



UNIVERSITI PUTRA MALAYSIA

DISTRIBUTION OF *Leptospira* sp. IN LIVESTOCK AND THE ENVIRONMENT IN KELANTAN AFTER A MASSIVE FLOOD

MOHAMMAD SABRI BIN ABDUL RAHMAN

FPV 2018 32



DISTRIBUTION OF *Leptospira* sp. IN LIVESTOCK AND THE ENVIRONMENT IN KELANTAN AFTER A MASSIVE FLOOD

By

MOHAMMAD SABRI BIN ABDUL RAHMAN

Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in fulfillment of the requirements for the Degree of Master of Veterinary Science

March 2018

COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs and all other artwork, is copyright of Universiti Putra Malaysia unless otherwise stated. Use may be made of any material contained within the thesis for non-commercial purposes from the copyright holder. Commercial use of material may only be made with the express, prior, written permission of Universiti Putra Malaysia.

Copyright © Universiti Putra Malaysia



DEDICATION

I would like to dedicate this thesis to my mother, Sa'adiyah Samad and my father, Abdul Rahman Autan. Without their kindness, generosity, and encouragement I would have been ploughing land in a remote village in Malaysia.



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Veterinary Science

**DISTRIBUTION OF *Leptospira* sp. IN LIVESTOCK AND ENVIRONMENT
IN KELANTAN AFTER A MASSIVE FLOOD**

By

MOHAMMAD SABRI ABDUL RAHMAN

March 2018

Chairman : Associate Professor Siti Khairani Bejo, PhD
Faculty : Veterinary Medicine

Leptospirosis caused by pathogenic leptospire is a worldwide health problem in animals and humans. Humans become infected through direct contact with infected animals or through indirect contact with contaminated water or soil. Outbreaks usually follow heavy rainfall and flooding, particularly in endemic areas. A cross-sectional study was carried out in Kelantan after a massive flood in 2014. The aims of this study were to determine the prevalence of leptospiral infection in livestock and to detect the presence of leptospire in water and soil in livestock farms after the massive flood. In-house immunoglobulin G enzyme-linked immunosorbent assay (IgG ELISA) using *Leptospira* sp. local isolate as antigen was also developed in this study.

Whole blood, serum, and urine from livestock and water and soil from livestock farms were collected in all 10 districts of Kelantan. Altogether, 1728 serum samples from 1024 cattle, 366 goats and 338 sheep were collected. Serum samples were tested for detection of anti-leptospiral antibody using microscopic agglutination test (MAT).

Whole blood samples were obtained from 280 cattle, 239 goats, and 116 sheep. Urine samples were collected from 21 cattle, 4 goats, and 6 sheep. In total, 635 whole blood samples and 31 urine samples were inoculated into semisolid Ellinghausen and McCullough, modified by Johnson and Harris (EMJH) medium containing 5-fluorouracil (5-FU) for isolation of *Leptospira* sp. The *Leptospira* sp. isolates were further identified using conventional methods (1M NaCl, 8-azaguanine and 13°C), serology (MAT) and molecular methods (multiplex polymerase chain reaction (mPCR) and DNA sequencing). All whole

blood and urine samples were also directly tested using mPCR. Water and soil sample were obtained from 28 livestock farms in Kelantan. The samples comprised of 62 soil samples and 62 water samples. The samples were filtered and inoculated into semisolid EMJH medium containing 5-FU for isolation of *Leptospira* sp. All isolates were further identified as animal samples mentioned above.

In-house IgG ELISA using local isolate of *L. kmetyi* serovar *malaysia* strain Bejo-Iso9 as antigen (ELISA-Bejo-Iso9) was developed in this study. One hundred and sixty (160) cattle sera were randomly selected. The selected serum samples comprised of 80 seropositive MAT and 80 seronegative MAT were tested with ELISA-Bejo-Iso9. The performance of ELISA-Bejo-Iso9 was compared with in-house IgG ELISA using reference strain 117123 (*hardjobovis*) antigen (ELISA-117123) and CUSABIO® commercial ELISA.

