Pertanika J. Trop. Agric. Sci. 40 (1): 99 - 110 (2017)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Detection of *Leptospira* spp. in Selected National Service Training Centres and Paddy Fields of Sarawak, Malaysia using Polymerase Chain Reaction Technique

Chai Fung Pui[1], Lesley Maurice Bilung[1]*, Lela Su'ut[2], Yee Ling Chong[1] and Kasing Apun[1]

[1]Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 UNIMAS, Kota Samarahan, Sarawak, Malaysia
[2]Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, 94300 UNIMAS, Kota Samarahan, Sarawak, Malaysia

ABSTRACT

Leptospirosis is a zoonotic disease which is caused by spirochetes from the genus *Leptospira*. It can be transmitted to humans through direct contact with infected animals or indirect contact with an environment contaminated by the urine of infected animals. The objective of this study was to study the status of leptospirosis in two selected National Service Training Centres (NSTCs) and two paddy fields of Sarawak. A total of 31 captured rats, 210 soil samples and 210 water samples were collected from these study sites. All the samples were inoculated into a modified semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) broth with 5-fluorouracil. For soil and water samples, a specific polymerase chain reaction (PCR) was conducted after a one-month incubation period. Kidney and liver samples from rats were incubated and PCR was carried out monthly during the three-month incubation period. Representative PCR-positive samples which targetted LipL32, 16S rRNA and rrs genes at 423 bp, 331 bp and 240 bp in pathogenic, intermediate and saprophytic *Leptospira*, respectively, were further sequenced. From the PCR analysis, intermediate *Leptospira* was detected in one (3.2%) rat species, *Rattus exulans*, that was captured in a paddy field. A total of six (2.9%) pathogenic *Leptospira*, one (0.5%) each from intermediate and saprophytic *Leptospira*, were present in soil samples from the study sites. Six (2.9%) water samples were contaminated by pathogenic *Leptospira*, four (1.9%) by intermediate *Leptospira* and seven (3.3%) by saprophytic *Leptospira*. All the contaminated environmental samples

ARTICLE INFO

Article history:
Received: 23 February 2016
Accepted: 20 September 2016

E-mail addresses:
chaifung85@gmail.com (Chai Fung Pui),
mblesley@unimas.my;
lesleymaurice@gmail.com (Lesley Maurice Bilung),
slela@unimas.my (Lela Su'ut),
ylchong@unimas.my (Yee Ling Chong),
kasing@unimas.my (Kasing Apun)
* Corresponding author
ISSN: 1511-3701

were collected from NSTCs except for two soil samples and four water samples from paddy fields that were infected by pathogenic *Leptospira*. Results from DNA sequencing analysis indicated that the dominant pathogenic, intermediate and saprophytic *Leptospira* species circulating in these study sites were *Leptospira noguchii*, *Leptospira wolffii* serovar Khorat and *Leptospira meyeri*, respectively. Although the prevalence of *Leptospira* is low, there is still a risk of infection to those who are involved in outdoor activities at training centres and paddy fields. Control and preventive measures are, therefore, important in tackling preventable diseases related to pathogenic *Leptospira*.

Keywords: Leptospira, rat, soil, water, National Service Training Centres, paddy fields

INTRODUCTION

Leptospirosis is a worldwide zoonotic disease caused by pathogenic *Leptospira*. This aerobic bacterium is highly motile and is observed as helical shaped under a dark-field microscope (Flores-Encarnación et al., 2014). More than 1.7 million cases of severe leptospirosis are reported worldwide annually, with an approximately 10% mortality rate (Chen et al., 2015). This disease is usually transmitted through direct contact with the urine of infected animals or indirect contact with an environment contaminated with the bacteria (Sumanta et al., 2015).

