors (Questionnaire design)

The main objective of the questionnaire was to establish whether seropositivity to *Leptospira* was associated with any risk factors (e.g., age, sex, occupation and exposure to animals).

A structured questionnaire was developed, based on validated questionnaires performed by others, and used in this study (Kawaguchi et al., 2008; Sarkar et al., 2002; Sasaki et al., 1993). The questions were categorised under six broad categories: general (age, sex, education and occupation); knowledge and symptoms of leptospirosis; exposure to animals; outdoor activities; potential sources of infection; and socio-economic status. All the questions, except for the source of drinking water and socio-economic status, were categorised as either a yes or no answer.

6.2.2.3 Questionnaire implementation

In each village, researchers contacted the household with the help of the village chief and requested permission for their participation in the study. On the day of blood collection a small introduction session was run to provide brief information
about the research and the disease. UNIMAS postgraduate students were selected and trained to administer the questionnaire. After the blood was collected the questionnaire was administered, which took approximately 20 to 30 minutes. All participants completed a consent form to agree to be involved in the study (Appendix 2).

6.2.3 Laboratory assays

6.2.3.1 MAT
Serum samples were tested by the MAT in the Leptospirosis Laboratory at the Institute for Medical Research, Kuala Lumpur, Malaysia. The MAT was performed using antigen from a collection of 17 live serovars of *Leptospira* that are common in Malaysia (Table 4.1). The MAT procedure followed was as described in Chapter 4. Initially sera were tested at a 1/100 dilution and the antigens that presented agglutination at that dilution were then titrated with the serovar reagents using a series of dilutions from 1/100 to 1/1600. Persons with a titre of 1:100 or more were considered as positive (cases).

6.2.3.2 Enzyme linked Immunosorbent assay (ELISA)
The ELISA was performed at the IMR according to the standard procedure provided by the CTK Biotech Data sheet (Cat No: E0331) to check for *Leptospira*-specific antibodies, IgG and IgM.
6.2.3.2.1 Preparation of the reagents

All reagents and control samples were brought to room temperature (28°C). The concentrated washing buffer was then diluted approximately 30 fold with water using the following methods:

20 mL of concentrated washing buffer was added to 580 mL of water and the solution mixed well.

Next, the washing buffer was warmed to 37°C to dissolve the precipitates that had appeared.

Subsequently, each reagent was mixed well before adding to the test wells. In the final step, the number of microwells needed was determined and appropriate information was marked on the ELISA working sheet. Positive and negative controls were run in duplicate on each plate.

6.2.3.2.2 Assay procedure

The first procedure was removal of the desired number of strips, which were secured in the microwell frame. Specimens were then added according to the designation on the ELISA working sheet. No reagents were added to the blank well. For the control wells, 100 µL of positive or negative control were added into the designated wells. For the test wells, 100 µL of sample diluent was added to each well along with 10 µL of the test specimen. Subsequently, the plates were gently rocked for 20 seconds and covered with sealant. The wells were then incubated at 37°C for 30 minutes. The incubation mixtures were carefully removed by emptying the solution into a waste container. Each well was then filled with diluted wash buffer and shaken gently for
20-30 seconds. The wash solution was then completely discarded by tapping the plate on absorbent paper. The above procedure was repeated a further four times. Then 100 µL of HRP-Protein-A conjugates was added to each well, except for the blank well, the plate covered and incubated at 37°C for 20 minutes. The plate was then washed five times as described previously. Next, 50 µL of TMB substrate A and 50 µL of TMB substrate B were added to each well, including the blank well. The wells were incubated at 37°C in the dark for 10 minutes. The reaction was stopped by adding 50 µL of stop buffer to each well and gently mixing for 30 seconds. During the process, it was important to make sure that all blue colour changed to yellow. Lastly, the absorbance (OD) of each well was measured in a microplate reader at a wavelength of 450 nm against the blank well within 15 minutes after the addition of the Stop Solution. For optimization of the assay result, a filter of 620-690 nm was used as a reference wavelength.

6.2.3.2.3 Interpretation of results

The cut-off value for a positive was set at 0.15 + N where N was the mean OD of the negative control wells. If the mean OD of the negatives was less than 0.05 then 0.05 was used as the cut-off value. The OD ratio was calculated for each specimen by dividing its OD value by the cut-off value.

The assay validations were carried out when the positive controls were ≥ 0.50 and when the negative controls were ≤ 0.10. A positive result was assigned when the specimen OD ratio was greater than 1.0 and a negative result if the OD ratio was lesser than 0.1. A negative result indicated that there was no detectable IgG/IgM antibody to *L. interrogans* in the specimen.
6.2.4 Statistical analyses

Data were entered into Microsoft Excel and statistical analyses were performed using SPSS version 22. The seroprevalence on the MAT test and their 95% confidence intervals (95%CI) were calculated. Categorical variables were summarised using percentages and compared using the Chi-square test. A multivariable logistic regression was performed to identify factors associated with seropositivity to leptospires. Odd ratios (ORs) and their 95% confidence intervals (CIs) were calculated for factors in the reduced subset model. Model building was performed by considering variables that were statistically significant (p values <0.20) in the univariable analyses, and including them in the preliminary multivariate logistic regression model. Once the preliminary main effects model was obtained (fully saturated model) a manual backward stepwise approach was used to remove non-significant variables and only variables with p values<0.05 were retained in the final model. Overall fit of the final logistic model was assessed with the use of the Hosmer-Lemeshow goodness-of-fit statistic. To determine possible interactions between the final set of factors interaction factors between each remaining variable were added one at a time to determine the impact of the interaction factor on the model. Findings were presented with Odds ratio (OR), 95%CI and p-value.

6.3 Results

Samples were collected from Kg. Sebayor (n=27), Kg. Etingan (n=40), Mt. Singai (n=70) and Wind and Fairy Caves (n=61) for this study. Of the 198 participants, 126
(63.64%) were women and 72 (36.36%) men (Table 6.1). The majority of the respondents were over the age of 55 years (Table 6.2).

**Table 6.1: Gender distribution of humans sampled at the four localities.**

<table>
<thead>
<tr>
<th>Locality</th>
<th>Gender</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Kg. Sebayor</td>
<td>17 (63.0%)</td>
<td>10 (36.0%)</td>
</tr>
<tr>
<td>Kg. Etingan</td>
<td>21 (52.5%)</td>
<td>19 (47.5%)</td>
</tr>
<tr>
<td>Mt. Singai</td>
<td>47 (67.1%)</td>
<td>23 (32.9%)</td>
</tr>
<tr>
<td>Wind &amp; Fairy Caves</td>
<td>41 (67.2%)</td>
<td>20 (32.8%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>126 (63.6%)</td>
<td>72 (36.4%)</td>
</tr>
</tbody>
</table>

**Table 6.2: Age distribution of participants in the study.**

<table>
<thead>
<tr>
<th>Locality</th>
<th>Age Group (years)</th>
<th>18-25</th>
<th>26-35</th>
<th>36-45</th>
<th>46-55</th>
<th>&gt;55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kg. Sebayor</td>
<td></td>
<td>0</td>
<td>6</td>
<td>3</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Kg. Etingan</td>
<td></td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Mt. Singai</td>
<td></td>
<td>7</td>
<td>6</td>
<td>9</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>Wind &amp; Fairy Caves</td>
<td></td>
<td>3</td>
<td>9</td>
<td>11</td>
<td>14</td>
<td>24</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>15</strong></td>
<td><strong>21</strong></td>
<td><strong>28</strong></td>
<td><strong>56</strong></td>
<td><strong>78</strong></td>
</tr>
</tbody>
</table>

6.3.1 **Seroological results**

An overall seroprevalence of 35.9% (95%CI 29.2-43.0) was obtained on the MAT. There was no significant difference ($\chi^2=3.04; df=3; P=0.38$) in the seroprevalence among the four sampled locations, with the highest seroprevalence (41%) (95%CI
28.6-54.3) obtained in people from the Wind and Fairy Caves. Individuals from Mount Singai had a seroprevalence of 38.6% (95%CI 27.2-51.0) (Table 6.3).

People from the Wind and Fairy Caves were 2.08 (95% CI 0.87-5.02) times more likely to have leptospiral antibodies than people residing in Kg. Etingan. Similarly people from Mt. Singai were 1.88 (95% CI 0.80-4.46) times more likely to have antibodies than people from Kg. Etingan. Kg. Sebayor had an odds of seropositivity of 1.5 (95%CI 0.51-4.39) compared to Kg. Etingan.

Table 6.3: Seroprevalence (MAT) to leptospirosis in humans from different localities.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Number Positive</th>
<th>Total</th>
<th>Seroprevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kg. Sebayor</td>
<td>9</td>
<td>27</td>
<td>33.3</td>
<td>16.5-54.0</td>
</tr>
<tr>
<td>Kg. Etingan</td>
<td>10</td>
<td>40</td>
<td>25.0</td>
<td>12.7-41.2</td>
</tr>
<tr>
<td>Mount Singai</td>
<td>27</td>
<td>70</td>
<td>38.6</td>
<td>27.2-51.0</td>
</tr>
<tr>
<td>Wind &amp; Fairy Caves</td>
<td>25</td>
<td>61</td>
<td>41.0</td>
<td>28.6-54.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>71</strong></td>
<td><strong>198</strong></td>
<td><strong>35.9</strong></td>
<td><strong>29.2-43.0</strong></td>
</tr>
</tbody>
</table>

Based on the results from the ELISA 33.3% (95%CI 26.8-40.4) of samples were seropositive to leptospires (Table 6.4). The highest percentage (41%; 95%CI 28.6-54.3) was again in residents from the Wind and Fairy Caves area. Only 5 of 40 respondents from Kampung Etingan (12.5%; 95%CI 4.2-26.8) were seropositive (Table 6.4). The seroprevalence in Kg Etingan was significantly less than that in Mt Singai and the Wind and Fairy Caves (p < 0.05).

Table 6.4: Seroprevalence (ELISA) of leptospirosis in humans according to their locality.
<table>
<thead>
<tr>
<th>Locality</th>
<th>Number Positive</th>
<th>Total</th>
<th>Seroprevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kg. Sebayor</td>
<td>9</td>
<td>27</td>
<td>33.3</td>
<td>16.5-54.0</td>
</tr>
<tr>
<td>Kg. Etingan</td>
<td>5</td>
<td>40</td>
<td>12.5</td>
<td>4.2-26.8</td>
</tr>
<tr>
<td>Mt. Singai</td>
<td>27</td>
<td>70</td>
<td>38.6</td>
<td>27.2-51.0</td>
</tr>
<tr>
<td>Wind &amp; Fairy Caves</td>
<td>25</td>
<td>61</td>
<td>41.0</td>
<td>28.6-54.3</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>198</td>
<td>33.3</td>
<td>26.8-40.4</td>
</tr>
</tbody>
</table>

Table 6.5: Seroprevalence (MAT) to Lepto 175 Sarawak in humans according to their locality.

Reactivity to serovar Lepto175 (Sarawak) was found in humans residing at all four locations (Table 6.5). Most of the serum samples collected from people residing at Kg. Sebayor, Mt. Singai and Wind & Fairy Caves that tested positive on the MAT analysis (Table 6.3) were positive to serovar Lepto175 (Sarawak) (62/71, 87.3%) (Table 6.5). Overall the seroprevalence to serovar Lepto175 (Sarawak) was significantly different among locations ($\chi^2=11.43; df=3; P=0.010$), being significantly lower in Kg. Etingan than in the other locations (Table 6.5).

Antibodies to 17 different serovars were detected in the serum samples collected from humans from the four locations sampled. Antibodies to serovar Lepto 175 Sarawak were most commonly detected being found in 62 (31.31%; 95%CI 24.9-38.3) of all samples. Thirty-three samples (16.7%; 95%CI 11.8-22.6) showed
reactivity to Djasiman, 15.7% (95%CI 10.9-21.5) were positive to Autumnalis and a similar percentage were positive to Canicola and 14.6% (95%CI 10.0-20.4) were positive to Australis (Table 6.6). The antibody titres of the seropositive samples varied from 1:100 to 1:400. More samples had a serum dilution of 1:100 (239) compared to other dilutions (1:200 in 70 samples and 1:400 in 18 samples).

Table 6.6: Distribution of seropositive (MAT) reactions to *Leptospira* spp. in humans according to serovar and their respective titres.

<table>
<thead>
<tr>
<th>Serovars</th>
<th>1:100</th>
<th>1:200</th>
<th>1:400</th>
<th>Total</th>
<th>% (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepto 175</td>
<td>42</td>
<td>17</td>
<td>3</td>
<td>62</td>
<td>31.3 (24.9-38.3)</td>
</tr>
<tr>
<td>Djasiman</td>
<td>28</td>
<td>4</td>
<td>1</td>
<td>33</td>
<td>16.7 (11.8-22.6)</td>
</tr>
<tr>
<td>Autumnalis</td>
<td>24</td>
<td>4</td>
<td>3</td>
<td>31</td>
<td>15.7 (10.9-21.5)</td>
</tr>
<tr>
<td>Canicola</td>
<td>23</td>
<td>8</td>
<td>-</td>
<td>31</td>
<td>15.7 (10.9-21.5)</td>
</tr>
<tr>
<td>Australis</td>
<td>18</td>
<td>9</td>
<td>2</td>
<td>29</td>
<td>14.6 (10.0-20.4)</td>
</tr>
<tr>
<td>Lai</td>
<td>13</td>
<td>5</td>
<td>3</td>
<td>21</td>
<td>10.6 (6.7-15.8)</td>
</tr>
<tr>
<td>Patoc</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>16</td>
<td>8.0 (4.7-12.8)</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>7.6 (4.3-12.2)</td>
</tr>
<tr>
<td>Hebdomadis</td>
<td>14</td>
<td>1</td>
<td>-</td>
<td>15</td>
<td>7.6 (4.3-12.2)</td>
</tr>
<tr>
<td>Javanica</td>
<td>13</td>
<td>2</td>
<td>-</td>
<td>15</td>
<td>7.6 (4.3-12.2)</td>
</tr>
<tr>
<td>Bataviae</td>
<td>9</td>
<td>2</td>
<td>-</td>
<td>11</td>
<td>5.6 (2.8-9.7)</td>
</tr>
<tr>
<td>Hardjo-bovis</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>5.0 (2.4-9.1)</td>
</tr>
<tr>
<td>Pomona</td>
<td>3</td>
<td>7</td>
<td>-</td>
<td>10</td>
<td>5.0 (2.4-9.1)</td>
</tr>
<tr>
<td>Celledoni</td>
<td>7</td>
<td>1</td>
<td>-</td>
<td>8</td>
<td>1.8 (1.8-7.8)</td>
</tr>
<tr>
<td>Serovars</td>
<td>1:100</td>
<td>1:200</td>
<td>1:400</td>
<td>Total</td>
<td>% (95%CI)</td>
</tr>
<tr>
<td>--------------------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>----------</td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>8</td>
<td>1.8 (1.8-7.8)</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>1.4 (1.4-7.1)</td>
</tr>
<tr>
<td>Copenhageni</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>0.8 (0.8-5.8)</td>
</tr>
</tbody>
</table>

### 6.3.2 Descriptive epidemiology

#### 6.3.2.1 Location

Seropositive humans were detected from all localities sampled (Section 6.3.1).