Microscopic agglutination test (MAT) results revealed that 11.75% (203/1728) of the livestock were seropositive for leptospiral infection. Serum samples from livestock were screened against 17 reference strains of pathogenic *Leptospira* sp., one reference strain of non-pathogenic *Leptospira* sp. and one local strain of pathogenic *Leptospira* sp. The districts of Gua Musang (4.12% ; 72/1728) , Kota Bharu (1.74% ; 30/1728), Kuala Krai (1.22% ; 21/1728) and Tanah Merah (1.10% ; 19/1728) showed the higher distribution of leptospiral antibodies in livestock compared to the other six districts. Those four districts were affected by the flood. Cattle had the highest serological prevalence of 8.39% (145/1728), while goats and sheep had 2.37% (41/1728) and 0.93% (17/1728) respectively. The predominant serovars detected were *L. hardjobovis* (3.70% ; 64/1728) and *L. hebdomadis* (2.08% ; 36/1728). There was a statistically significant association ($p < 0.05$) between samples collected from livestock that were exposed to the flood compared to those that were not exposed. This result indicates the potential risk associated between flooding and leptospirosis. *Leptospira* sp. was only isolated from cattle's urine in Gua Musang. All whole blood samples were negative for *Leptospira* sp. There were 14 (11.29% ; 14/124) and seven (5.65% ; 7/124) *Leptospira* sp. were isolated from soil and water respectively.

Direct detection using mPCR showed that 0.63% (4/635) of whole blood and 3.23% (1/31) of urine samples were positive for *Leptospira* sp. Pathogenic *Leptospira* sp. were detected in two blood samples and one urine sample of cattle. Non-pathogenic *Leptospira* sp. were detected in two blood samples of goat. All positive samples were from livestock exposed to the flood.

Conventional methods revealed varies in the result and failed to differentiate the isolates into pathogenic and non-pathogenic *Leptospira* sp. Microscopic agglutination test (MAT) showed that the isolates reacted to *L. autumnalis*, *L. hebdomadis*, *L. pyrogenes*, *L. bataviae*, *L. patoc* and *L. wolffii*. However,

mPCR showed that all isolates were non-pathogenic *Leptospira* sp. Further identification using DNA sequencing found that, all the isolates were identified as *L. wolffii*, an intermediate species of *Leptospira*. The presence of *L. wolffii* in water, soil and animal provides evidence of transmission of the organism within the environment and animal hosts.

The sensitivity and specificity of ELISA-Bejo-Iso9 were 53.75% and 98.75% respectively. The sensitivity and specificity of ELISA-117123 were 73.25% and 98.75% respectively. The sensitivity and specificity of CUSABIO® commercial ELISA were 80.00% and 97.50% respectively. Based on the sensitivity and specificity obtained, it shows that commercial ELISA has good performance compared than both in-house ELISA.

In conclusion, this study revealed the role of flooding in the transmission and distribution of leptospires to susceptible livestock and represents a threat to public health. The detection of *Leptospira* sp. in livestock and environment is needed to ensure necessary action to be taken to minimise the occurrence of the disease after the flood. As leptospirosis is endemic in Malaysia, a very specific test is needed to diagnose leptospirosis. ELISA-Bejo-Iso9 was shown to be specific and may be suitable to use for serological diagnosis of leptospiral infection in livestock.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia,
sebagai memenuhi keperluan untuk Ijazah Master Sains Veterinar

**DISTRIBUSI *Leptospira* sp. DALAM TERNAKAN DAN ALAM SEKITAR DI
KELANTAN SELEPAS BANJIR BESAR**

Oleh

MOHAMMAD SABRI ABDUL RAHMAN

Mac 2018

Pengerusi : Professor Madya Siti Khairani Bejo, PhD
Fakulti : Perubatan Veterinar