Leptospirosis has been associated with occupation and is reported to commonly occur among rice farmers, sewer workers, fishermen or military personnel (Alavi et al., 2014). However, recreational activities such as water sport have been recorded as a risk factor as well (Narita et al., 2005). In Malaysia, leptospirosis outbreaks originating from National Service Training Centres (NSTCs) and paddy fields have been documented. The National Service Training Programme was introduced by the Malaysian government with the aim of enhancing patriotism among youths and encouraging national integration and unity. In January 2010, a contaminated pond in the Junaco Park National Service Training Camp, Sibu, Sarawak was closed due to the presence of *Leptospira* (Bernama, 2010). In April 2012, a trainee died due to leptospirosis in the Terkok National Service Training Centre in Sungai Siput, Perak (Bernama, 2012). An outbreak of leptospirosis during high-risk fisheries activities in a neglected swamp was also documented in Pauh, Perlis in the same year. Further investigation showed that 87.5% of water samples collected from the swamp was contaminated with pathogenic *Leptospira*. It was discovered that this neglected swamp had previously been a paddy field that could no longer be cultivated (Baharudin et al., 2012).

To the best of our knowledge, no detection study on *Leptospira* in NSTCs and paddy fields of Sarawak have been previously published. Due to the potential risk of *Leptospira* infection in these localities, the objectives of this study were (i) to screen for *Leptospira* spp. in selected NSTCs and paddy fields of Sarawak, and

(ii) to identify the dominant *Leptospira* spp. circulating in these localities.

MATERIALS AND METHODS

Sampling

Sampling was conducted from May 2014 to January 2015. A total of 31 captured rats, 210 soil samples and 210 water samples were collected from two NSTCs and two paddy fields in Kuching and Miri, Sarawak. Sampling was conducted at NSTCs approved by the National Service Training Department and camp managers of the respective NSTCs. The two paddy fields chosen were situated in villages with most of the population engaged in agricultural activities, namely paddy field cultivation.

In the NSTCs, cage traps were placed in open fields, trainees' hostels, canteens, store rooms, jungle areas and along the lakes in the camps with salted fish and banana as bait. Soil samples were collected from the jungle areas and open field whereas water samples were collected from lakes, water streams and drain effluent from the canteens in the camps. Cage traps were set at random in the paddy fields and barns. Soil samples were collected from the paddy fields whereas water samples were collected from the paddy fields, streams or rivers nearby.

Collection of Rat Samples

Rectangular cage traps were used to capture the rats. All the trapped rats under the family Muridae were identified based on the morphological characteristics recorded by Payne and Francis (2007). They were then euthanised humanely and dissected aseptically. Kidney and liver samples were inoculated into a modified semisolid Ellinghausen-McCullough-Johnson-Harris (EMJH) broth with 100 µg/mL 5-fluorouracil. The enriched samples were incubated aerobically at room temperature for three months. PCR was conducted every month to detect the presence of *Leptospira* spp. (Houemenou et al., 2013).

Collection of Soil and Water Samples

Soil and water samples were collected using 50 mL sterile falcon tubes. About 20 g of soil samples were mixed vigorously with sterile distilled water before they were allowed to settle for 15 min. They were passed through sterile a 0.2-µm pore size membrane filter (Sartorious AG, Germany). Approximately 50 mL of water samples were filtered using the same type of membrane filter. Next, about 1 mL of the samples was inoculated into a modified semisolid EMJH broth with 100 µg/mL 5-fluorouracil. The enriched samples were incubated aerobically at room temperature for one month prior to PCR amplification (Ridzlan et al., 2010).

Detection of *Leptospira* spp.

The Wizard™ Genomic DNA Purification Kit (Promega Corporation, USA) was used to extract DNA before conducting specific PCR amplification. Different primer pairs were used to target LipL32, 16S rRNA and rrs genes in pathogenic, intermediate and saprophytic *Leptospira*, respectively (Pui

et al., 2015). The reaction mixtures (25 µL) included 5 µL of 5x PCR buffer, 0.2 mM of dNTP mix, 0.4 µM of each primer pair, 2.0 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (Promega Corporation, USA) and 5 µL of DNA template. The cycling conditions were initial denaturation at 95°C for 2 min; 35 cycles each of denaturation at 95°C for 1 min, primer annealing at 55°C for 30 s and extension at 72°C for 1 min; further extension at 72°C for 5 min and indefinite holding period at 4°C. Electrophoresis was run on 2% agarose gel in 1x TBE buffer at 90V for 75 min.