#### 6.3.2.2 Gender

Males had a slightly higher seroprevalence (MAT) (38.4%; 95%CI 27.2-50.5) than females (34.4%; 95%CI 26.1-43.4), although this difference was not significant ($\chi^2=0.31; df=1; P=0.57; OR 1.12, 95%CI 0.61, 2.04$).

#### 6.3.2.3 Age

Overall the seroprevalence (MAT) varied between age groups ($\chi^2= 9.38; df=4; P=0.052$). Results were not significantly different but in regards to age as a risk factor ($P=0.052$), while this is approaching significance. People in the 36-45 year age group had the highest seroprevalence (53.6; 95%CI 33.9-72.5) followed by the 18-25 age group (46.7; 95%CI 21.3-73.4) (Table 6.7). People 34 to 45 years old were 2.45 times (95%CI 1.01-5.91) more likely to have leptosiral antibodies than those older than 55 years of age.
6.3.2.4 Educational status

The seroprevalences based on the results of the MAT were not significantly different among the different educational levels ($\chi^2 = 0.38; df=3; P=0.95$).

6.3.2.5 Occupation

Overall the seroprevalence (MAT) was not significantly different among the different occupations ($\chi^2 = 4.12; df=3; P=0.25$). However people who were working around the forest (54.5%; 95%CI 23.4-83.3) and national service participants (50%; 95%CI 27.2-72.8) had the highest seroprevalence (Table 6.7).

6.3.2.6 Symptoms of clinical disease

People with a fever were significantly more likely to be seropositive (OR 3.83, 95%CI 2.07-7.07; $\chi^2 = 19.2; df=1; P=0.001$) than those without fever. People who had been treated for fever were also significantly more likely to be seropositive than those not treated (OR 3.39, 95%CI 1.84-6.27; $\chi^2 = 7.52; df=1; P=0.006$).

Patients with skin wounds also had a significantly higher seroprevalence (58.8%; 95%CI 40.7-75.4) than those without skin wounds (31.1%; 95%CI 24.1-38.8) (OR 5; 95%CI 2.34-10.68; $\chi^2 = 9.41; df=1; P=0.002$) (Table 6.7).

6.3.2.7 Owning pets or farm animal contact

Owning a pet animal, such as a dog or cat, did not increase the likelihood of a seropositive reaction ($\chi^2 = 1.27; df=1; P=0.25$). In contrast contact with farm
animals (OR 2.2, 95%CI 1.22-3.99; $\chi^2 = 6.89$; $df=1$; $P=0.009$) increased the probability of leptospiral infection (Table 6.7).

6.3.2.8 Presence of rats

The presence of rats in or near the participant’s residences ($\chi^2 = 60.6$; $df=1$; $P=0.001$) or the sighting of rats more than 3 times within a day of the survey ($\chi^2 = 53.0$; $df=1$; $P=0.001$) increased the probability of seropositivity in the residents (Table 6.7).
Table 6.7: Relationship between seropositivity (MAT) to *Leptospira* spp. and host and environmental factors.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total</th>
<th>Seropositive</th>
<th>Seroprevalence (95%CI)</th>
<th>OR (95%CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Locality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wind &amp; Fairy Caves</td>
<td>61</td>
<td>25</td>
<td>41.0 (28.6-54.3)</td>
<td>2.08 (0.87-5.02)</td>
<td>0.38</td>
</tr>
<tr>
<td>Mt. Singai</td>
<td>70</td>
<td>27</td>
<td>38.6 (27.2-51.0)</td>
<td>1.88 (0.80-4.46)</td>
<td></td>
</tr>
<tr>
<td>Kg. Sebayor</td>
<td>27</td>
<td>9</td>
<td>33.3 (16.5-54.0)</td>
<td>1.50 (0.51-4.39)</td>
<td></td>
</tr>
<tr>
<td>Kg. Etingan</td>
<td>40</td>
<td>10</td>
<td>25.0 (12.7-41.2)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>73</td>
<td>28</td>
<td>38.4 (27.2-50.5)</td>
<td>1.19 (0.65-2.16)</td>
<td>0.575</td>
</tr>
<tr>
<td>Female</td>
<td>125</td>
<td>43</td>
<td>34.4 (26.1-43.4)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-25</td>
<td>15</td>
<td>7</td>
<td>46.7 (21.3-73.4)</td>
<td>1.86 (0.61-5.69)</td>
<td>0.052a</td>
</tr>
<tr>
<td>26-35</td>
<td>21</td>
<td>3</td>
<td>14.3 (3.0-36.3)</td>
<td>0.35 (0.10-1.31)</td>
<td></td>
</tr>
<tr>
<td>36-45</td>
<td>28</td>
<td>15</td>
<td>53.6 (33.9-72.5)</td>
<td>2.45 (1.01-5.91)</td>
<td></td>
</tr>
<tr>
<td>46-55</td>
<td>56</td>
<td>21</td>
<td>37.5 (24.9-51.5)</td>
<td>1.27 (0.62-2.61)</td>
<td></td>
</tr>
<tr>
<td>&gt;55</td>
<td>78</td>
<td>25</td>
<td>32.0 (21.9-43.6)</td>
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<td></td>
</tr>
<tr>
<td>Variables</td>
<td>Total</td>
<td>Seropositive</td>
<td>Seroprevalence (95%CI)</td>
<td>OR (95%CI)</td>
<td>P</td>
</tr>
<tr>
<td>------------------------</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Schooling</td>
<td>57</td>
<td>21</td>
<td>36.8 (24.4-50.7)</td>
<td>0.78 (0.16-3.82)</td>
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</tr>
<tr>
<td>Primary</td>
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<td>37.2 (24.1-51.9)</td>
<td>0.79 (0.16-3.93)</td>
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<tr>
<td>Secondary</td>
<td>83</td>
<td>28</td>
<td>33.7 (23.7-44.9)</td>
<td>0.68 (0.14-3.25)</td>
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</tr>
<tr>
<td>Tertiary</td>
<td>7</td>
<td>3</td>
<td>42.9 (9.9-81.6)</td>
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</tr>
<tr>
<td><strong>Occupation</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cleaner</td>
<td>46</td>
<td>16</td>
<td>34.8 (21.4-50.2)</td>
<td>1.12 (0.55-2.30)</td>
<td>0.25</td>
</tr>
<tr>
<td>Work around forest</td>
<td>11</td>
<td>6</td>
<td>54.5 (23.4-83.3)</td>
<td>2.52 (0.73-8.78)</td>
<td></td>
</tr>
<tr>
<td>National Service</td>
<td>20</td>
<td>10</td>
<td>50.0 (27.2-72.8)</td>
<td>2.10 (0.81-5.47)</td>
<td></td>
</tr>
<tr>
<td>Farmer/Dairy farmer</td>
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<td>39</td>
<td>32.2 (24.0-41.3)</td>
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</tr>
<tr>
<td><strong>Fever</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>90</td>
<td>47</td>
<td>52.2 (41.4-62.9)</td>
<td>3.83 (2.07-7.07)</td>
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</tr>
<tr>
<td>No</td>
<td>108</td>
<td>24</td>
<td>22.2 (14.8-31.2)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Variables</td>
<td>Total</td>
<td>Seropositive</td>
<td>Seroprevalence (95% CI)</td>
<td>OR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>----------------------------</td>
<td>-------</td>
<td>--------------</td>
<td>-------------------------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td><strong>Treatment for fever</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>65</td>
<td>32</td>
<td>49.2 (36.6-61.9)</td>
<td>3.39 (1.84-6.27)</td>
<td>0.006</td>
</tr>
<tr>
<td>No</td>
<td>133</td>
<td>39</td>
<td>29.3 (21.8-37.8)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td><strong>Skin wounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>34</td>
<td>20</td>
<td>58.8 (40.7-75.4)</td>
<td>5.00 (2.34-10.68)</td>
<td>0.002*</td>
</tr>
<tr>
<td>No</td>
<td>164</td>
<td>51</td>
<td>31.1 (24.1-38.8)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td><strong>Presence of dog or cat</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>130</td>
<td>43</td>
<td>33.8 (25.1-41.9)</td>
<td>0.71 (0.39-1.29)</td>
<td>0.259</td>
</tr>
<tr>
<td>No</td>
<td>68</td>
<td>28</td>
<td>41.2 (29.4-53.8)</td>
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<td></td>
</tr>
<tr>
<td><strong>Presence of Farm animals</strong></td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Yes</td>
<td>98</td>
<td>44</td>
<td>44.9 (34.8-55.3)</td>
<td>2.20 (1.22-3.99)</td>
<td>0.009*</td>
</tr>
<tr>
<td>No</td>
<td>100</td>
<td>27</td>
<td>27.0 (18.6-36.8)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td><strong>Presence of rats</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>121</td>
<td>69</td>
<td>57.0 (47.7-66.0)</td>
<td>50.8 (11.6-212.0)</td>
<td>0.001*</td>
</tr>
<tr>
<td>No</td>
<td>77</td>
<td>2</td>
<td>2.6 (0.3-9.1)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Variables</td>
<td>Total</td>
<td>Seropositive</td>
<td>Seroprevalence (95%CI)</td>
<td>OR (95%CI)</td>
<td>P</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>-------</td>
<td>--------------</td>
<td>------------------------</td>
<td>------------</td>
<td>------</td>
</tr>
<tr>
<td>Sited rats more than 3 times per day by participants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>96</td>
<td>59</td>
<td>61.5 (51.0-71.2)</td>
<td>12.0 (7.7-24.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>No</td>
<td>102</td>
<td>12</td>
<td>11.8 (6.2-19.6)</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Overseas travel</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>18</td>
<td>5</td>
<td>27.8 (9.7-53.5)</td>
<td>0.66 (0.2-1.9)</td>
<td>0.453</td>
</tr>
<tr>
<td>No</td>
<td>180</td>
<td>66</td>
<td>36.7 (29.6-44.2)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*variables (with P-values<0.20) were offered to the multivariable logistic regression model.*

Table 6.8: Final logistic regression model.

<table>
<thead>
<tr>
<th>Description of variables</th>
<th>B</th>
<th>SE*</th>
<th>Wald*</th>
<th>Sig*</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin wounds</td>
<td>1.116</td>
<td>0.493</td>
<td>5.132</td>
<td>0.023</td>
<td>3.05 (1.16-8.01)</td>
</tr>
<tr>
<td>Presence of farm animals</td>
<td>0.921</td>
<td>0.374</td>
<td>6.056</td>
<td>0.014</td>
<td>2.51 (1.21-5.23)</td>
</tr>
<tr>
<td>Presence of rats</td>
<td>3.878</td>
<td>0.747</td>
<td>26.977</td>
<td>0.000</td>
<td>48.3 (11.19-208)</td>
</tr>
<tr>
<td>Constant</td>
<td>-4.288</td>
<td>0.767</td>
<td>31.216</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

*SE=Standard error; *Wald=a test that a coefficient is zero based on the Wald statistic; *Sig=Significance for the Wald statistic; *OR=Odds ratios
In the final multivariable logistic regression model the presence of skin wounds (OR 3.05), farm animals (OR 2.51) and rats (OR 11.2) were all significantly associated with seropositivity. The logistic regression model for *Leptospira* antibodies exhibited a good overall fit (Hosmer and Lemeshow Chi-square value 2.484, \( P=0.648 \)).

### 6.4 Discussion

In this study over one third of the sampled individuals were seropositive to leptospires (35.9%; 95%CI 29.2-43.0). Other serological surveys of the general population have reported a seroprevalence up to 25% (Poeppl et al., 2013). However the current study targeted a population where it could be expected that the seroprevalence was higher than the overall general population. Another targeted study of residents in the Rajang Basin of Sarawak similarly reported an elevated seroprevalence of 30.6% (Suut et al., 2011). Studies in other tropical countries, including Barbados, have reported a seroprevalence of 29.8% in various occupational groups and in 1987 a seroprevalence as high as 25% was reported in patients hospitalised in Pakistan (Rao et al., 2003). From a global perspective a seroprevalence that is this high is unusual, even in areas where leptospirosis is rampant, although a seroprevalence of 30 to 50% has been reported in high risk groups (Rao et al., 2003).

In the current study inhabitants residing in the vicinity of the Wind and Fairy Caves had a higher, although not significant, risk of seroconversion to leptospires than residents from the other surveyed locations. Wind and Fairy Caves are surrounded by forest and residents would be expected to more likely to come into contact with
wildlife (rats, squirrels and bats) or leptospire-contaminated water or soil than residents from urban areas. In Chapter 5 a high proportion of rats, squirrels and bats from these regions were shown to have antibodies to leptospires. These could be the result of a current infection but also could be the result of a previous infection as titres can remain for up to 20 years (Blackmore et al., 1984).

In this study Serovar Lepto 175 was the dominant strain (31.3%) affecting people. Although its virulence has not been confirmed and it is currently classified as an intermediate strain, it has a high level of 16S rRNA gene sequence similarity to *Leptospira wolffii* sv. Khorat strain Khorat-H2 (99.1%) (Chapter 3). *Leptospira wolffii* has been linked to clinical leptospirosis and has been isolated from humans and other animals in Thailand, India and Iran (Balamurugan et al., 2013; Zakeri et al., 2010) and this serovar could potentially cause an outbreak in the near future. Serovars Autumnalis (15.7%), Canicola (15.7%) and Lai (10.6%) were also commonly found in this study with evidence of infection to most serovars present in all four locations.