Leptospirosis yang disebabkan oleh bakteria *Leptospira* sp. yang patogenik adalah masalah kesihatan dalam haiwan dan manusia di seluruh dunia. Manusia dijangkiti melalui hubungan langsung dengan haiwan yang dijangkiti atau melalui sentuhan tidak langsung dengan air atau tanah yang tercemar. Wabak biasanya berlaku selepas hujan lebat dan banjir, terutamanya di kawasan yang endemik. Kajian rentas telah dijalankan di Kelantan selepas banjir besar pada tahun 2014. Tujuan kajian ini adalah untuk menentukan jangkitan leptospiral dalam ternakan dan untuk mengesan kehadiran *Leptospira* sp. dalam air dan tanah di ladang ternakan selepas banjir besar. Esei imunoserapan berkait enzim imunoglobulin G (IgG ELISA) secara 'in-house' juga dibangunkan dengan menggunakan isolat tempatan.

Sampel darah, serum, dan air kencing telah diambil dan dikumpulkan daripada ternakan, air dan tanah dari ladang ternakan di 10 daerah di Kelantan. Secara keseluruhan, jumlah sampel serum ialah 1728 yang terdiri daripada 1024 dari lembu, 366 dari kambing dan 338 daripada bebiri. Sampel serum telah diuji untuk mengesan antibodi anti-leptospiral menggunakan ujian aglutinasi mikroskopik (MAT).

Sampel darah yang telah diperolehi daripada 280 ekor lembu, 239 ekor kambing dan 116 ekor bebiri. Sampel kencing telah dikumpulkan dari 21 ekor lembu, 4 ekor kambing dan 6 ekor bebiri. Secara keseluruhannya, 635 sampel darah dan 31 sampel air kencing telah disuntikkan ke dalam medium separa pepejal Ellinghausen dan McCullough, yang diubahsuai oleh Johnson dan Harris (EMJH) yang mengandungi 5-fluorouracil (5-FU) untuk pengasingan

Leptospira sp. *Leptospira* sp. isolat telah dikenal pasti dengan menggunakan kaedah konvensional (1M NaCl, 8-azaguanine dan 13°C), serologi (MAT) dan molekular (reaksi rangkaian polimerase multipleks (mPCR) dan penjujukan DNA). Semua sampel darah dan sampel air kencing juga diuji secara langsung menggunakan mPCR. Sampel air dan tanah diperoleh dari 28 ladang ternakan di Kelantan. Sampel terdiri daripada 62 sampel tanah dan 62 sampel air. Sampel itu ditapis dan disuntik ke medium separa pepejal EMJH yang mengandungi 5-FU untuk pengasingan *Leptospira* sp. Semua *Leptospira* sp. isolat dikenal pasti sebagai sampel haiwan yang disebutkan di atas.

IgG ELISA secara 'in-house' menggunakan isolat tempatan *L. kmetyi* serovar *malaysia* strain Bejo-Iso9 sebagai antigen (ELISA-Bejo-Iso9) telah dibangunkan dalam kajian ini. Seratus enam puluh (160) sampel serum daripada lembu dipilih secara rawak. Sampel serum yang dipilih terdiri daripada 80 seropositif MAT dan 80 seronegatif MAT dan diuji dengan menggunakan ELISA-Bejo-Iso9. Prestasi ELISA-Bejo-Iso9 dibandingkan dengan IgG ELISA secara 'in-house' menggunakan isolat rujukan 117123 (*hardjobovis*) sebagai antigen (ELISA-117123) dan komersial ELISA CUSABIO®.