Identification of *Leptospira* spp.

The amplicons from representative positive samples (one rat, five soil and nine water samples) were subjected to sequencing by commercial facility (First BASE Laboratories Sdn Bhd, Malaysia). The sequencing data were compared with GenBank database using nucleotide BLAST from the National Centre for Biotechnology Information (NCBI).

RESULTS

The prevalence of *Leptospira* spp. in rat, soil and water samples as detected by PCR is summarised in Table 1. PCR detection indicated that only one (3.2%) rat sample captured from a paddy field was infected by intermediate *Leptospira*. Among the soil samples, six (2.9%) were contaminated by pathogenic *Leptospira*, one (0.5%) by intermediate *Leptospira* and one (0.5%) by saprophytic *Leptospira*. All of these contaminated soil samples were collected from the NSTCs except for two from the paddy fields that were infected by pathogenic *Leptospira*. A total of six (2.9%) pathogenic *Leptospira*, four (1.9%) intermediate *Leptospira* and seven (3.3%) saprophytic *Leptospira* were recovered from the water samples. They were all present in the NSTCs except for four from the paddy

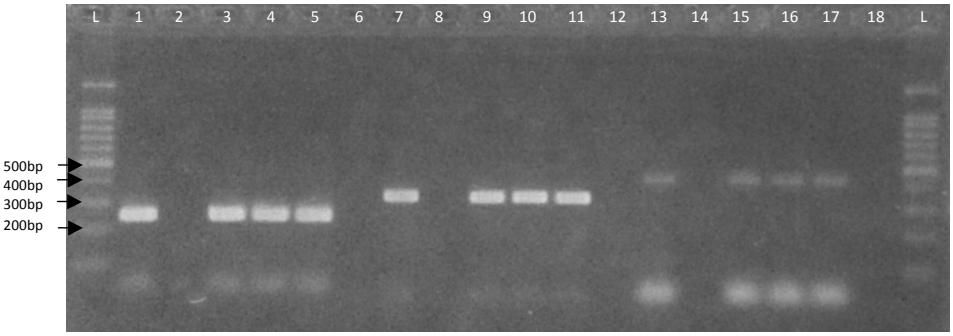

Figure 1. Representative gel image from PCR amplification. Lanes L show the 100 bp DNA ladder. *Leptospira* meyeri strain Sant-1 (lane 1), Leptospira wolffii serovar Khorat strain Khorat-H2 (lane 7) and *Leptospira noguchii* strain LT796 (lane 13) are positive controls. Lanes 2, 8 and 14 are negative controls. Lanes 3 to 5 show positive PCR amplicons specific to saprophytic *Leptospira* at 240 bp. Lanes 9 to 11 show the positive PCR amplicons specific to intermediate *Leptospira* at 331 bp. Lanes 15 to 17 show the positive PCR amplicons specific to pathogenic *Leptospira* at 423 bp. Lanes 6, 12 and 18 are negative PCR amplicons.

Table 1
Prevalence of Leptospira Spp. in Rat, Soil and Water Samples Collected from NSTCS and Paddy Fields in Sarawak

Sample	Study sites	Pathogenic		Intermediate		Saprophytic	
		No.[a]	%[b]	No.[a]	%[b]	No.[a]	%[b]
Rat	National Service Training Centres	0/15	0.0	0/15	0.0	0/15	0.0
	Paddy fields	0/16	0.0	1/16	6.3	0/16	0.0
	Total	0/31	0.0	1/31	3.2	0/31	0.0
Soil	National Service Training Centres	4/150	2.7	1/150	0.7	1/150	0.7
	Paddy fields	2/60	3.3	0/60	0.0	0/60	0.0
	Total	6/210	2.9	1/210	0.5	1/210	0.5
Water	National Service Training Centres	2/150	1.3	4/150	2.7	7/150	4.7
	Paddy fields	4/60	6.7	0/60	0.0	0/60	0.0
	Total	6/210	2.9	4/210	1.9	7/210	3.3

Note: anumber of positive samples/total number of samples collected; b, prevalence (in %) of positive samples among the samples collected

fields that were contaminated by pathogenic *Leptospira*. The representative gel image is shown in Figure 1.