In this study the seroprevalence in males (37.5%; 95%CI 26.4-49.7) was slightly higher than that in females (34.9%; 95%CI 26.6-43.9). This could be attributed to the fact that most of the work done outside of the home environment in this region was performed by men. Furthermore, jobs that revolve around the forest, such as woodcutting and hunting, tend to be male-dominated positions because of the harsh environment involved.

Although in the univariable analyses people between the ages of 36 and 45 and 18 to 25 years were at a greater likelihood of being seropositive compared to people older than 55, age did not remain in the final multivariable logistic regression model. The
greater risk found in the univariable analysis for people between 18 and 25 years of age may be associated with this group be dominated by National Service Participants and researchers who would be expected to have greater exposure to the forest and hence wild animals than other groups.

Of 90 respondents who reported to have suffered from a fever within three months of sampling, approximately half (52.2%; 95%CI 41.4-62.9) were seropositive to leptospires. Similarly just under half (49.23%) of those who received treatment for fever were also diagnosed with leptospirosis, while 20 of 34 (58.82%; 95%CI 40.7-75.4) who had skin wounds were seropositive. This supports the importance of infection entering via skin lesions and the presence of fever as a common symptom of leptospiral infection (Bovet et al., 1999; Sethi et al., 2010). Individuals with wounds have the potential to be infected if they are involved in activities likely to expose them to leptospires such as through agricultural pursuits, forestry businesses or wildlife and rodent interactions. In a previous case control study in the Seychelles, leptospirosis was positively associated with activities in forests, which may be related to an increase in environmental exposure to the pathogen (Bovet et al., 1999).

The presence of domesticated pets did not increase the risk of infection significantly in this study. In contrast exposure to farm animals and rodents presented a major risk of infection. These findings highlight the role of peri-urban rats as the principal source of leptospiral transmission as reported by others (Collares-Pereira et al., 2000; Felt et al., 2011; Matthias et al., 2008; Szyfres, 1976). However, surprisingly rodent-associated serovars, such as Icterohaemorrhagiae (1.8% positive) and Copenhageni (0.8%), did not appear to be of major importance in this study. In
contrast, Canicola (15.7%), which is mostly carried by dogs (Schreiber et al., 2005), presented itself as a major threat in this study.

Overseas travel, involvement in water-related activities and camping around forests (data not shown) were not linked to seropositivity, however the number of residents engaging in these pursuits was small. As has been documented in previous studies, a number of outbreaks in Borneo and around the world have occurred after patients have been exposed to jungle environments or been involved in aquatic sports (Ashford et al., 2000; Barcellos and Sabroza, 2001; Hathaway and Blackmore, 1981; Meng et al., 2009).

The key findings of this study indicate that residents of forested areas having skin wounds, contact with farm animals and the presence of rats are at a greater risk of leptospirosis. This is further compounded if the subjects work with animals or depend on the forest for their livelihood. Skin wounds appear to be the primary mode of infection, with fever as the main symptom, meaning anyone with skin-cuts who complains of subsequent fever should be considered as a potential case of leptospirosis. This research also identified the potential role of rats as a source of leptospiral infection for residents, highlighting the need to make rodent control a priority in disease management. In the multivariable logistic model the findings that owning farm animals or the presence of rats in the vicinity of the participant’s house, together with the results of other studies (Alonso-Andicoberry et al., 2001; Waitkins, 1986), highlight the importance of these species in leptospiral infection of humans. In contrast, as water-related activities did not increase the risk of seropositivity, it suggests that the local waterways may not be contaminated with leptospires at a level likely to cause infection in humans.
6.5 Potential causes of bias and confounding factors in the study

This study was designed to gain information on the seroprevalence to leptospires from a population living and working around wildlife inhabitants. The population was biased, as individuals younger than 18 years of age were not sampled, which may have resulted in overestimation of the seroprevalence by targeting individuals more likely to have exposure to the pathogen through occupational exposure. It is possible that there was also a degree of selection bias with participants who were more concerned about leptospirosis, fever or rats being more likely to participate. It was not possible to quantify the impact of this potential selection bias.

6.6 Conclusions

Although the findings of this study indicate that the intermediate strain Lepto 175 Sarawak might be responsible for asymptomatic infections in Sarawak, there is a need for increased awareness of the risks associated with living close to places where wildlife inhabit and measures should be developed to minimise exposure to these animals. The presence of rodents appears to be a strong risk factor in this instance, and it would be pertinent to keep the population of these animals under control in urban and peri-urban areas. While pets may currently not pose a major threat to the health of local villagers, these animals may become hosts of leptospires acquired from peri-urban wildlife such as rats and bats.
6.7 Future research

Information obtained from this study should be used in the future to design more focused and specific research. Further blood sampling, serology and isolation of the strains from the community and hospital based leptospirosis confirmed cases should be performed to determine whether the infections are current or indicative of previous infection. By doing so, it is also possible to establish, albeit roughly, which particular strains are consistently active in the vicinity. Ideally a prospective cohort study should be conducted with the screening of participants over a period of five to ten years to determine the incidence of infection. If possible, children less than 18 years should be included in a future study as they sometimes accompany their parents into the forest and Everard et al., (1989) suggested that children as young as seven may be infected with leptospirosis. A prospective study should also include a control group from the same villages so as to enable more thorough epidemiological analyses to be conducted.
CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

7.1 Introduction

The focus of this study was to determine the seroprevalence of leptospirosis in the community living around several wildlife habitats and in small wildlife (mammals) located in these wildlife reserves. The impetus for this work came from anecdotal evidence of outbreaks of leptospirosis in and around wildlife reserves in Malaysia and a high seroprevalence to Leptospira in humans and wildlife as discussed in Chapter 2.

Leptospirosis is a major zoonosis that has serious effects on the livestock industry, as well as implications for public health (Bharti et al., 2003). It is endemic in tropical regions and has been associated with the monsoonal seasons and flooding. With the recent growth in eco-tourism, it is pertinent to identify those species of wildlife which may act as potential carriers of the pathogen and to develop strategies to minimise contact between human communities and these wildlife. In Malaysia, there is a potential for an outbreak in Sarawak due to the risk factors prevalent there. However the available data on the transmission of leptospirosis between wild animals and humans is limited (Caley and Ramsey, 2001), despite the significance of the disease in humans. This thesis was designed to further the current knowledge on the likely transmission of leptospires between small wildlife and primates and people who were living in or were associated with wildlife habitats. To investigate
this aim, the focus of the thesis was to survey rodents, bats and primates, including humans, to quantify the seroprevalence of different leptospiral serovars.

7.2 Discussion

7.2.1 General Aims and Chapter Summaries

This thesis is made up of seven parts. The opening chapter and the second chapter provided an overview of the current situation of leptospirosis in the world and gave a brief history of the international research that had been conducted to combat this disease and also explored the link between leptospirosis and eco-tourism in Malaysia, and discussed the possible economic impacts of the disease.

The following chapter discussed the novel strain of Leptospira, Serovar 175 Sarawak. This new serovar was found to be genetically similar to Leptospira wolffii and was classified as an intermediate leptospire, although its pathogenicity has yet to be confirmed. In Malaysia, several other researchers have isolated and identified serovars of Leptospira from humans and animals (El Jalii and Bahaman, 2004). During the 1950’s a group of researchers successfully isolated leptospiral serovars Hebdomadis, Grippotyphosa and Canicola from three species of Malaysian rodents and a serogroup of Icterohaemorrhagiae from Rattus whiteheadi from Sabah (Wisseman et al., 1955). In 2004, researchers discovered a new pathogenic serovar, Lai, from a patient who had returned from Langkawi Island in West Malaysia (Wagenaar et al., 2004). As late as 2009, a new serovar, L. kmetyi Bejo-Iso 9, was isolated from a soil sample in the southern state of Johor (Slack et al., 2009).

In Chapter 4 the tests performed on a small number of captive and free-ranging primate species found in the region surrounding Kuching, Sarawak were described.
Few researchers had previously studied Asian primates, although anti-leptospiral agglutinins had been reported in various primate species conducted in other countries (Bengis et al., 2004; Cantu et al., 2008; Cole and Landres, 1995; Diesch et al., 1970; Hathaway et al., 1981; Kilbourn et al., 2003). Studies by Kilbourn et al., (2003) on free-ranging orangutans in Sabah found a high prevalence of antibodies against *L. interrogans* serovars, notably Grippotyphosa and Autumnalis, as well as *Leptospira wolffii*. In the current work a positive titre of Lepto 175 Sarawak was demonstrated in a proboscis monkey – the first such report in this species.

The results of screening from various small mammal species from the same area for leptospiral antibodies were reported in Chapter 5. The animals screened included rats, squirrels and bats, which have often been implicated as reservoir hosts for the disease. The strong presence of Lepto 175 Sarawak in many of these animals was confirmed. A higher seroprevalence of *Leptospira* in rats and bats was found in this research, confirming the results of other researchers (Cox et al., 2005; Matthias et al., 2005; Smythe et al., 2002a; Tulsiani et al., 2011).

The focus of Chapter 6 shifted to the human population residing either in forested areas or in established communities within the vicinity. The results highlighted the presence of antibodies to Lepto 175 Sarawak in the residents and it was hypothesised that rats were most likely responsible for the spread of this serovar. Studies carried out in Sabah Malaysia, reported a high seroprevalence (25.75%) in people living within the periphery of a national park, presumably due to exposure/contact with wild mammals (Russ et al., 2003). Recently researchers from Universiti Malaysia Sarawak (UNIMAS) and the Sarawak Health Department have conducted research in the Rejang Basin area, Sarawak reporting that 31% of humans sampled were seropositive.
for leptospirosis and infection was associated with farming and/or water activities (Stoye, 2012; Suut et al., 2011).

7.2.2 Epidemiology

Leptospires are known to infect various mammals and colonise their kidneys (Vashi et al., 2010) with rats and bats appearing to be the main reservoir hosts for the disease shedding leptospires into the environment via their urine. Some mammals, however, have been shown to be susceptible to specific serovars (Faine, 1962a). As the main routes of infection are through mucous membranes, abraded skin or the genital tract, infection usually results from contact with urine from an infected animal or leptospire-contaminated soil and water (Faine, 1994). In this study in at-risk communities in Sarawak, it was found that many patients had cuts on their skin highlighting this route of infection. Furthermore many were identified to have had received treatment for fever. One of the major clinical symptoms of leptospirosis is fever, and the non-specific nature of this symptom is one reason why the disease can be difficult to diagnose. Blood (serum) needs to be collected from patients suspected of having leptospirosis and tested using the MAT and ELISA. In Malaysia, only the Institute of Medical Research has the facilities for performing the MAT due to this test’s laborious nature. In contrast ELISAs can be performed at most major hospitals and it is recommended that diagnostic facilities for leptospirosis be expanded in Malaysia, particularly in higher risk areas such as Sarawak. Cross-referencing of results from the MAT and ELISA are needed to determine the infecting serovar of *Leptospira*, which in turn can help identify the potential reservoir host for the bacterium.
Several measures can be adopted to restrict the spread of leptospirosis, with pest control being one of the most important (Mohamed-Hassan et al., 2012). As rats are critical in the spread of the disease (Collares-Pereira et al., 2000), it is vital that communities be educated about the role played by these animals. In areas of human settlement, rats are often seen close to rubbish or food waste. This poses an occupational risk for cleaners and garbage collectors. Reducing the rat population and stopping indiscriminate dumping is one way to minimise the risk of leptospirosis. Poor pest management was reported to be a key factor in the 2012 leptospirosis outbreak in Sibu city, Sarawak (Thayaparan et al., 2013b).

Contact with bats should also be avoided as these have been shown in this study, and in the studies of others (Tulsiani, 2010), to be carriers of leptospires. Most bats live in jungles and seldom come into contact with people, but with increased deforestation and the rise of eco-tourism, humans are increasingly likely to have contact with them. With land clearing bats can now be found on the fringes of human settlements with some roosting in buildings. Herbivorous bats are also commonly associated with fruit trees in people’s homes. The bat’ urine contaminates the ground, which allows leptospires to pass into the bodies of other animals, such as rats, perpetuating a sylvatic cycle of infection (Tulsiani, 2010).

The Malaysian government has implemented restrictions on bat hunting (Epstein et al., 2009), making it difficult for bats to be culled as a pre-emptive measure for control of the disease. However the culling of such mammals, although potentially beneficial for restricting the spread of leptospires to humans, would impact negatively on the ecosystem (Chivian, 2002; Horan et al., 2010).
The rise of ecotourism in East Malaysia has increased the potential for tourists to have direct contact with wildlife infected with leptospires or soil and groundwater contaminated with the bacterium. In the current study eight of 12 captive and free-ranging primates at Matang Wildlife Centre and Bako National Park were seropositive to leptospires and could be potential sources of infection for visitors to these establishments. Quite often, the symptoms of leptospirosis manifest after the tourists have returned to their country due to the incubation period (4-14 days) (Mortimer, 2005; Reisberg et al., 1997). As leptospirosis is not really well-understood by many western physicians, misdiagnosis may result and inappropriate treatment be prescribed.

Wildlife species that are undergoing rehabilitation should also be screened annually for leptospires to avoid introducing the disease into the wild populations when they are eventually released.

7.2.3 Leptospirosis status in Sarawak

Leptospirosis is subjected to mandatory reporting in Sarawak due to its pathogenic nature. In fact, almost all states in Malaysia have had problems keeping leptospirosis in check (Hakim, 2011). Figures obtained from the country’s health department show a recent increase in the number of confirmed cases of leptospirosis in Sarawak, as well as a high case fatality rate (Hakim, 2011) and several well-documented outbreaks have been reported (Thayaparan et al., 2013b). Access to better diagnostic methods may help to reduce the case fatality rate of leptospirosis in Malaysia, however conversely it may also result in increased reporting. The recent increase in the number of cases in Sarawak could be due to the implementation of mandatory reporting for the disease in 2010 (Hakim, 2011) as well as increased awareness by medical practitioners, rather than a real increase in the incidence of disease. With the
discovery of the new serovar Lepto 175 Sarawak in a sample of water collected from Sarawak, there will be increased pressure on the State health authorities to control the disease. Although, serovar Lepto 175 Sarawak is currently considered to be an intermediate strain, its characteristics are similar to that of *Leptospira wolffii*, which has been identified as the main cause of recent outbreaks in the neighbouring country of Thailand as well as in India and Iran (Slack et al., 2008; Zakeri et al., 2010).