Hasil ujian agglutinasi mikroskopik (MAT) menunjukkan bahawa 11.75% (203/1728) ternakan adalah seropositif untuk jangkitan leptospiral. Sampel serum dari ternakan telah diuji terhadap 17 patogenik *Leptospira* sp., satu *Leptospira* sp. yang tidak patogenik dan satu isolat tempatan *Leptospira* sp. yang patogenik. Daerah Gua Musang (4.12% ; 72/1728), Kota Bharu (1.74% ; 30/1728), Kuala Krai (1.22% ; 21/1728) dan Tanah Merah (1.10% ; 19/1728) menunjukkan pengagihan antibodi leptospiral lebih tinggi dalam ternakan berbanding enam daerah lain. Empat daerah itu terjejas oleh banjir. Lembu mempunyai prevalensi serologi tertinggi sebanyak 8.39% (145/1728), manakala kambing dan bebiri masing-masing mempunyai 2.37% (41/1728) dan 0.93% (17/1728). Serovar yang dikesan paling tinggi adalah *L. hardjobovis* (3.70% ; 64/1728) dan *L. hebdomadis* (2.08% ; 36/1728). Terdapat perbezaan yang signifikan secara statistik ($p < 0.05$) antara sampel yang dikumpulkan dari ternakan yang terdedah kepada banjir berbanding dengan yang tidak didedahkan. Hasil ini menunjukkan potensi risiko yang berkaitan antara banjir dan leptospirosis. Satu *Leptospira* sp. isolat terasing dari air kencing lembu di Gua Musang. Keseluruhan sampel darah adalah negatif untuk *Leptospira* sp. Terdapat 14 (11.29% ; 14/124) dan tujuh (5.65% ; 7/124) *Leptospira* sp. isolat diasingkan masing-masing daripada tanah dan air.

Pengesanan langsung menggunakan mPCR menunjukkan bahawa keseluruhan sampel darah 0.63% (4/635) dan 3.23% (1/31) sampel air kencing adalah positif kepada *Leptospira* sp. *Leptospira* sp. telah dikesan dalam dua sampel darah dan satu sampel air kencing daripada lembu.

Leptospira sp. yang patogenik juga telah dikesan dalam dua sampel darah daripada kambing. Semua sampel positif adalah daripada ternakan yang terdedah kepada banjir.

Kaedah konvensional mendedahkan keputusan yang variasi dan gagal untuk membezakan isolat menjadi spesies *Leptospira* yang patogenik dan tidak patogenik. Ujian aglutinasi mikroskopik (MAT) menunjukkan bahawa isolat bertindak balas terhadap *L. autumnalis*, *L. hebdomadis*, *L. pyrogenes*, *L. bataviae*, *L. patoc* dan *L. wolffii*. Walaubagaimanapun, mPCR menunjukkan bahawa semua isolat adalah *Leptospira* sp. yang tidak patogenik. Pengenalpastian lebih lanjut menggunakan penjujukan DNA mendapati bahawa semua isolat telah dikenalpasti sebagai *L. wolffii*, iaitu spesies perantaraan *Leptospira*. Kehadiran *L. wolffii* di dalam air, tanah dan haiwan membuktikan bahawa transmisi organisma ini terdapat di antara alam sekitar dan haiwan.

Kepekaan dan kekhususan ELISA-Bejo-Iso9 masing-masing 53.75% dan 98.75%. Kepekaan dan kekhususan ELISA-117123 masing-masing adalah 73.25% dan 98.75%. Kepekaan dan kekhususan ELISA komersial CUSABIO® masing-masing adalah 80.00% dan 97.50%. Berdasarkan kepekaan dan kekhususan yang diperolehi, ia menunjukkan bahawa komersial ELISA mempunyai prestasi yang baik berbanding dengan ELISA secara 'in-house'.

Kesimpulannya, kajian ini menunjukkan peranan banjir dalam transmisi dan distribusi *Leptospira* sp. kepada ternakan yang terdedah dan merupakan ancaman kepada kesihatan awam. Pengesanan *Leptospira* sp. dalam ternakan dan alam sekitar diperlukan untuk memastikan tindakan yang perlu diambil untuk meminimumkan terjadinya penyakit selepas banjir. Oleh kerana leptospirosis adalah endemik di Malaysia, ujian yang sangat khusus diperlukan untuk mendiagnosis leptospirosis. ELISA-Bejo-Iso9 sangat khusus dan mungkin sesuai digunakan untuk diagnosis serologi jangkitan leptospiral dalam ternakan.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my heartiest gratitude, deepest thanks and appreciation to my supervisor, Assoc. Prof. Dr. Siti Khairani Bejo for her kind assistance, guidance and advice throughout my research work and valuable suggestions that helped to shape my research skills.