A total of 15 rats comprising four different rat species were captured in two NSTCs in Sarawak, where *Sundamys muelleri* and *Niviventer cremoriventer* dominated at 46.7% and 33.3%, respectively. Ten rats were male and the remaining five were female. Adult rats made up 93.3% of the captured population. Based on a PCR analysis, none of the rats were infected by *Leptospira* spp. However, it was found that the soil samples in the two NSTCs were contaminated by pathogenic, intermediate and saprophytic *Leptospira* at 2.7%, 0.7% and 0.7%, respectively. Out of the 150 water samples collected, 1.3%, 2.7% and 4.7% of them were positive for the gene specific to pathogenic, intermediate and saprophytic *Leptospira*.

In the two paddy fields of Sarawak, a total of 16 individual rats comprising eight species were captured. *Rattus rattus* and *Rattus exulans* dominated by 50.0% and 18.8%, respectively. Out of the 16 rats, 12 were male and 11 were adult rats. None of the rats was infected by pathogenic and saprophytic *Leptospira*. However, one *Rattus exulans* was positive to intermediate *Leptospira*. This infected rat was an adult male rat. Pathogenic *Leptospira* was found to have contaminated 3.3% of the soil samples. The water samples from paddy fields were contaminated by pathogenic *Leptospira* at 6.7%.

From the PCR analysis, the DNA from 12 pathogenic strains, six intermediate strains and eight saprophytic strains of *Leptospira* were amplified. At least one positive sample from each study site was sent for DNA sequencing, subject to the availability of sample sources. The representative positive samples included seven pathogenic *Leptospira*, five intermediate *Leptospira* and three saprophytic *Leptospira*. Nucleotide BLAST results (Table 2) indicated that *Leptospira noguchii* was the dominant pathogenic *Leptospira* present in these study sites. *Leptospira wolffii* serovar Khorat was the dominant intermediate *Leptospira* whereas *Leptospira meyeri* was the dominant saprophytic *Leptospira*. The results showed similarity ranging from 78% to 99%.

DISCUSSION

In this study, *Sundamys muelleri* and *Niviventer cremoriventer* dominated the rat population in the selected NSTCs of Sarawak. This was different from the rat species found in West Malaysia, where 88.1% out of the total 268 rats captured in NSTCs in Terengganu, Kelantan, Malacca and Selangor were *Rattus tiomanicus* (Mohamed-Hassan et al., 2012). In a study where 90 rodents were trapped in Cotonou, West Africa, *Rattus rattus* was the rat species with the greatest distribution as it spread inland and was present in small towns and villages (Houmenou et al., 2013). In reality, different species of rodents tend to be selective of their own habitat. Nonetheless, the rodent species diversity may change as rodent habitats are destroyed due to agricultural intensification, urbanisation or deforestation (Paramasvaran et al., 2013).