### 7.2.4 Risk factors in the spread of leptospirosis

Leptospirosis is often regarded as an occupational disease. Previous research has identified farmers, meat industry workers, cleaners and veterinarians as being at high-risk of acquiring the disease due to the nature of their work (Waitkins, 1986). In recent years the popularity of adventure travel and eco-tourism in Asia has meant that Western tourists are now considered to also have a higher risk of acquiring leptospirosis. Poor knowledge about disease, control measures and prevention could also be considered a factor in cases of human infection (Waitkins, 1986). Communities with poor sanitation have also been shown to have a higher risk of infection, primarily due to the presence of rodents (Costa et al., 2014). In the current study in Sarawak, a high seroprevalence was observed in communities living near forested areas (Chapter 6) and the presence of livestock or rats near the participant’s property increased the likelihood of infection. The seroprevalence was higher in males than in females and this is likely to be associated with occupation and personal hygiene habits rather than an innate higher susceptibility in males (Ashford et al., 2000; Ciceroni et al., 2000; Kawaguchi et al., 2008).
7.3 Need for further studies

This research has uncovered evidence of a high leptospiral seroprevalence in proboscis monkeys (Chapter 4), although only few animals were sampled. This is despite their arboreal lifestyle and the fact that they only feed on vegetation. Other primates, such as macaques, were found to have high titres to the novel serovar Lepto 175 Sarawak. It is known that macaques are scavengers and may have been exposed to the bacterium after rummaging through leftovers and garbage. Further study as to whether macaques may be perpetuating a sylvatic cycle of infection needs to be undertaken. The possibility of non-human primates acting as reservoir hosts also needs to be explored.

In Chapter 6, the survey of communities living near forested areas has produced some interesting, albeit inconclusive results. It seems likely that many of the people surveyed may have acquired the infection several years ago and a prospective cohort study is required to determine the incidence of infection. Follow-up studies should also determine which serovars induce antibodies that remain the longest in humans to help confirm the extent of leptospirosis in humans residing in Sarawak.

7.4 Conclusions

This study has highlighted that leptospiries are a potential threat to public health in Sarawak, and a number of risk factors associated with human infection were identified.

Educating local communities about the disease and methods to control it, particularly the role of rodents in the disease, is critical. More also needs to be done
to prevent exposure to wildlife so as to reduce the infection rates in these animals. At present, there is a significant potential for a major outbreak of leptospirosis on the outskirts of Kuching and measures should be adopted to minimise this risk.
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APPENDIX 1- Leptospirosis Risk Assessment Survey in Malaysian Patients and Participants Questionnaire

(Confidential Data)

Instructions: Please tick or circle the most appropriate options and/or fill in the necessary information as required

A. Interviewer details

i. Interviewer’s Name

ii. Mobile #

B. Respondent’s details

i. Participant/Patient

ii. Ethnic group:

iii. City:

iv. State /Sub district

vi. Contact details:

1. Participant/Patient Sex:

   a. Female       b. Male

2. Participant/Patient Age
3. What level of education did you complete?

   a. No schooling
   
   b. Primary school
   
   c. Secondary school
   
   d. Tertiary education

4. What is your main occupation?

   a. Farmer
   
   b. Wildlife researcher/ work around forest
   
   c. Veterinarian
   
   d. Military personnel/ National service participant
   
   e. Cleaner

   Others please specify………………………….

**SYMPTOMS AND SIGNS OF LEPTOSPIRAL INFECTION**

5. Have you had a fever within the last 3 months?

   a. Yes
   
   b. No

   If yes which hospital/clinic did you visit?  …………………………………

6. Do you have/had any skin wounds on your hands or legs within the last 3 months
7. Have you received any treatment for leptospirosis in the past year:
   a. Yes  b. No

If yes please provide the name of the hospital/clinic……………………………

EXPOSURE TO ANIMALS OR PETS

8. Do you have any pet animal/s at home?
   a. Yes  b. No

If yes what type of pets do you have?  ………………………………………....

9. Do you have any farm animal/s at home/farm?
   a. Yes  b. No

If yes what type of farm animal/s?  ……………………………………….....

10. Do you have a problem with rats around your house/farm?
    a. Yes  b. No

11. Have you seen rats around your house or farm more than three times in one day?
    a. Yes  b. No

OUTDOOR ACTIVITIES AND POTENTIAL EXPOSURE TO LEPTOSPIRA
12. Have you traveled overseas within the last month?
   a. Yes  c. No
   If yes which country/countries did you visit? ...........................................

13. Have you undertaken any water sports (eco-challenge activities like boating, canoeing, running and cycling along stagnated water areas) within the last three months?
   a. Yes  c. No
   If yes what sport? .................................................................
   In which area? .................................................................

14. Have you been camping within the last three months?
   a. Yes  b. No
   If yes in which area? .................................................................

15. Have you been swimming in a lake within the last 3 months?
   a. Yes  b. No
   If yes in which lake/area? .................................................................

16. Have you done any water rafting activities in the last 3 months?
   a. Yes  b. No
   If yes in which area? .................................................................

POTENTIAL SOURCE OF INFECTION
17. What is your usual source of drinking water?

a. Stream water  
b. Tube well  
c. Municipal chlorinated tap water  
d. Rainwater  
e. Other please specify………………………………………………..

18. Do you usually drink treated/boiled water?

a. Yes  
b. No  

Thank you for completing this survey. Leptospirosis is a zoonotic disease found in wildlife, domestic animals and humans. This disease is caused by a bacterium called a Leptospira. With the rise of ecotourism and related activities, recreational exposure to leptospira has emerged as a concern to global health. This research is attempting to identify those risk factors that can lead to infection of humans with leptospirosis in Malaysia. Your participation is greatly appreciated to allow this research to be completed successfully.
Leptospirosis is a disease found in wildlife, domestic animals and also humans. It is caused by a type of bacteria or germ called a Leptospira. This disease is believed to be common in Malaysia but little work has been done in this country to identify factors that lead to the disease. This research is designed to confirm if there is a link between wildlife and disease in humans in Malaysia. Your participation in this research is greatly appreciated.

1. I confirm that I meet the criteria for participation in this study, namely:
   □ I am over the age of 18 years.

2. I agree voluntarily to take part in this study.

3. I have read the Leptospirosis Information Sheet provided and have been given a full explanation of the purpose of this study, of the procedures involved and of what is expected of me. The researcher has answered all my questions and has explained the possible problems that may arise as a result of my participation in this study.

4. I understand I am free to withdraw from the study at any time without needing to give any reason.

5. I understand I will not be identified in any publication arising out of this study.

6. I understand that my name and identity will be stored separately from the data, and these are accessible only to the investigators. All data provided by me will be analysed anonymously using code numbers.

7. I understand that all information provided by me is treated as confidential and will not be released by the researcher to a third party unless required to do so by law.

8. I agree to be contacted by phone two weeks from now for a brief follow up survey. I would like to be contacted at the following phone number ______________________ (daytime / evening) for the purpose of this survey.

9. I would like to receive a copy of the feedback from the study. Please contact me at ____________________________
10. ☐ I consent to be photographed during the sample collection for presentation purposes.
☐ I am not willing for this session to be photographed.

Signature of Participant: __________________________  Date: ........../....../......

(Name)

Signature of Investigator: __________________________
  Date: ........../....../......
SHORT COMMUNICATION

Serological Prevalence of Leptospiral Infection in Wildlife in Sarawak, Malaysia

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ABSTRACT

Leptospirosis is a zoonotic disease caused by pathogenic leptospiral bacteria, which are transmitted directly or indirectly from animals to humans or animal to animal. The first phase of this proposed study was carried out to determine the extent of exposure to leptospirosis in wild mammals surrounded by human settlements around wildlife or tourism area (Wind Cave, Fairy Cave, Bako National Park and Matang Wildlife Center). This study reports an incident of leptospirosis among primates (three captive and two free ranging), rats, bats, squirrels and mongoose around Kuching, Sarawak area, which has been screened for Leptospirosis. Blood samples were obtained to determine the presence of antibodies through the microscopic agglutination test (MAT) using eighteen serovars of Leptospira commonly found in Malaysia as antigens. It was observed that four out of the five monkeys (80%), rats (9/4) (44%), bats (20/5) (20.8%), squirrels 4/4 (100%) and mongoose (1) (100%) reacted against one or more serovars of Leptospira. In this study antibody of five serovars of Leptospira interrogans Copenhegeni, Leptospira interrogans Lai, Leptospira interrogans Pomona, Leptospira interrogans Pyrogenes, Lepto 175* were detected. Serovars Copenhegeni, Lai, Pomona and Pyrogenes were considered pathogenic for different mammals including human beings. No information about serovars lepto 175 and further studies going on. This is providing information on the possible zoonotic importance of mammalian species in maintaining this disease in Sarawak. The transmission of leptospires in rats reported several incidents and between primates, bats, squirrels, mongoose and human is not reported elsewhere but this could create new reservoir and transmission routes and may affect the tourism, conservation effort and public health.

Keywords: Leptospirosis, wildlife, mammals, Sararawak, Borneo

Leptospirosis can affect both humans and animals throughout the world resulting in morbidity and mortality (Russ et al. 2003; WHO Headquarters, Geneva, 2006). The important epidemiological feature of leptospirosis in domestic animals and wildlife can lead to economic loss and potentially spread to the human communities.

Leptospirosis can be transmitted in human by direct contact with infected blood, tissues, organs or urine of infected hosts or through indirect contact with contaminated formites, soil, mud, fresh water, vegetation and food stuffs or working in places infected with rodents (Terpstra 2003; Zavitsanou & Babatsikou 2008). Transmission can also occur via the direct penetration of the leptospires through the conjunctiva or surface epithelium (Russ et al. 2003). The role of rats as a source of human infection was discovered in 1917 (Levett 2001) and subsequently some researchers have identified that flying foxes can carry pathogenic leptospires in Australia (Cox, Smythe, & Leung 2005; Smythe et al. 2002). The bacteria can cause polymorphic disease conditions in wild, domestic animals and in human (Terpstra 2003). However, to date there has been little research on the role of wildlife in outbreak throughout the world. Due to the current significant levels of reforestation occurring and the involvement of humans in the jungle, there is the potential for exposure of humans to new serovars of leptospires.

Trapping of monkeys, rats, bats, squirrels and mongoose were carried out around Wind Cave, Fairy Cave, Bako National Park and Matang Wildlife Center to determine the extent of exposure to leptospirosis in wild mammals around human settlements. This study reports an incident of leptospirosis among primates (three captive and two free ranging), rats, bats, squirrels and mongoose around Kuching, Sarawak area, which has been screened for Leptospirosis. Blood samples were obtained to determine the presence of antibodies through the microscopic agglutination test (MAT) using eighteen serovars of Leptospira commonly found in Malaysia as antigens. It was observed that four out of the five monkeys (80%), rats (9/4) (44%), bats (20/5) (20.8%), squirrels 4/4 (100%) and mongoose (1) (100%) reacted against one or more serovars of Leptospira. In this study antibody of five serovars of Leptospira interrogans Copenhegeni, Leptospira interrogans Lai, Leptospira interrogans Pomona, Leptospira interrogans Pyrogenes, Lepto 175* were detected. Serovars Copenhegeni, Lai, Pomona and Pyrogenes were considered pathogenic for different mammals including human beings. No information about serovars lepto 175 and further studies going on. This is providing information on the possible zoonotic importance of mammalian species in maintaining this disease in Sarawak. The transmission of leptospires in rats reported several incidents and between primates, bats, squirrels, mongoose and human is not reported elsewhere but this could create new reservoir and transmission routes and may affect the tourism, conservation effort and public health.

Keywords: Leptospirosis, wildlife, mammals, Sararawak, Borneo

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Cave, Fairy Cave, Bako National Park and Matang Wildlife Center. Rats, squirrels and bats were trapped alive, anaesthetized and blood extracted by cardiac puncture. Serum samples were kept at -20°C until perform the Microscopic Agglutination Test (MAT). To screen the status of Leptospira, three captive primates (Macaca nemestrina, Hyllobates muelleri, Macaca fascicularis) and two free ranging primates (Presbytis cristata, Nasalis larvatus) have chosen for this study. 5 ml of blood collected in plain tube for the serum. After 15 min plain tube centrifuged at 15,000 RPM for 5 minutes and serum separated and stored under -20°C for MAT. The mongoose caught accidently in cage trap included into this screening and collected 5ml blood from cephalic vein and serum separated and stored under -20°C before screen for leptospirosis antibodies.

The Microscopic Agglutination Test was performed according to Faine (1982) to check for Leptospira-specific antibodies from non-human primates, rats, bats, squirrels and mongoose. Serum was tested against 18 serovars using the following serovars: Australis, Autumnalis, Bataviae, Canicola, Copenhageni, Celledoni, Shermani, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Patoc, Pomona, Pyrogenes, Sejroe, Lai and Lepto 175 (Sarawak) commonly found in Malaysia. Serum positive at titre 1:50 was further titrated until 1:1600. Sera were considered to be positive if the titre was ≥ 1:50 by MAT.

A total of five primates, nine rats, 20 bats, four squirrels, and one mongoose were caught during the first phase of sample collection around wildlife area. Four out of the five monkeys (80%) Macaca nemestrina, Hyllobates muelleri, Presbytis cristata, Nasalis larvatus showed the positive titre and Macaca fascicularis did not show any titre. Rats (9/4) (44%), bats (20/5) (20.8%), squirrels (Callosciurus notatus) (4/4) (100%) and mongoose (Herpestes brachyurus) (1) (100%), were positive for leptospiral antibodies from their serum. From the positive cases 72% were showed the positive titer with Lepto 175, 33% with Lai, 17% with Pomona, 11% with Copenhageni and 11% with Pyrogenes. Detailed information about antibodies indentified in different species shown below.

Table 1. Findings of antibodies of different leptospira serovars in particular species.