I thank to my co-supervisors, Assoc. Prof. Dr. Zunita Zakaria and Prof. Dr. Latiffah Hassan for their guidance, patience and knowledge which they have imparted throughout my research work. A special thanks goes to Prof Dato' Dr. Abdul Rani Bahaman, Assoc. Prof. Dr. Goh Yong Meng and Dr. Siti Zubaidah for their invaluable insight.

My warmest thanks to all staffs from Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM (En. Azri Roslan, Cik Krishnammah, Cik Nur Rabiataladawiyah, Pn. Rasimah), Department of Veterinary Services, Kelantan (Dr. Bainun, En. Zakaria, En. Che Mat), Veterinary Regional Laboratory, Kota Bharu and all farmers who involved for their technical assistance.

Last but not least, I would like to convey my deepest gratitude to my beloved family for their endless support and encouragement to do my very best in whatever I do. To everyone especially Alif, Rasul, Fahmi, Adilah, Shazreena, Bashiru, Ghada, Sattar, Krishnan, Kiven, Goh, Azri, Danish, Fadzly, Akmal, Diyana, Najhah, Ammar, Sofie and Abdul Rahman who had provided me with continuous help, support, and encouragement as well as priceless memories throughout the entire duration of my research, thank you.

I certify that a Thesis Examination Committee has met on 9 March 2018 to conduct the final examination of Mohammad Sabri bin Abdul Rahman on his thesis entitled "Distribution of *Leptospira* sp. in Livestock and the Environment in Kelantan After a Massive Flood" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Veterinary Science.

Members of the Thesis Examination Committee were as follows:

Abdul Rahman bin Omar, PhD

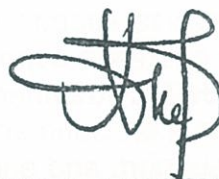
Professor
Institute of Bioscience
Universiti Putra Malaysia
(Chairman)

Mohd Zamri bin Saad, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Aziah binti Daud, PhD

Associate Professor
Universiti Sains Malaysia
Malaysia
(External Examiner)



NOR AINI AB. SHUKOR, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 26 April 2018

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for degree of Master of Veterinary Science. The members of Supervisory Committee are as follows:

Siti Khairani Bejo, PhD

Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Latiffah Hassan, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Zunita Zakaria, PhD

Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD

Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by graduate student

I hereby confirm that:

- this thesis is my original work;
- quotations, illustrations and citations have been duly referenced;
- This thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- intellectual property from the thesis and copyright of the thesis are fully-owned by Universiti Putra Malaysia, as according to Universiti Putra Malaysia (Research) Rules 2012;
- written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writings, seminar papers, manuscripts, posters, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- there is no plagiarism or data falsification / fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature: _____ Date: _____

Name and Matric No: Mohammad Sabri Bin Abdul Rahman, GS43790

Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

Signature: _____

Name of
Chairman of
Supervisory
Committee:

Associate Professor
Dr. Siti Khairani Bejo

Signature: _____

Name of
Member of
Supervisory
Committee:

Professor
Dr. Latiffah Hassan

Signature: _____

Name of
Member of
Supervisory
Committee:

Associate Professor
Zunita Zakaria

TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iv
ACKNOWLEDGEMENTS	vii
APPROVAL	viii
DECLARATION	x
LIST OF TABLES	xv
LIST OF FIGURES	xvii
LIST OF ABBREVIATIONS	xix
CHAPTER	
1 INTRODUCTION	1
1.1 Justification	2
1.2 Objectives	3
1.3 Hypotheses	3
2 LITERATURE REVIEW	4
2.1 Leptospirosis	4
2.2 Biology and classification of leptospires.	4
2.3 Differences between saprophytic and pathogenic leptospires	6
2.4 Mode of transmission to human and animal	7
2.5 Pathogenesis and immunity	8
2.6 Leptospirosis in Malaysia	10
2.6.1 Leptospirosis in humans	11
2.6.2 Leptospirosis in animals	12
2.6.3 Leptospire in water and soil	14
2.7 Flooding and leptospirosis	15
2.8 Approaches to the diagnosis of leptospirosis	16
2.8.1 Isolation of organism	17
2.8.2 Microscopic agglutination test (MAT)	17
2.8.3 Enzyme-linked immunosorbent assay (ELISA)	18
2.8.4 Polymerase chain reaction (PCR)	18