From the present results, none of the rats trapped from the two NSTCs in Sarawak were positive for *Leptospira* spp. This contradicts the finding by Mohamed-Hassan et al. (2010). In their study on 168 rats captured in NSTCs in Kelantan and Terengganu, 17.9% showed positive sera against leptospiral antibodies. For the soil and water samples from the two NSTCs, low prevalence of *Leptospira* spp. was recorded. Soil samples in NSTCs showed contamination at 2.7%, 0.7% and 0.7% by pathogenic, intermediate and saprophytic *Leptospira*, respectively while 1.3%, 2.7% and 4.7% of the water samples were contaminated by pathogenic, intermediate and saprophytic *Leptospira*, respectively. These results supported the findings of Ridzlan et al. (2010), who reported the presence of pathogenic *Leptospira* in three out of 145 (2.1%) environmental soil and water samples collected from NSTCs in Kelantan and Terengganu. Previously, two series of screening programmes for *Leptospira* were carried out in 2010 at all NSTCs in northern and eastern regions of Peninsular Malaysia by a public health laboratory in Malaysia. During the first screening programme, 18% (21/115) of water samples from 13 NSTCs were positive for pathogenic *Leptospira*. A total of 13% (16/123) of water samples from nine NSTCs were detected to contain pathogenic *Leptospira* in the second screening programme (Hasanatunnur

Table 2
Nucleotide Sequence Similarity of Leptospira spp. Isolates Targetting lipL32 and 16S Ribosomal RNA Genes Based on Leptospira spp. Reference Strains from GenBank Entries

Sample ID	Study site	Sample	Accession number	Descriptio	Query length (bp)	Query coverage (%)	Maximum identity (%)
Pathogenic *Leptospira* targetting outer membrane protein, lipL32 gene							
CFP11	NSTC	Water	KC800989.1	*Leptospira interrogans* serovar Autumnalis strain RTCC 2802	434	99	89
CFP12	NSTC	Water	KF297610.1	*Leptospira weilii* clone lipL32-122069	446	81	92
CFP16	NSTC	Soil	AY461920.1	*Leptospira noguchii* strain LT796	438	97	90
CFP22	PF	Water	AY461920.1	*Leptospira noguchii* strain LT796	447	98	88
CFP26	PF	Water	AY461920.1	*Leptospira noguchii* strain LT796	427	99	79
CFP23	PF	Soil	AY461920.1	*Leptospira noguchii* strain LT796	445	98	88
CFP27	PF	Soil	AY461920.1	*Leptospira noguchii* strain LT796	439	98	78
Intermediate *Leptospira* targetting 16S ribosomal RNA geneles collected							
CFG29	PF	Rat	NR_044042.1	*Leptospira wolffii* serovar Khorat strain Khorat-H2	342	97	95
CFG9	NSTC	Water	AB758753.1	L*eptospira* sp. MS341	338	97	98
CFG11	NSTC	Water	NR_044042.1	*Leptospira wolffii* serovar Khorat strain Khorat-H2	339	98	98
CFG21	NSTC	Water	KP031573.1	*Leptospira* sp. Neco007	361	93	97
CFG12	NSTC	Soil	NR_044042.1	*Leptospira wolffii* serovar Khorat strain Khorat-H2	340	97	99
Saprophytic *Leptospira* targetting 16S ribosomal RNA gene							
CFS3	NSTC	Water	NR_103924.1	*Leptospira biflexa* serovar Patoc strain 'Patoc 1 (Ames)' strain Patoc 1 (Ames)	256	97	98
CFS5	NSTC	Water	JQ988852.1	*Leptospira meyeri* strain Semaranga_DB49	259	98	97
CFS11	NSTC	Soil	JQ988852.1	*Leptospira meyeri* strain Semaranga_DB49	253	98	99

et al., 2011). Elsewhere, 0.5% (1/220) of stripped field mice, *Apodemus agrarius*, was detected with *Leptospira* in two Korean-operated military training sites (O'Guinn et al., 2010). In 2014, American Marines were reported to be sick due to l*eptospirosis* after attending jungle warfare training at Camp Gonsalves, Japan (Stewart, 2010).