<table>
<thead>
<tr>
<th>Order/Species</th>
<th>sv.lep175*</th>
<th>sv.copen.</th>
<th>sv.lai.</th>
<th>sv.pom.</th>
<th>sv.pyro.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macaca nemestrina</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hyllobates muelleri</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trachypithecus cristatus</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nasalis larvatus</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sundamys muelleri</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ratus argentiventer</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cynopterus brachyotis</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Penthetor lucasi</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Nycteris tragata</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hipposideros cervinus</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Callosciurus notatus</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Herpestes brachyurus</td>
<td>+</td>
<td>-</td>
<td>+</td>
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</table>

Sv.copen- copenhageni, sv.lai.- lai, sv.pom.-pomona, sv.pyro.-pyrogenes, svlep175.- lepto175. *Leptospirosis Lepto 175 (Sarawak) strain was isolated from Sarawak environmental source and no further information on this strain and further studies is going on in Institute for Medical research (IMR) Kuala Lumpur.

Malaysia is endemic to Leptospira (Sejvar et al. 2003). In recent decade Malaysia is becoming famous for Eco-tourism and many tourist from various countries are visiting for Eco-challenge activities and wildlife tourism. Screening the wildlife around the wildlife area clearly showing there is a source of infection around those areas and it could cause direct transmission to humans or it could transmit from animals to human. Rats are well known
carrier of leptospirosis and spread among other animals and human. But bats, squirrels, mongoose and monkeys are not known about their carrier status and need more studies to explore their carrier status. A positive result on proboscis monkey is alarming the situation and its first time reported this case in Malaysia. The information on Lepto 175 (Sarawak) is unknown and its endemic in Sarawak Malaysia. The status of its pathogenic or not is not known and further studies is going on by Institute for Medical Research Kuala Lumpur, but the serovars Lai is pathogenic and its major cause of zoonotic spread from adventure activities, such as rafting, cannonining, and swimming in river (Sejvar et al. 2003). Past history of the human cases such as in 1984 there were two teams of cave explorers from British confirmed with Leptospira infection after their return from Mulu cave, Sarawak (Mortimer 2005). Again in 1985 another two British tourist confirmed with Leptospira after their return from Sarawak. After the 2000 Eco-challenge in Sabah, several participants from USA, UK, Australia and New Zealand are confirmed with Leptospira infection (Sejvar et al. 2003). This all incidents clearly indicating that Leptospira infection might be going to affect the tourism industry in future. It is best to find out the source of infection and take the preventive measures from Leptospira infection before getting any major outbreak.

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Leptospirosis, an emerging zoonotic disease in Malaysia

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Abstract

Leptospirosis is an endemic disease in Malaysia and recently has received increasing attention mainly due to several recent incidents that have resulted in human mortality which have alarmed health professionals in Malaysia. The increasing incidence of leptospirosis in forested regions is associated with the bacteria infecting small wild mammals other than rats. Infection in wildlife could result in the introduction of new serovars to humans and domesticated animals. More research on leptospirosis and the screening of wildlife and humans near wildlife habitats is required to have a better understanding of the involvement of wildlife in the disease.

Key words: infectious disease, leptospirosis, zoonotic disease, wildlife

INTRODUCTION

Leptospirosis, caused by infection with Leptospira interrogans, is a worldwide disease affecting both humans and animals resulting in morbidity and mortality.1 The agent can be transmitted both directly and indirectly.2,3 In 1917, the role of rats as a source of human infection was first discovered,4,5 and subsequently some previous studies have demonstrated that other wild mammals can also act as potential carriers,6,7 including flying foxes.7,8 However, to date there has been little research conducted on the role of wildlife in outbreaks. As a result of the current significant level of deforestation and the increasing anthropogenic activities in the forested habitats and jungle, humans are at a greater risk of being exposed to new serovars of leptospires. Leptospirosis in wildlife can have negative consequences for biodiversity, human and livestock health, animal welfare and the economy of a country.9,10 This review will examine the impact of leptospirosis on wildlife, the current status of surveillance and the options to strengthen policies in Malaysia.

Morphology and taxonomic classification

Leptospires are spirochaetes in the order Spirochaetales, family of Leptospiraceae and include two genera, Leptospira and Leptonema.11 Leptospira are obligate aerobes with an optimum growth temperature ranging from 28°C to 30°C.12,13 The genus Leptospira was divided into two species based on serological classification: Leptospira interrogans, which comprises all the pathogenic strains and Leptospira biflexa, the environmental saprophytic strains.14-16 Based on the microscopic agglutination test (MAT), leptospires can be further divided into over 250 serovars. Serovars that are antigenically similar have been aggregated into serogroups.14 Furthermore both pathogenic and non-pathogenic serovars can be classified within the same species. A combination of methods is now used to confirm the species of Leptospira.

Pathogenesis

The most adverse clinical and pathological signs usually occur in young animals, especially when their maternally-derived immunity is waning.17 However, the common signs of Leptospira infection in farm animals are abortions, stillbirths, decreased milk production and a failure to thrive.18,20 In all species, congenital infection and its sequelae are well reported. The most important difference between infection of animals and humans is the presence of chronic carriers.
in animals through reservoirs in the kidneys and genital tract.20-23

Epidemiology of leptospirosis

Leptospires are widespread and their abundance is due to their ability to infect a range of animal species, including humans, as well as the ability to survive outside the host, if environmental conditions are favourable.24-26 The main sources of infection are urine of infected or carrier animals, contaminated surface water, mud and soil.5 Transmission can happen as a result of direct or indirect contact with infected animals or their secretions.4 The major route of infection is via mucous membranes, however carnivores can be infected through the ingestion of leptospiral-infected carcasses.27 Usually leptospires appear in the blood four to 10 hours after infection and can be detectable in the blood from a few hours to seven days.28-30 Clinical signs may not occur in every case but severe fever is an important sign of acute leptospirosis. Animals that have recovered from leptospirosis may become carriers with the organism present in the renal tubules for periods of days to years,31,32 and subsequently leptospires are shed in urine into the environment.32

Impact on humans, domestic animals, wildlife and ecotourism

Infectious diseases are transmitted globally through animal and human movements due to eco-tourism, wildlife research, reintroduction, rehabilitation, hunting, pet trade, laboratory and food industry demands and farming.33,34 These movements and activities are major contributing factors for the transfer of leptospirosis to animals and humans and the spread of the disease to new areas.

Research on leptospirosis has highlighted that rodents (20%), marsupials (35%) and bats (35%) are most likely to spread the disease.35 Marsupials and chiropterans have been implicated as more significant reservoir hosts of leptospires pathogenic to humans than previously was recognized.35 Studies on leptospirosis involving bats have produced equivocal results,7 however the fact that leptospires can potentially be spread through bats is a major concern due to their abundance, their ability to travel long distances and their potential to expose humans to infected urine. Ground-dwelling species, such as rodents foraging under bat roosts, could also encounter Leptospira-contaminated urine and spread the organism into urban areas.36 From the limited research on wildlife, several pathogenic serovars have been isolated with the most prominent serovars including *grippotyphosa* and *pomona* from white-tailed deer (*Odocoileus virginianus*) in North America.37 Surveillance of leptospirosis is important to determine the emergence of new strains, which have the potential to cause an outbreak. Although it is not economically viable to survey all wildlife species, it is important to regularly screen particular species, which live on the periphery of forests and have the potential to interact with humans (e.g. wild rats, carnivores and bats). Screening these ‘sentinels’ provides useful cost-effective information on the health status of a broader range of species.

Leptospirosis can cause serious economic loss to the livestock industries38,39 and is a major cause of abortions in cattle.40,41 As the disease is common in domestic animals and wildlife, control in domestic animals can also have a positive impact on the disease in wildlife.

Worldwide there have been more than 184 distinct serovars of *L. interrogans* belonging to 20 serogroups identified.42 In Southeast Asia, the most common serovars associated with disease of domestic animals and humans are *icterohaemorrhagiae, autumnalis, canicola, pomona, patoc, grippotyphosa, australis* and *poi*.43,44 The first three are the most important serovars with respect to veterinary and public health perspectives. Fever is an important and common presentation of tropical diseases and may sometimes be the only manifestation of a serious illness. Diagnosis is often difficult because of the many diagnostic possibilities, and symptoms, which may often be non-specific, and because of many doctors’ unfamiliarity with the spectrum of tropical diseases. Treating the disease appropriately will reduce the risk to public health, and this can only come following a correct diagnosis. With the recent popularity of adventure travel and triathlete competitions, there is an increased awareness of the potential for leptospiral infection amongst those who partake in such activities.45,46

**Laboratory diagnostic methods used in Malaysia to diagnose leptospirosis**

Diagnosis of leptospirosis depends on adoption of appropriate laboratory methods as the clinical presentation can vary greatly. The diagnostic method selected depends on the available samples and the purpose of testing. Identification of the infecting serovar is important
both epidemiologically and clinically, since this may assist in determining the source and likely outcome of infection. Different assays have been developed in an attempt to diagnose leptospirosis accurately, but the majority are unsuitable for use in developing countries due to their requirement for the maintenance of multiple strains or expensive equipment. Several methods are used in the diagnosis of leptospirosis in Malaysia. These include the simple Microscopic Agglutination Test (MAT); Polymerase Chain Reaction (PCR) test, as well as the Enzyme-linked Immunosorbent Assay (ELISA). The MAT is inexpensive, but time consuming and laborious, and is only carried out at the Institute for Medical Research (IMR) Kuala Lumpur, MKAK (Makmal Kesihatan Awam Kebangsaan) Sungai Buloh Malaysia for community outbreaks and also at some universities undertaking research on leptospirosis. ELISAs are performed at hospitals with the necessary facilities and the IMR and MKAK. Currently culture of *Leptospira* is only performed at the IMR. Diagnosis of animal leptospirosis is only undertaken at the Veterinary Research Institute, Ipoh and the Wildlife Department, Kuala Lumpur (PERHILITAN). Environmental samples can be tested in MKAK, Sungai Buloh, Malaysia.

**Treatment, control and prevention of leptospirosis**

A range of antibiotics are used to treat hosts with leptospirosis, with IV C-Penicillin (2M units 6 hourly for 5-7 days) being commonly used in severe adult human cases. The less severe cases are usually treated orally with antibiotics such as doxycycline, tetracycline, ampicillin or amoxicillin. A trial on the penicillin derivative, doxycycline, on the prevention of infection and clinical disease was conducted in the North Andaman Islands in 1999. Although the findings indicated that doxycycline prophylaxis did not prevent leptospiral infection in an endemic area, such treatment did have a significant protective effect in reducing the morbidity and mortality during outbreaks.

Cases of naturally occurring leptospirosis in wildlife have not been documented and little is known about the immunological features of the disease in wild mammals. However researchers have demonstrated antibodies to several serovars that have been found in a range of wildlife species (non-human primates, bats, rats, squirrels and mongoose) in Kuching Sarawak, Malaysia. These animals are capable of being infected with one or more leptospiral serovars and can then serve as reservoir hosts for these serovars. These animals have an asymptomatic and transient infection with no obvious clinical signs of disease.

**History of leptospirosis and geographical distribution**

Leptospirosis has been documented worldwide, but formal reporting systems vary widely. Frequently the disease gains public attention when outbreaks occur in association with natural disasters, such as flooding in Nicaragua in 1995 or among foreign travellers and extreme athletes. Southeast Asia is an endemic area for leptospirosis, and infection in humans has been reported throughout the region. Seventy percent of the major pathogenic serovars have been isolated from Asia. Although leptospirosis has been around for millions of years, it is only in recent times that the bacterial cause of this disease has been identified. In 1886 Adolf Weil published an account detailing the icteric form of leptospirosis, which has since taken on his name. However, his observations had already been postulated by others, including Hippocrates. It has been hypothesised that leptospirosis was responsible for an epidemic among the natives of the Massachusetts coast just before the arrival of the Pilgrims in 1620. What Weil described was the icteric form of leptospirosis with jaundice. In contrast the milder forms were hard to diagnose due to a lack of advanced bacteriology. Spirochaetes continued to cause problems for the later part of the 19th century, and it was only in 1907 that Stimson managed to isolate a leptospire from a patient. His subsequent research highlighted that the bacteria were concentrated in the renal tubules and were shaped like question marks. This gave rise to the name “Spirocheta interroogans”, which has remained.

**Status of leptospirosis in South East Asia**

Leptospirosis is emerging as a serious concern to public health in Southeast Asia. The disease has been recognised in patients from Indonesia with clinical jaundice and non-malarial fever, patients with clinical jaundice from Laos and Vietnam, and patients with haemorrhagic fever in Cambodia. In addition, a cross-sectional community-based study was conducted in Laos to obtain estimates of the background
sequence typing of pathogenic Bandicota bengalensis for the outbreak and was being maintained by confirmed serovar Autumnalis was responsible in Pahang and reported the lack of specificity in the sampled dogs. It was possible that the dogs acquired their infection from rats, which were responsible for an outbreak in north-eastern Thailand between 1999 and 2003. During that period, Thaipadungpanit et al. developed a scheme of multi-locus sequence typing of pathogenic Leptospira and confirmed serovar Autumnalis was responsible for the outbreak and was being maintained by Bandicota bengalensis (bandicoot rats).

Besides infecting local residents, Leptospira can also cause illness in visitors to tropical regions, particularly those associated with eco-tourism and adventure travel. Van Crevel et al. investigated leptospirosis in 32 Dutch travellers between 1987 and 1991 and found 28 of them had returned from Southeast Asia with the majority visiting Thailand and 21 of these had taken a rafting tour. From this research it was recommended that doctors should consider leptospirosis in their differential diagnosis whenever a patient with fever returned from the tropics. Due to the increasing number of reported cases of leptospirosis in the western world, it has been recommended that travellers try to avoid high-risk aquatic activity in the tropics and undertake chemoprophylaxis if they do take part in water-sports in Southeast Asia.