3	BACTERIOLOGICAL AND SEROLOGICAL PREVALENCES OF LEPTOSPIRAL INFECTION IN LIVESTOCK IN KELANTAN FOLLOWING A MASSIVE FLOOD	20
3.1	Introduction	20
3.2	Materials and methods	21
3.2.1	Animals	21
3.2.2	Isolation of <i>Leptospira</i> sp. from blood and urine	23
3.2.3	Identification of <i>Leptospira</i> sp. isolated from blood and urine	23
3.2.4	Serological detection of leptospiral infection in livestock	28
3.2.5	Statistical analysis	31
3.3	Results	31
3.3.1	Bacteriological prevalence of leptospiral infection in livestock in Kelantan	31
3.3.2	Serological prevalence of leptospiral infection in livestock in Kelantan	37
3.4	Discussion	42
3.5	Conclusion	46
4	DETECTION OF <i>Leptospira</i> sp. IN LIVESTOCK AND ENVIRONMENT IN KELANTAN FOLLOWING A MASSIVE FLOOD	47
4.1	Introduction	47
4.2	Materials and methods	48
4.2.1	Animals	48
4.2.2	Direct detection of <i>Leptospira</i> sp. from blood and urine using multiplex polymerase chain reaction (mPCR)	48
4.2.3	Environmental samples	50
4.2.4	Isolation of <i>Leptospira</i> sp. from water and soil	51
4.2.5	Identification of <i>Leptospira</i> sp. isolated from water and soil	52
4.2.6	Statistical analysis	53
4.3	Results	53
4.3.1	Direct detection of <i>Leptospira</i> sp. from blood and urine using multiplex polymerase chain reaction (mPCR)	53
4.3.2	Isolation and identification of <i>Leptospira</i> sp. from water and soil	57
4.4	Discussion	64
4.5	Conclusion	67

5	DETECTION OF LEPTOSPIRAL INFECTION IN CATTLE IN KELANTAN USING IN-HOUSE IMMUNOGLOBULIN G (IgG) ELISA	68
5.1	Introduction	68
5.2	Materials and methods	69
5.2.1	Serum sample	69
5.2.2	In-house immunoglobulin G (IgG) ELISA	69
5.2.3	Commercial ELISA	70
5.2.4	Statistical analysis	70
5.3	Results	71
5.3.1	Establishment of ELISA-Bejo-Iso9 and ELISA-117123	71
5.3.2	Performance of ELISA-Bejo-Iso9, ELISA-117123 and CUSABIO® commercial ELISA	74
5.4	Discussion	76
5.5	Conclusion	78
6	SUMMARY, GENERAL CONCLUSION, AND RECOMMENDATION FOR FUTURE RESEARCH	79
	REFERENCES	82
	APPENDICES	98
	BIODATA OF STUDENT	119
	LIST OF PUBLICATIONS	120

LIST OF TABLES

Table	Page
2.1 Reservoirs of different serovars present in animals.	5
2.2 Test methods available for diagnosis of leptospirosis and their purpose.	16
3.1 Blood samples collected from livestock (cattle, goat and sheep) in 10 districts of Kelantan.	22
3.2 Urine samples collected from livestock (cattle, goat and sheep) in 10 districts of Kelantan.	22
3.3 Panel of reference hyperimmune sera with respective titre used in MAT for identification of <i>Leptospira</i> sp. isolates.	25
3.4 16S rRNA primer and LipL32 primer used in mPCR.	26
3.5 PCR reaction volumes consisting of 20.00 µl PCR working concentration and 5.00 µl DNA template.	27
3.6 PCR protocol comprised of initial denaturation, denaturation, annealing, extension and final extension with specific temperature, time and cycle.	27
3.7 Serum samples collected in 10 districts of Kelantan comprised of 1.024 cattle, 366 goats and 338 sheep.	29
3.8 Panel of antigens comprised of 19 leptospiral serovars used in MAT.	30
3.9 <i>Leptospira</i> sp. isolated from urine samples of livestock.	32
3.10 Microscopic agglutination test (MAT) seropositive results by sampling location, animal species, <i>Leptospira</i> sp. serovar and MAT titre.	37
3.11 Frequency of leptospiral serovars detected in livestock in Kelantan tested by using MAT with 19 leptospiral antigens.	39
3.12 Distribution of anti-leptospiral antibodies in livestock in 10 districts of Kelantan with predominant leptospiral serovar in each district.	41
3.13 2x2 table for MAT results and flood status.	42