From these results, it can be concluded that although the prevalence of *Leptospira* in these NSTCs is low, the risk is still present. Prior to 2015, there was an intake of more than 100,000 trainees annually at the 93 NSTCs in Malaysia. These trainees were exposed to physical activities like canoeing, abseiling and obstacle courses, which are conducted near the ponds in NSTCs. These activities would have put them at risk for leptospiral infection from urine-contaminated soil and water (Mohamed-Hassan et al., 2010). The National Service programme was suspended in 2015 for a year by the Malaysian government but according to the latest press release (The Star Online, 2016). Defence Minister Datuk Seri Hishammuddin Tun Hussein relaunched a new and improved National Service Training Programme (PLKN2.0) on 24 April, 2016. Therefore, the camp management of NSTCs should always take preventive measures to ensure the safety of the camp environment for any future activities in the camp.

Rodents breed rapidly in paddy fields and this may lead to uncontrolled population growth (Fadzlina et al., 2013). Paddy fields are favourable places for their survival because they can obtain sufficient food in this habitat (Vedhagiri et al., 2010). Out of the 16 rats captured from the paddy fields, only one was infected by intermediate *Leptospira*. Nonetheless, pathogenic *Leptospira* was found in two soil samples and four of the water samples collected. This highlighted the presence of *Leptospira* spp. in the selected paddy fields of Sarawak. Yasouri et al. (2013) reported the presence of 47.0% (54/115) saprophytic *Leptospira* and 33.0% (38/115) pathogenic *Leptospira* in 36 soil samples, 67 water samples and 12 faeces samples collected from a paddy field in Iran. *Leptospira* was also detected in a paddy field in Nakhornratchasrima Province in Thailand (Tangkanakul, 2000).

Leptospirosis outbreaks related to paddy farming is known to be common. Until 1960, more than 200 deaths due to leptospirosis were reported annually in Japan; most of the victims were farmers working in paddy fields (Saito et al., 2013). Consequently, occupation has been perceived as a significant risk factor of *Leptospira* spp. Most leptospirosis infection related to occupation has involved rice field workers, fish farmers, veterinarians, sewer workers and soldiers (Tansuphasiri et al., 2006). In 2011, a large leptospirosis outbreak involving traditional and full-time paddy farmers was reported in Anuradhapura district of Sri Lanka. Paddy farming is the main source of income for these farmers.

In Malaysia, a total of 7,806 cases and 92 deaths resulted from leptospirosis in 2014, while 4,457 cases with 71 deaths were reported in 2013. The distribution of leptospirosis cases by occupation in

Malaysia indicated that farmers contributed to 6% of the total cases in 2013 and 7% in 2014 (Ministry of Health Malaysia, 2015). It can be concluded that the presence of *Leptospira* spp. in the two paddy fields of Sarawak put the farmers at risk of infection to leptospirosis. Hence, the villagers must take preventive measures such as wearing protective clothing and rubber boots to minimise contact with a contaminated environment as suggested by Koay et al. (2004).

CONCLUSION

This study gave an insight into the leptospirosis status in selected NSTCs and paddy fields of Sarawak, Malaysia. Since there is a lack of information on the real status of leptospirosis in these localities, this study serves as a source of important surveillance data for the public health sector in Malaysia, especially Sarawak. Knowing that there is a risk of infection to humans, appropriate preventive measures must be taken by the authorities, the public health sector and the public. Some of the effective preventive measures include usage of protective boots by paddy field farmers and prohibition of using or drinking water from ponds in National Service training centres. Future studies on the risk of exposure to *Leptospira* spp. in other agricultural sites such as land farms and oil palm estates in relation to wet or dry seasons can be conducted for more comprehensive surveillance data.

ACKNOWLEDGEMENTS

This study was funded by the Ministry of Higher Education Malaysia under the Fundamental Research Grant Scheme FRGS/SG03(01)/970/2013(11) and a UNIMAS internal grant under the PhD Student Fund F07(DPP21)/1191/2014(21). Special acknowledgement goes to the National Service Training Department and the camp managers of selected NSTCs for approval to collect samples from the NSTCs in Sarawak. Appreciation also goes to the paddy farmers for allowing us to collect samples from their fields. The authors appreciate the assistance given by lab assistants and undergraduates of the Department of Molecular Biology in Faculty of Resource Science and Technology, Universiti Malaysia Sarawak.