In Thailand, researchers conducted a serological survey of workers who had been involved in cleaning a pond in Khumuang, Buriram Province. Multivariable logistic regression indicated that wearing long pants or skirts was protective against leptospiral infection, while the presence of more than two wounds on the body was associated with infection. In Malaysia isolation of Leptospira from black rats (Rattus rattus) was reported in 1928 by Fletcher. Many subsequent investigations have demonstrated a high prevalence of infection in humans in Malaysia, with Robinson and Kennedy, reporting 31 cases among British army personnel in Malaysia. From 1953 to 1955, 30 pathogenic leptospiral serovars were identified by Alexander from both military personnel and civilians. Their studies demonstrated a high sero-prevalence in humans throughout Malaysia. The highest distribution was found in labourers working in rubber estates and those working with the sewage, drainage, forestry and town cleaning industries. In the 1950s and 1960's a comprehensive study of leptospirosis in Malaya was undertaken that included testing various mammals from a range of environments, as well as assessing occupational risks to humans. The results suggested that rats were the main maintenance hosts for leptospirosis, despite the presence of infection across many animal species. Over one hundred (104) strains were isolated and identified. Bahaman et al. conducted a cross-sectional serological survey of domestic animals in West Malaysia and found that approximately one quarter of the animals examined had agglutinating antibodies to L. interrogans. Cattle, buffaloes and pigs were all observed to have a high seroprevalence with temperate cattle breeds appearing more susceptible to infection than local breeds. A subsequent study found that the bacteriological seroprevalence in cattle and buffaloes was 14.4%. A new serovar was isolated from a bovine kidney, while six other serovars were isolated for the first time from Malaysian cattle. Serovar hardjo was shown to be maintained in Malaysian cattle. Recently leptospirosis has been reported in detention centres for refugees in Malaysia. An outbreak at Jurong Detention Centre was contained by the health authorities, but the death of six Burmese at an undisclosed detention centre in September 2009 has raised fresh concerns for several Malaysian NGOs. In July 2010, cases of leptospirosis were reported nation-wide and eight people who took part in a search and rescue operation in Lubok Yu, Maran, Pahang died from the disease, causing...
Leptospirosis

Other cases were reported in Kedah during July 2011, with one fatality at Lata Bayu.74 As recent as March 2012, a national service trainee died of suspected leptospirosis at Sungai Siput in Perak, Malaysia.75 This resulted in a suspension of water based activities at all National Service Training camps.

According to data from the Ministry of Health the prevalence of leptospirosis increased dramatically from 2004 to 2009 (Table 1), however the number of deaths from leptospirosis did not change from 2004 to 2007, although it increased markedly to 47 and 62 deaths in 2008 and 2009, respectively. The highest numbers of cases were reported in the state of Perak during 2005, 2006, 2008 and 2009 with 71, 93, 289 and 280 cases, respectively (Table 1). In 2004, more cases32 were reported in Sarawak than in other years and in 2007 the highest numbers of cases (184) were reported in Pahang. The states of Sarawak, Selangor and Terengganu showed a gradual increase in the number of cases over this period. No cases were reported in WP Labuan owing to its small geographical area with no forest habitat and the fact that it is surrounded by sea-water.

Case fatality rates (CFR) over the period from 2004 to 2009 varied from 1.8% to 7.6% (Figure 1) with an average of 4.44%. During this period a total of 5,267 cases of leptospirosis were reported nationwide with 234 known fatalities. Perak had the highest CFR for this period (6.81%), followed by Sarawak (6.42%) and Perlis (6.25%). Approximately one-fifth of all cases of leptospirosis for this period were documented in Perak state, of which 71 proved fatal (Figure 2). The almost threefold increase in the number of cases in 2007 and subsequently may be due to improved diagnostic techniques.

FIG. 1: Case Fatality Rate (CFR) of leptospirosis in Malaysia from 2004 to 2010.

FIG. 2: Leptospirosis CFR in different Malaysian states from 2004 to 2009.
Table 1: Status of leptospirosis in humans in Malaysia from 2004 to 2009

<table>
<thead>
<tr>
<th>State</th>
<th>2004</th>
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<th>2006</th>
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<th>2008</th>
<th>2009</th>
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<td>Deaths</td>
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Sources: (3)
or a greater awareness of the disease. This may also have contributed to reducing the CFR from 2004. It is also possible that climate change in the last four years of this period played a part in influencing the pattern of infection, as outbreaks of leptospirosis usually follow after a major flood during monsoon seasons.

**Status of leptospirosis in Sabah and Sarawak**

As well as Peninsular Malaysia, scientific research has also focused on Malaysian Borneo. There have been reports of leptospirosis in humans in Sabah and Sarawak, notably an outbreak following the 2000 Eco-challenge event in Sabah.\(^78,79\) This highlighted the risks of eco-tourism and adventure travel, as most of the victims were western athletes. In the Eco-challenge event, participants competed in jungle trekking, swimming and kayaking in freshwater and caving. A team of researchers from the CDC Atlanta contacted 189 athletes from 27 countries following the outbreak.\(^79\) Eighty athletes met their case definition, with 29 cases being hospitalized, although no fatalities were reported. Another study in Sabah revealed that swimming in the Segama River appeared to be a risk factor for infection and 20 athletes reported taking doxycycline prophylactically and this seemed to have a protective effect, with a preventive efficacy of 55%.

Studies carried out in Sabah reported a high seroprevalence (25.75%) in people living within the periphery of a national park, presumably due to exposure/contact with wild mammals.\(^9\) Leptospirosis has also been reported in a caver from the USA after he returned from Gunung Mulu National Park, in Sarawak.\(^52\) Recently researchers from Universiti Malaysia Sarawak (UNIMAS) and Sarawak Health Department have conducted research in the Rejang Basin area, Sarawak. They found that 30.6% of humans sampled were seropositive for leptospirosis and infection was associated with farming and/or water activities.\(^80\) In Bakun in Sarawak, a Chinese national working on the hydro-electric dam project site was hospitalised with a serious condition suspected to be leptospirosis, and the subsequent death of 10 workers involved in the translocation of animals from the Bakun Hydroelectric Dam water catchment area to higher ground were speculated to be due to either leptospirosis or melioidosis.\(^81,82\)

In 2011, 186 cases of leptospirosis were reported in humans in Sarawak including 13 deaths, compared with 49 cases in the previous year.\(^83\) The CFR associated with this outbreak (6.9%) was higher than that in previous years. On the 12\(^{th}\) December 2011, an outbreak occurred at RSAT Army camp at Penrissen Batu 8, Kuching, Sarawak with five army recruits showing symptoms of leptospirosis and the disease was subsequently confirmed serologically. The source of infection was identified as drinking and bathing activities in the small river near the camp.\(^84\) The Kuching Divisional Health Office was notified of another suspected leptospiral outbreak on the 30\(^{th}\) December 2011 by the Sarawak General Hospital. The incident involved two army recruits from Blok G10, Kem Semenggok, Kuching and both were serologically positive for leptospirosis.\(^85\) The health authority of Sarawak confirmed a leptospirosis outbreak in the drains along the Tiong Hua Road in Sibu during June 2012.\(^86\) In the state of Sarawak, the number of cases of leptospirosis was similar from 2004 to 2010, however in 2011 and 2012 there was a large increase in the number (Figure 3).

The four-fold spike in the number of cases in Sarawak is a valid cause for concern for health authorities in both Kuching and Kuala Lumpur. For 2011 alone, the CFR was 6.9%, above the six-year average of 6.42% (2004 and 2009). The number of cases (186) reported in 2011 alone was already equivalent to 60% of the combined total from 2004 to 2009. The record high of 271 documented cases the following year may be attributed to mandatory reporting procedures being implemented, or increased awareness of the symptoms of leptospirosis by medical practitioners. These also reflect on the living conditions of residents in various areas of Sarawak.

**Conclusions**

Over the years, leptospirosis has presented itself intermittently to the Malaysian health authorities and further research has consequently been conducted. The biggest issue for doctors has been the confusing nature of symptoms presented by affected humans. Since leptospirosis is a febrile illness and several tropical diseases manifest in their early stages with fever, it is easy to misdiagnose the disease unless further laboratory testing is undertaken. This can result in delayed commencement of appropriate treatment (single dose doxycycline therapy). Fortunately with advances in medical technology,
diagnosis of leptospirosis has become more accurate. Currently there is much research being undertaken on leptospirosis, however due to poor coordination of this research the actual incidence of disease is not truly reflected in Malaysia. There is a need for cooperation between researchers from veterinary and medical backgrounds to determine the real status of leptospirosis. Loss of biodiversity is a possible outcome of this disease as wildlife comes under constant threat and increasing pressure to survive.

Emerging zoonotic infectious diseases represent a growing threat and danger to wildlife as well as humans. Real-time surveillance through integrated human, veterinary and wildlife disease systems will reduce the time taken to recognise disease and implement suitable disease control practices.

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Leptospiral agglutinins in captive and free ranging non-human primates in Sarawak, Malaysia

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Abstract

Aim: The proposed study was carried out to determine the extent of exposure to leptospirosis in non-human primates.

Materials and Methods: Trapping of non-human primates was carried out opportunistically around the Bako National Park and the Matang Wildlife Center in the vicinity of human settlements and tourism areas of Sarawak. Blood samples were obtained from the saphenous vein to determine the presence of antibodies by the Microscopic Agglutination Test (MAT) to 17 serovars of *Leptospira* commonly found in Malaysia.

Results: This study reports the screening of twelve primates (eight captive and four free ranging) for leptospirosis. Eight of the 12 monkeys (66.6%; 95% CI 34.9-90.1) reacted against one or two serovars of *Leptospira* (Lai and Lepto 175). The serovar Lai is considered pathogenic for different mammals, including humans. *Leptospira* Lepto 175 has been identified as an intermediate strain and further studies are being undertaken on this serovar.

Conclusion: These results are important as primates may act as reservoirs of *Leptospira* spp. for humans, which may potentially affect tourism (economic loss), conservation efforts and public health.

Keywords: leptospirosis, MAT, seroprevalence, wildlife, zoonotic disease.

Introduction

*Leptospira* have been detected from wildlife in many countries, however their role as reservoirs is still poorly understood [1-3]. Leptospirosis can result in economic losses in domesticated animals and has the potential to be an important zoonotic disease of humans [4]. Leptospires were first isolated from rats in 1917 and it is widely acknowledged that rodents are a key source of infection for humans [5]. However, recently Australian and Peruvian researchers have reported that bats can also carry pathogenic *Leptospira*, [1-2, 6], although their role as carriers is not fully understood. Other wildlife, including primates, can also act as potential carriers of these pathogens [7-10]. However to date there has been little research conducted on free ranging wildlife. Leptospirosis in wildlife can affect biodiversity, human and livestock health, animal welfare and consequently the national economy [4].

Recently leptospirosis has been recognised as a re-emerging public health problem in Malaysia [11]. At present Malaysian wildlife disease surveillance is poorly coordinated and emerging zoonotic infectious diseases represent a growing threat. Tourism is a major contributor to the economy of Malaysia with 24.6 million tourists visiting the country annually. It has been estimated that approximately one million tourists are involved in eco-tourism activities and this group is particularly at increased risk of exposure to infectious diseases [12-13].

In recent years outbreaks of leptospirosis in Malaysia have been documented around the wildlife reserves and parks resulting in confirmation of a high number of confirmed cases and associated mortalities. Wildlife tourism is an important source of revenue in Malaysia, particularly in the state of Sarawak and leptospirosis has the potential to impact on this. The current research reports on the carriage of *Leptospira* by opportunistically sampled non-human primates in Sarawak.

Materials and Methods

Ethical approval: All procedures were performed with the approval of the Animal Ethics Committee of the Murdoch University (W2376/10) and Sarawak Forestry cooperation (NCCD.907.4.4 (V)-235).

Study area: Trapping of monkeys was carried out around Bako National Park and Matang Wildlife Centre. Bako National Park is located 37 km from Kuching, Sarawak, East Malaysia (Figure-1). It is Sarawak’s oldest national park, covering an area of 2,727 hectares and is located at the tip of the Muara Tebas Peninsula [14]. Although it is one of the smallest national parks in Sarawak, it contains almost most types of vegetation found in Borneo along with long-tailed macaques (*Macaca fascicularis*), silver-leaf monkeys (*Trachypithecus cristatus*), proboscis monkey (*Nasalis
Serological and molecular detection of *Leptospira* spp. from small wild mammals captured in Sarawak, Malaysia

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**ABSTRACT**

**Aims:** Leptospirosis is endemic to tropical regions of the world and is re-emerging as a new danger to public health in Southeast Asia, including Malaysia. The purpose of this particular study was to determine the common leptospiral serovars present in small wild mammals living around wildlife reserves and disturbed forest habitats and human communities.

**Methodology and results:** The samples of blood and kidneys of small rodents, bats and squirrels were analyzed. Antibodies to different serovars of leptospires were detected in 73 of 155 wild small mammals captured (47.0%; 95% CI 39.0-55.3%). The seroprevalence for rats (57.9%; 95% CI 44.1-70.9) was slightly higher than that for squirrels (42.9%; 95% CI 24.5-62.8) and bats (40%; 95% CI 28.5-52.4). Seropositive animals were detected in all 5 localities sampled. Antibodies to serovar Lepto 175 Sarawak were detected in 30 (24.7%) rats, 11 (9.0%) squirrels and 27 (52.9%) bats. Of 155 kidney samples from individual animals only 17 were positive for *Leptospira* on a molecular study (10.97%, 95% CI 6.5-17). The majority of the positive results were from plantain squirrels (53%; 95% CI 27.8, 77), Müller’s rat (35%; 95% CI 14.2, 61.7) and brown spiny rats (12%; 95% CI 1.5, 36.4).

**Conclusion, significance and impact of study:** This particular study should generate concerns and lead to the health authorities expanding disease control measures in the region as there are significant levels of human activity at all five locations where the animals were sampled. The pathogenesis of serovar Lepto 175 Sarawak also needs to be monitored closely, considering its similarities to the pathogenic *Leptospira wolffii*.

**Keywords:** Leptospirosis, zoonotic disease, bats, rodents, wildlife

**INTRODUCTION**

Leptospirosis is endemic to tropical regions of the world and is re-emerging as a new threat to public health in Southeast Asia, including Malaysia. Although studies have been conducted in Malaysia for more than 70 years (Hanson, 1982), the threat leptospirosis poses is still not well understood. Malaysian jungles are home to numerous species of wildlife and peri-domestic animals. These include bats, squirrels and rats, as well as primates. Bats and rats are known to harbour leptospiral serovars in their bodies and pass them to other species including humans (Roth, 1964; Faine *et al.*, 1999; Richardson and Gauthier, 2003; Matthias *et al.*, 2005; Vashi *et al.*, 2010).

Rodents have long been associated with leptospirosis as reservoir or maintenance hosts and verminous rodents are considered a key source for the distribution of leptospires in an urban setting (Faine *et al.*, 1999; Slack *et al.*, 2006; Priya *et al.*, 2007; Harris, 2009; Lau *et al.*, 2010a; Lau *et al.*, 2010b; Tulsiani, 2010; Costa *et al.*, 2014). Rats have been shown to be carriers of leptospires throughout the world and are important reservoirs of infection for animals and humans (Roth, 1964; Priya *et al.*, 2007). Recently bats have also been implicated in the spread of leptospirosis to humans (Mortimer, 2005; Vashi *et al.*, 2010).

With deforestation becoming more commonplace in many tropical environments, including Sarawak, Malaysia, there is increased likelihood of contact between humans and wildlife due to the disturbance of natural habitats. The increasing popularity of eco-tourism raises another risk for the disease. Such tourism brings visitors to Malaysia close to nature and native wildlife, some of which may be reservoir hosts for leptospirosis, as well as potentially interfering with an already-fragile ecosystem.

The purpose of this study was to determine the common leptospiral serovars present in small wild mammals living around wildlife reserves, disturbed forest...
habitats and human communities around Kuching, Sarawak, Malaysia.

MATERIALS AND METHODS

Study and sampling sites

The sampling of animals was undertaken in the wider ecological ranges of Kuching, and Kota Samarahan area, including Universiti Malaysia Sarawak (UNIMAS) area, Matang Wildlife Centre, Kubah National Park, Bako National Park, Wind and Fairy Cave Reserves and Mount Singai Conservation Area (Figure 1). Sampling sites were chosen to cover different ecological environments (e.g. disturbed habitats and secondary forests located around human settlements) to gain an accurate representation of the possible transmission of leptospires. All locations sampled in the current study were within approximately 30 kilometres of Kuching, the capital city of Sarawak. A common trend in most of the human leptospirosis outbreaks in the Kuching region has been exposure to wildlife and contact with water contaminated by leptospires (Thayaparan et al., 2013a). Bako National Park, Matang-Kubah Wildlife Centre, Mount Singai and the Wind and Fairy caves are known to contain several wildlife species (Hall et al., 2004; Khan et al., 2007; Thayaparan et al., 2013a) that may be potentially accountable for transmitting leptospires (Abdullah, 2011). UNIMAS is located in an area where the natural environment has been disturbed, similar to other areas of Sarawak. Certain parts of the UNIMAS campus are also known to be waterlogged and have patches of forested environment, increasing the possibility of exposure of students at the campus to leptospires.

Animal capturing and ethical aspects

During the investigation 155 individual small mammals were captured and sampled. The species sampled in this study included any small mammals (rats, squirrels, tree shrew and bats) were captured in the selected areas over a one-year period from January 2011 to March 2012.

The rats, squirrels and tree shrews were trapped using cage traps (Baeumler and Brunner, 1988). A maximum of 50 cage traps were set around the border of a wildlife area and human settlement, with the intention of trapping small mammals at one sampling time. A mixture of one-part raisins and two parts peanut butter with rolled oats, banana and dry fish was used as bait and placed in each trap (Hickie and Harrison, 1930; Wood, 1984). Traps were opened from 6 am and checked once the afternoon and following morning. Any by-catch was immediately released at the site of trapping. If pregnant or lactating small mammals were captured these were also released immediately at the captured site. Any trapped rats, squirrels and tree shrews were collected and taken to the UNIMAS laboratory for processing. The cage was covered with cloth and kept in the animal house until processing. Each small mammal was housed in individually ventilated cages. During the period of transportation small mammals were fed sunflower seeds, cereals, cooked corn kernels and cheese and provided with water ad libitum.

The rodents were anaesthetised using Ketamine + Xylazine (50–75 mg/kg + 10 mg/kg IP) (Karwacki et al., 2001). Once the rats were anaesthetised 1-3 mL of blood was collected via cardiac puncture using a 25G needle and a 5 mL syringe. After blood collection, the rats were euthanased with barbiturate (sodium pentobarbitone at 150-200 mg/kg) via either an intracardiac or intravenous (IV) injection. After euthanasia the animals were necropsied and kidney samples collected. Blood samples were left at room temperature for 30 min to clot and were then centrifuged at 3,000 RPM for 1 min. The serum was removed and stored at −20 °C until testing in the laboratory.

All the bats were wild-caught either using mist nets and harp traps and terrestrial small mammals using cage traps. Mist net is a lightweight net that was placed in the flight path of the bats, for example across walking or animal trails, over streams, adjacent to fruiting trees and at the mouths of caves. Bats that were caught in the nets were carefully removed from the net to ensure their delicate wings were not injured. Harp traps were also set across trails or over small streams. Three harp traps and five mist nets were placed along bat flight paths for a maximum of six hours between 6 pm and 11 pm. Nets were checked every 30 min and any bats caught were removed from the traps. After removal each bat was placed in an individual cloth bag. The cloth bags were hung and tied in a handmade wicker basket. The basket was covered with a cloth and placed in a safe quiet place to minimise disturbance. If any birds were trapped these were removed and released immediately.

Bats or small mammals were anaesthetised to reduce the stress of handling and to minimise the risk of the handler.
being bitten. Each animal was anaesthetised with an intramuscular injection of ketamine (6–7 mg/kg) and medetomidine (60–70 µg/kg) (Plowright et al., 2008). A blood sample (1 to 3 mL) was collected by cardiac puncture from each bat using a 5 mL syringe and a 25G needle. Prior to collection the hair was sterilized with cotton wool dipped in 70% alcohol. The bats were placed in a fabric pouch and placed in a plastic airtight container. Cotton balls were thoroughly saturated with isoflurane in the barrel of a six ml syringe (after removing the plunger). The syringe barrel containing the isoflurane saturated cotton balls were then placed into the container and the lid closed. Bats were usually anaesthetised within seconds and death was confirmed by auscultation with a stethoscope. Kidney samples were removed for culturing and molecular analysis. Carcasses were stored in 99% ethanol for future use by UNIMAS students and kept as museum voucher specimens following Abdullah et al. (2010). All personnel who handled bats had been vaccinated against rabies and all samples were treated as potentially infectious.

Trapping of wildlife was approved by the Murdoch University Animal Ethics Committee (Animal ethics No: W2376/10) and Sarawak Forestry Department, Sarawak Malaysia (Permit No: NCCD.907.4.4 (V)-235).

Diagnostic methods

Microscopic Agglutination Test (MAT)

The Microscopic Agglutination Test (MAT) was performed according to Faine (1982) to check for Leptospira-specific antibodies to 17 serovars commonly found in Malaysia: Australis, Autumnalis, Bataaviae, Canicola, Icterohaemorrhagiae, Celledoni, Pyrogenes, Hardjo, Tarassovi, Patoc, Djasiman, Lai, Copenhegeni, Leptospira Javanica, Pomona, Pyrogenes, Grippotyphosa, Australis, Autumnalis, Bataviae, Canicola, and 10.0 pmol f

Molecular analysis

Culture and isolation and preparation of genomic DNA from kidney samples

Kidney samples from all animals were cultured for leptospires. A cross-section of the cortex of one kidney was removed using a sterile scalpel and this section was cut into small pieces and inoculated into two EMJH and representative 16S rRNA (1331 bp) gene sequences were aligned with members of the genus Leptospira were aligned with CLUSTAL W (Thompson et al., 1997). By using MEGA 5 (Tamura et al., 2011), distances of aligned 16S rRNA gene sequences were estimated by the Jukes-Cantor method (Jukes and Cantor, 1969), bootstrapped 1000 times and the tree topology was determined by the neighbour-joining method (Slack et al., 2006; Slack et al., 2008; Slack et al., 2009). The final phylogenetic tree was rooted by using Turniaevella parva serovar Parva strain H as an out-group and bootstrap values (1000) were displayed as percentages (Thaipadungpanit et al., 2007).
RESULTS

In total 155 animals were sampled including 70 bats, 57 rodents, 20 squirrels, and eight treeshrew. The rats trapped were Müller’s rat \((\text{Sundamys muelleri}) (n=29)\), ricefield rat \((\text{Rattus argentiventer}) (n=20)\), brown spiny rat \((\text{Maxomys rajah}) (n=7)\), and whitehead’s rat \((\text{Maxomys whiteheadi}) (n=1)\). All squirrels were either Low’s squirrels \((\text{Sundasciurus lowii}) (n=8)\) or plantain squirrels \((\text{Callosciurus notatus}) (n=19)\) and captured treeshrew \((\text{Tupaia tana}) (n=8)\). The bats netted and trapped were dusky fruit bats \((\text{Penthetor lucasi}) (n=20)\), short-nosed fruit bats \((\text{Cynopterus brachyotis}) (n=22)\), spotted-winged fruit bats \((\text{Balionycteris maculata}) (n=13)\), fawn roundleaf bats \((\text{Hipposideros cervinus}) (n=5)\), Bornean horseshoe bats \((\text{Hipposideros borneensis}) (n=2)\), bicolored roundleaf bats \((\text{Hipposideros bicolor}) (n=2)\), hollow-faced bat \((\text{Nycteris tragata}) (n=1)\), intermediate horseshoe bat \((\text{Rhinolophus affinis}) (n=1)\), dusky roundleaf bat \((\text{Hipposideros ater}) (n=1)\), lesser woolly horseshoe bat \((\text{Rhinolophus sedulus}) (n=1)\), \(p\)apillosa woolly bat \((\text{Kerivoula papillosa}) (n=1)\) and dayak roundleaf bat \((\text{Hipposideros dyacorum}) (n=1)\). The largest number of animals was caught in the locality of UNIMAS (40) followed by Wind Cave and Fairy Cave areas (36), Bako National park (31), Matang and Kubah (30) and Mount Singai (18) (Tables 1 and 2).

Table 1: Species, location and MAT results for animals other than bats caught.

<table>
<thead>
<tr>
<th>Species</th>
<th>UNIMAS</th>
<th>Mt. Singai</th>
<th>Wind &amp; Fairy</th>
<th>Matang &amp; Kubah</th>
<th>Bako</th>
<th>Total</th>
<th>% Positive</th>
<th>MAT (+)</th>
<th>% Positive (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Sundamys muelleri})</td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>29</td>
<td>34.1</td>
<td>20</td>
<td>68.9 (42.9, 84.7)</td>
</tr>
<tr>
<td>(\text{Rattus argentiventer})</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>20</td>
<td>23.5</td>
<td>9</td>
<td>45.0 (23.1, 68.5)</td>
</tr>
<tr>
<td>(\text{Maxomys rajah})</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>7</td>
<td>8.2</td>
<td>4</td>
<td>57.1 (18.4, 90.1)</td>
</tr>
<tr>
<td>(\text{Maxomys whiteheadi})</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1.2</td>
<td>0</td>
<td>0.0 (0, 97.5)</td>
</tr>
<tr>
<td>(\text{Sundasciurus lowii})</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1.2</td>
<td>1</td>
<td>100.0 (2.5, 100)</td>
</tr>
<tr>
<td>(\text{Callosciurus notatus})</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>6</td>
<td>19</td>
<td>22.4</td>
<td>10</td>
<td>52.6 (28.9, 75.6)</td>
</tr>
<tr>
<td>(\text{Tupaia tana})</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>8</td>
<td>9.4</td>
<td>1</td>
<td>12.5 (0.3, 52.7)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
<td><strong>13</strong></td>
<td><strong>17</strong></td>
<td><strong>16</strong></td>
<td><strong>19</strong></td>
<td><strong>85</strong></td>
<td><strong>100.0</strong></td>
<td><strong>45</strong></td>
<td><strong>52.9 (41.8, 63.9)</strong></td>
</tr>
<tr>
<td>% Positive</td>
<td>23.5</td>
<td>15.3</td>
<td>20.0</td>
<td>18.8</td>
<td>22.4</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Species, location and MAT results for bats caught.

<table>
<thead>
<tr>
<th>Species</th>
<th>UNIMAS</th>
<th>Mt. Singai</th>
<th>Wind &amp; Fairy</th>
<th>Matang &amp; Kubah</th>
<th>Bako</th>
<th>Total</th>
<th>% Positive</th>
<th>MAT (+)</th>
<th>% Positive (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Penthetor lucasi})</td>
<td>5</td>
<td>2</td>
<td>11</td>
<td>0</td>
<td>2</td>
<td>20</td>
<td>28.5</td>
<td>12</td>
<td>60.0 (36.1, 80.9)</td>
</tr>
<tr>
<td>(\text{Cynopterus brachyotis})</td>
<td>10</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>5</td>
<td>22</td>
<td>31.4</td>
<td>9</td>
<td>40.9 (20.7, 63.6)</td>
</tr>
<tr>
<td>(\text{Balionycteris maculata})</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>13</td>
<td>18.5</td>
<td>3</td>
<td>23.1 (5.0, 53.8)</td>
</tr>
<tr>
<td>(\text{Hipposideros cervinus})</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>5</td>
<td>7.1</td>
<td>2</td>
<td>40.0 (5.3, 85.3)</td>
</tr>
<tr>
<td>Other bat species</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>10</td>
<td>14.2</td>
<td>2</td>
<td>20.0 (2.5, 55.6)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
<td><strong>5</strong></td>
<td><strong>19</strong></td>
<td><strong>14</strong></td>
<td><strong>12</strong></td>
<td><strong>70</strong></td>
<td><strong>100.0</strong></td>
<td><strong>28</strong></td>
<td><strong>40.0 (28.5, 52.4)</strong></td>
</tr>
<tr>
<td>% Positive</td>
<td>23.5</td>
<td>15.3</td>
<td>20.0</td>
<td>18.8</td>
<td>22.4</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

woolly bat \((\text{Kerivoula papillosa}) (n=1)\) and dayak roundleaf bat \((\text{Hipposideros dyacorum}) (n=1)\). The largest number of animals was caught in the locality of UNIMAS (40) followed by Wind Cave and Fairy Cave areas (36), Bako National park (31), Matang and Kubah (30) and Mount Singai (18) (Tables 1 and 2).