REFERENCES

Alavi, S. M., & Khoshkho, M. M. (2014). Seroprevalence study of leptospirosis among rice farmers in khuzestan province, South west iran, 2012. *Jundishapur journal of microbiology, 7*(7), e11536-e11536.

Baharudin, B., Napiah, R. M., Yasin, M. S., Othman, A., & Abdullah, Z. (2012, July). Outbreak of leptospirosis in Pauh, Arau, Perlis. In *Abstract of UMT 11th International Annual Symposium on Sustainability Science and Management*. Terengganu, Malaysia.

Bernama. (2010, August 13). Contaminated pond closed. *The Sun Daily*. Retrieved from http://www.thesundaily.my/node/139575.

Bernama. (2012, March 31). All water training at PLKN camps suspended-JLKN. *Borneo Post Online*. Retrieved from http://www.theborneopost.com/2012/03/31/all-water-training-at-plkn-camps-suspended-jlkn-latest/.

Chen, H. W., Weissenberger, G., Atkins, E., Chao, C. C., Suputtamongkol, Y., & Ching, W. M. (2015). Highly sensitive loop-mediated isothermal amplification for the detection of Leptospira. *International Journal of Bacteriology, 2015*(2015), 1-6. doi: http://dx.doi.org/10.1155/2015/147173.

Fadzlina, A. S., Nasyriq, A. M. N., Noor Faezah, M. N., Lee, L. H., Cheah, Y. K., & Sukardi, S. (2013). Detection of PLC-ζ from testis of Rattus argentiventer (rice-field rat) using RT-PCR and qRT-PCR. *IOSR Journal of Pharmacy and Biological Sciences, 8*(3), 65–74.

Flores-Encarnación, M., Ramírez-Águila, A., González-Luna, B., Carreño-López, R., Cabrera-Maldonado, C., García-García, S. …Ixtepan-Tejero, C. (2014). Presence of Leptospira in aquatic environments. *Basic Research Journal of Medicine and Clinical Sciences, 3*(8), 80–87.

Hasanatunnur, A., Norliziana, M. A., Roziah, A., Zulhainan, H., & Naim, A. K. (2011). Screening for pathogenic Leptospira from water samples at Pusat Latihan Khidmat Negara (PLKN) in northern and eastern region of Peninsular Malaysia. *Malaysian Journal of Public Health Medicine, 11*(1), 23.

Houemenou, G., Ahmed, A., Libois, R., & Hartskeerl, R. A. (2013). Leptospira spp. prevalence in small mammal populations in Cotonou, Benin. *International Scholarly Research Notices Epidemiology, 2013*, 1-8

Koay, T. K., Nirmal, S., Noitie, L., & Tan, E. (2004). An epidemiological investigation of an outbreak of leptospirosis associated with swimming, Beaufort, Sabah. *Medical Journal of Malaysia, 59*(4), 455–459.

Ministry of Health Malaysia (2015). *Epidemiology and current situation of leptospirosis in Malaysia*. Persidangan Kesihatan Persekitaran Pihak Berkuasa Tempatan 2015. Retrieved from http://jkt.kpkt.gov.my/resources/index/pdf/Persidangan_2015/persidangan%20kesihatan/Leptospirosis_in_Malaysia.pdf.

Mohamed-Hassan, S. N., Bahaman, A. R., Mutalib, A. R., & Khairani-Bejo, S. (2012). Prevalence of pathogenic Leptospires in rats from selected locations in Peninsular Malaysia. *Research Journal of Animal Sciences, 6*(1), 12–25.

Mohamed-Hassan, S. N., Bahaman, A. R., Mutalib, A. R., & Khairani-Bejo, S. (2010). Serological prevalence of leptospiral infection in wild rats at the National Service Training Centres in Kelantan and Terengganu. *Tropical Biomedicine, 27*(1), 30–32.

Narita, M., Fujitani, S., Haake, D. A., & Paterson, D. L. (2005). Leptospirosis after recreational exposure to water in the Yaeyama Islands, Japan. *The American Journal of Tropical Medicine and Hygiene, 73*(4), 652–656.

O'Guinn, M. L., Klein, T. A., Lee, J. S., Richards, A. L., Kim, H. C., Ha, S. J. …Song, J. W. (2010). Serological surveillance of scrub typhus, murine typhus, and leptospirosis in small mammals captured at firing points 10 and 60, Gyeonggi province, Republic of Korea, 2001-2005. *Vector-Borne and Zoonotic Diseases, 10*(2), 125–133.

Paramasvaran, S., Sani, R. A., Krishnasamy, Amal, N. M., Hassan, L., Mohd Zain, S. N., Latifah, I. …Selvanesan, S. (2013). Distribution and morphological measurements of wild and urban rodents from four habitats in the states of Selangor and Negeri Sembilan, Malaysia. *Malaysian Journal of Veterinary Research, 4*(2), 1–12.

Payne, J., & Francis, C. M. (2007). A *field guide to the mammals of Borneo*. Malaysia: The Sabah Society.

Pui, C. F., Bilung, L. M., Su'ut, L., & Apun, K. (2015). Prevalence of Leptospira species in environmental soil and water from national parks in Sarawak, Malaysia. *Borneo Journal of Resource Science and Technology, 5*(1), 49–57.

Ridzlan, F. R., Bahaman, A. R., Khairani-Bejo, S., & Mutalib, A. R. (2010). Detection of pathogenic Leptospira from selected environment in Kelantan and Terengganu, Malaysia. *Tropical Biomedicine, 27*(3), 632–638.

Saito, M., Villanueva, S. Y. A. M., Chakraborty, A., Miyahara, S., Segawa, T., Asoh, T. …Yoshida, S. I. (2013). Comparative analysis of Leptospira strains isolated from environmental soil and water in the Philippines and Japan. *Applied and Environmental Microbiology, 79*(2), 601–609.

Stewart, J. (2014, November 3). Oki Marines fall ill after training in murky waters. *Marine Corps Times*. Retrieved from http://www.marinecorpstimes.com/story/military/pentagon/2014/11/03/oki-marines-fall-ill-after-training-in-murky-waters/18402307.

Sumanta, H., Wibawa, T., Hadisusanto, S., Nuryati, A., & Kusnanto, H. (2015). Spatial analysis of Leptospira in rats, water and soil in Bantul District, Yogyakarta Indonesia. *Open Journal of Epidemiology, 5*(01), 22–31.

Tangkanakul, W. (2000). Risk factors associated with leptospirosis in Northeastern Thailand, 1998. *The American Journal of Tropical Medicine and Hygiene, 63*(3), 204–208.

Tansuphasiri, U., Thipsuk, C., Phulsuksombati, D., & Chanyasanha, C. (2006). Duplex PCR-hybridization based detection of pathogenic Leptospira in environmental water samples obtained from endemic areas in northeast region of Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health, 37*(4), 729–741.

Kumar, M. (2016, April 24). Hisham launches revamped National Service programme. *The Star Online*. Retrieved from http://www.thestar.com.my/news/nation/2016/04/24/hisham-launches-revamped-national-service-programme/

Vedhagiri, K., Natarajaseenivasan, K., Prabhakaran, S. G., Selvin, J., Narayanan, R., Shouche, Y. S. … Ratnam, S. (2010). Characterization of *Leptospira borgpetersenii* isolates from field rats (*Rattus norvegicus*) by 16S rRNA and lipL32 gene sequencing. *Brazilian Journal of Microbiology, 41*(1), 150–157.

YYasouri, S. R., Moghadam, R. G., & Ghane, M. (2013). Identification of pathogenic and saprophytic Leptospira spp. from the rice fields of Tonekabon Township using PCR technique. *Advanced Studies in Biology, 5*(10), 437–445.