Serological and descriptive analysis

The seroprevalence for rats \((57.9\%; 95\% \text{ CI } 44.1-70.9)\) was slightly higher than that for squirrels \((42.9\%; 95\% \text{ CI } 24.5-62.8)\) and bats \((40\%; 95\% \text{ CI } 28.5-52.4)\), however these differences were not significant \((\chi^2 = 4.28; df=2; P=0.117)\).

The highest seroprevalence was found in Müller’s rat \((68.9\%; 95\% \text{ CI } 42.9-84.7)\) followed by brown spiny rat \((57.1\%; 95\% \text{ CI } 18.4-90.1)\), plantain squirrel \((52.6\%; 95\% \text{ CI } 42.1-63.9)\).
The highest seroprevalence in bats was observed in the dusky fruit bat (60%; 95% CI 36.1-80.9), followed by the short-nosed fruit bat (40.9%; 95% CI 20.7-63.6) and spotted-winged fruit bat (23.1%; 95% CI 5.0-53.8) (Tables 1 and 2). There was no significant difference in the seroprevalence between the different animal species ($\chi^2 = 24.9; df=18; P=0.126$).

Some seropositive animals were detected in all of the localities sampled. The highest prevalence was found at Mount Singai (64.7%; 95%CI 38.3-85.8) followed by Matang and Kubah (56.7%; 95%CI 37.4-74.5), Wind Cave and Fairy cave (47.4%; 95%CI 30.4-64.5), followed by Bako National Park (45.2%; 95%CI 27.3-64.0) and UNIMAS area (35%; 95%CI 20.6-51.7) (Table 3). There was no significant difference in the seroprevalence between the five sampling location ($\chi^2 = 4.28; df=4; P=0.117$), however significantly more seropositive animals were detected at Mount Singai than at UNIMAS (OR 3.4; 95% CI 1.04, 11.17).

For the analysis of locality UNIMAS was selected as the referent to ensure that all OR were greater than 1. Small mammals from Mount Singai were 3.4 times (95% CI 1.04-11.17) more likely to have leptospiral antibodies than animals captured from the UNIMAS area. Animals from Matang and Kubah were 2.4 times (95% CI 0.92, 6.42) more likely to have antibodies than those from the UNIMAS area (not significant). Wind Cave & Fairy Cave and Bako National Park had odd ratios of 1.7 and 1.5 respectively (95% CI 0.66, 4.18; 0.59, 4.0). There was little difference between the risk of animals from UNIMAS and those from Matang and Bako being seropositive (95% confidence intervals including the value 1.0) (Table 3).

### Table 3: Seroprevalence to leptospires in small mammals according to their locality.

<table>
<thead>
<tr>
<th>Locality</th>
<th>No of seropositive</th>
<th>Seroprevalence (95% CI)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNIMAS</td>
<td>14</td>
<td>35.0 (20.6, 51.7)</td>
<td>1.0</td>
</tr>
<tr>
<td>Mt. Singai</td>
<td>11</td>
<td>64.7 (38.3, 85.8)</td>
<td>3.4 (1.04, 11.17)</td>
</tr>
<tr>
<td>Wind &amp; Fairy Caves</td>
<td>17</td>
<td>47.2 (30.4, 64.5)</td>
<td>1.7 (0.66, 4.18)</td>
</tr>
<tr>
<td>Matang &amp; Kubah</td>
<td>17</td>
<td>56.7 (37.4, 74.5)</td>
<td>2.4 (0.92, 6.42)</td>
</tr>
<tr>
<td>Bako NP</td>
<td>14</td>
<td>45.2 (27.3, 64.0)</td>
<td>1.5 (0.59, 4.0)</td>
</tr>
</tbody>
</table>

### Table 4: Seroprevalence to leptospires in small mammals and their odd ratios.

<table>
<thead>
<tr>
<th>Animals</th>
<th>MAT Positive</th>
<th>Seroprevalence (95% CI)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rats</td>
<td>33</td>
<td>57.9 (44.1, 70.9)</td>
<td>1.0</td>
</tr>
<tr>
<td>Squirrels &amp; Treeshrew</td>
<td>12</td>
<td>42.9 (24.5, 62.8)</td>
<td>0.55 (0.24, 0.99)</td>
</tr>
<tr>
<td>Bats</td>
<td>28</td>
<td>40.0 (28.5, 52.4)</td>
<td>0.48 (0.22, 1.36)</td>
</tr>
</tbody>
</table>

Among the Rodentia (rats), Scandentia (Squirrel, treeshrew) and Chiroptera (bats), rats were selected as the referent animal group. The odds of disease in Scandentia and Chiroptera compared to Rodentia were 0.55 (95% CI 0.24, 0.99) and 0.48 (95% CI 0.22, 1.36), indicating these animals were less likely to be seropositive than rats; although the Chiroptera result was not significant (Table 4).

Antibodies to 10 different serovars were detected in rodents and six different serovars in bats. Antibodies to serovar Lepto 175 Sarawak were detected in 30 (24.7%) rats, 11 (9.0%) squirrels and 27 (52.9%) bats. Antibodies to serovar Icterohaemorrhagiae were detected in 20 (16.5%) rodentia and scandentia; sv. Australis in 13 (10.6%) blood samples and sv. Autumnalis in 12 (9.6%) samples. Serovars Australis and Lai were detected in one dusky fruit bat and one short-nosed fruit bat respectively. Antibodies to serovar Pyrogenes were detected in 10 (19.6%) samples from dusky fruit bats and short-nosed fruit bats. The antibody titers for seropositive animals varied from 1:50 to 1:800. More animals had a serum dilution of 1:100 (n=65) than other dilutions (1:50 in 20 serum samples; 1:200 in 30 serum samples and 1:400 in 8 samples, while only one squirrel had a dilution of 1:800) (Table 5).

### Culture and molecular analysis

None of the culture sample maintained in the lab produced positive results and majority of the specimens were contaminated and after four weeks all the samples were discarded. Out of 155 kidney samples from individual animals, only 17 were positive for *Leptospira* on the molecular study (10.97%, 95% CI 6.5, 17). The majority of the positive results were from plantain squirrels (53%; 95% CI 27.8, 77), Müller’s rat (35%; 95% CI 14.2, 61.7) and brown spiny rats (12%; 95% CI 1.5, 36.4). No other animals were positive on the PCR (Table 6).
Table 5: Titres of antibodies to different serovars found in small mammals.

<table>
<thead>
<tr>
<th>Serovar</th>
<th>Positive</th>
<th>1:50</th>
<th>1:100</th>
<th>1:200</th>
<th>1:400</th>
<th>1:800</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lepto 175 Sarawak</td>
<td>68</td>
<td>23</td>
<td>27</td>
<td>12</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Australis</td>
<td>15</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Autumnalis</td>
<td>12</td>
<td>2</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td>21</td>
<td>5</td>
<td>13</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Javanica</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Patoc</td>
<td>25</td>
<td>8</td>
<td>14</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Copenhageni</td>
<td>6</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pomoma</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lai</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total/%</strong></td>
<td><strong>172/100</strong></td>
<td><strong>56/32.5</strong></td>
<td><strong>77/44.7</strong></td>
<td><strong>30/17.4</strong></td>
<td><strong>8/4.6</strong></td>
<td><strong>1/0.6</strong></td>
</tr>
</tbody>
</table>

Table 6: PCR results from the kidneys of small mammals and their potential PCR using Neighbouring method.

<table>
<thead>
<tr>
<th>Place</th>
<th>Species</th>
<th>PCR</th>
<th>Serovar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt. Singai</td>
<td>Callosciurus notatus</td>
<td>Positive</td>
<td><em>L. pomona</em></td>
</tr>
<tr>
<td></td>
<td>Sundamys mueller</td>
<td>Positive</td>
<td><em>L. icterohaemorrhagiae</em></td>
</tr>
<tr>
<td>UNIMAS</td>
<td>Sundamys mueller</td>
<td>Positive</td>
<td><em>L. lai</em></td>
</tr>
<tr>
<td>Wind Cave &amp; Fairy Caves</td>
<td>Callosciurus notatus</td>
<td>Positive</td>
<td><em>L. pomona</em></td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Positive</td>
<td><em>L. pomona</em></td>
</tr>
<tr>
<td></td>
<td>Maxomys rajah</td>
<td>Positive</td>
<td><em>L. icterohaemorrhagiae</em></td>
</tr>
<tr>
<td></td>
<td>Maxomys rajah</td>
<td>Positive</td>
<td><em>L. icterohaemorrhagiae</em></td>
</tr>
<tr>
<td></td>
<td>Sundamys mueller</td>
<td>Positive</td>
<td><em>L. lai</em></td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Positive</td>
<td><em>L. pomona</em></td>
</tr>
<tr>
<td></td>
<td>Sundamys mueller</td>
<td>Positive</td>
<td><em>L. icterohaemorrhagiae</em></td>
</tr>
<tr>
<td>Matang &amp; Kubah</td>
<td>Sundamys mueller</td>
<td>Positive</td>
<td><em>L. icterohaemorrhagiae</em></td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Positive</td>
<td><em>L. pomona</em></td>
</tr>
<tr>
<td>Bako National Park</td>
<td>Callosciurus notatus</td>
<td>Positive</td>
<td><em>L. pomona</em></td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Positive</td>
<td><em>L. pomona</em></td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Positive</td>
<td><em>L. pomona</em></td>
</tr>
<tr>
<td></td>
<td>Sundamys mueller</td>
<td>Positive</td>
<td><em>L. icterohaemorrhagiae</em></td>
</tr>
<tr>
<td></td>
<td>Callosciurus notatus</td>
<td>Positive</td>
<td><em>L. pomona</em></td>
</tr>
</tbody>
</table>

DISCUSSION

The results indicate that small mammals from Mount Singai were 3.4 times more likely to have leptospiral antibodies than animals from the UNIMAS area. These findings highlight potential risk for acquiring leptospirosis to villagers living around Mount Singai and eco-tourists and recreationists visiting the forested mountain. The studies undertaken indicated a greater proportion of rats were seropositive than bats, squirrels and tree shrews, highlighting the importance of these species as carriers or reservoirs of leptospires.

Deforestation, which is becoming commonplace across Malaysia (Aiken and Leigh, 1992; Jomo et al., 2004; Abdullah, 2011), can promote the emergence of
infectious diseases, such as leptospirosis, by placing humans into contact with novel reservoirs or infectious agents (Arief, 2013; Matthias et al., 2005). Bats respond to the destruction of their habitat at the level of populations and communities, making their spatial and temporal dynamics particularly sensitive to anthropogenic activity (Calisher et al., 2006). Matthias et al. (2005) detected Leptospira serovar Icterohaemorrhagiae from one bat in the Peruvian Amazon region and proposed a rodent-bat cycle of infection. (Vashi et al., 2010) added further support to the growing awareness of the role played by bats in the transmission of this pathogen to humans and other animals.

Foods in Southeast Asia, including Malaysia, are characterized by flowering and fruit production, substantially contributing to the abundance of rodents (Nakagawa et al., 2007). A ricefield rat captured by Mohamed-Hassan et al. (2012) at a military training camp in West Malaysia was identified as a carrier of leptospires. Rats are also reservoirs of a number of other parasites and infectious pathogens, including the agent of plague, Yersinia pestis (Zahedi et al., 1984).

Studies have shown that rodents and bats are reservoirs for leptospirosis (Matthias et al., 2005; Vashi et al., 2010). In the current study the positive PCR result from kidney samples are considered to be indicative of a carrier status of this pathogen in squirrels and rodents. In contrast animals positive on the MAT are not necessarily carriers as Leptospira-specific antibodies can be detected in convalescent sera and positivity to an MAT indicates past or current infection but not necessarily renal shedding (Faine et al., 1999). In the current study, serovar Pomona was most prevalent among the plantain squirrels, being detected in kidneys collected from animals from four out of the five regions sampled. Müller’s rats were identified as carriers of both serovar Laï and Icterohaemorrhagiae, with the latter also found in brown spiny rats. Many small mammals also displayed high levels of antibodies to the newly discovered strain sv Lepto 175 Sarawak, with plantain squirrels and Müller’s rats being more commonly affected with this serovar. This Lepto 175 Sarawak was also found in a large number of dusky fruit bats tested. At present serovar Lepto 175 Sarawak is best considered an intermediate strain, as its lethal capacity is unknown.

According to (Thayaparan et al., 2013b) Lepto 175 was shown to have a close similarity to Leptospira wolffi group, which has been isolated from Thailand, Iran and India (Zakeri et al., 2010; Balamurugan et al., 2013). Antibodies to serovar Icterohaemorrhagiae, a well-known pathogen implicated in many fatal cases of leptospirosis in humans, was the fourth-most common serovar detected after Australis and Autumnalis. Pyrogenes seemed to be more common in bats while Autumnalis was found mainly in rodents. Rice field rats tested positive for at least two known pathogenic strains. An epidemic of leptospirosis has recently been reported in Malaysian Borneo, with Sarawak recording a nearly four-fold increase in the number of cases in 2011 as compared to 2010. A joint study by UNIMAS and the state health department in 2011 in the Rejang basin found that 31% of humans sampled were seropositive for leptospirosis and infection was associated with farming and/or water activities (Suut et al., 2011).

CONCLUSIONS AND RECOMMENDATIONS

This particular study has generate concerns that should lead to the health authorities to expand their disease control measures as there are significant levels of human activity at all five locations where the animals were sampled. Visitors and eco-tourists should be advised to protect themselves and avoid direct contact with contaminated soil or water. Local nearby villagers who are workers at the Universiti Malaysia Sarawak and Universiti Teknologi MARA in Kota Samarahan should be screened to avoid major health risks among the students and academic staff. Wherever possible, pest control measures should be implemented to contain the rat population, particularly considering that these rodents appear to be the main source of infection in these locations.

Wildlife that is undergoing rehabilitation at captive facilities should be screened for Leptospira periodically before their eventual re-introduction to the natural environment. This helps prevent them from transmitting the disease to other species in the wild or to humans involved in their release.

The pathogenesis of serovar Lepto 175 Sarawak also needs to be monitored closely considering its similarities to Leptospira wolffi. The latter has been isolated from both humans and animals by scientists in Thailand, Iran and India. It remains to be seen if serovar Lepto 175 Sarawak will be as virulent as Leptospira wolffi and hence extra vigilance is required and the Malaysian health authorities need to implement disease control measures to prevent the occurrence of a potential epidemic.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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