4.1	Environmental samples comprised of 62 water samples and 62 soil samples collected in 28 farms in Kelantan.	51
4.2	Blood samples from goat in Gua Musang and cattle in Kuala Krai and Kota Bharu tested positive for <i>Leptospira</i> sp. by using mPCR correspond with MAT titre.	54
4.3	Direct detection of <i>Leptospira</i> sp. from urine samples using mPCR.	56
4.4	Positive <i>Leptospira</i> sp. isolation from water and soil samples in Kelantan.	58
4.5	Differentiation between pathogenic and saprophytic leptospires from 21 <i>Leptospira</i> sp. isolates using 1M NaCl, 8-azaguanine and 13°C methods.	59
4.6	<i>Leptospira</i> sp. isolates reaction towards 17 hyperimmune sera by using MAT.	60
5.1	Area Under the Curve (AUC) generated from ROC curve analysis at three different serum dilutions for ELISA-Bejo-Iso9.	73
5.2	Area Under the Curve (AUC) generated from ROC curve analysis at three different serum dilutions for ELISA-117123.	73
5.3	Sensitivity and specificity of ELISA-Bejo-Iso9 on three cut-off dilutions. TP: True Positive; FN: False Negative; TN: True Negative; FP: False Positive.	74
5.4	Sensitivity and specificity of ELISA-117123 on three cut-off dilutions. TP: True Positive; FN: False Negative; TN: True Negative; FP: False Positive.	75
5.5	2x2 table between MAT (positive and negative) and commercial ELISA (positive and negative).	75
5.6	Performance of commercial ELISA based on sensitivity, specificity, positive predictive value and negative predictive value.	75

LIST OF FIGURES

Figure	Page
2.1 High-resolution scanning electron micrograph of <i>Leptospira interrogans</i> serovar <i>copenhageni</i> (Bharti <i>et al.</i> , 2003). (A) Note characteristic hooked ends ; (B) At high magnification the surface of the spirochete seems ruffled and beaded.	5
2.2 Mode of transmission of leptospirosis to human and animals.	8
2.3 Antibody response towards leptospiral infection against time.	10
3.1 Pathogenic reference control <i>L. hardjobovis</i> strain 117123 examined under dark-field microscopy 20x magnification. (A) Without 1M NaCl; (B) After two hours incubation with 1M NaCl (spherical form – see arrows).	33
3.2 Saprophytic reference control <i>L. patoc</i> strain Patoc I examined under dark-field microscopy 20x magnification. (C) Without 1M NaCl; (D) After two hours incubation with 1M NaCl (remain the original form).	33
3.3 Identification of <i>Leptospira</i> sp. isolate from urine of cattle in Gua Musang using mPCR. (M) 100bp DNA ladder; (1) negative control (RNase free water); (2) positive control reference strain <i>L. hardjobovis</i> ; (3) <i>Leptospira</i> sp. isolate from urine of cattle.	34
3.4 16S rRNA gene sequence-based phylogeny of <i>L. wolffii</i> strain UPM-G.Musang 5, <i>L. wolffii</i> strain Khorat-H2 ^T , representative <i>Leptospira</i> sp. and <i>Leptonema illini</i> serovar Habaki as an outgroup.	36
3.5 Frequency of seropositive samples by MAT titre.	40
3.6 Distribution of anti-leptospiral antibodies in livestock in 10 districts of Kelantan.	41
4.1 Direct detection of <i>Leptospira</i> sp. in blood from cattle and goat using mPCR. (M) 100bp DNA ladder; (3)-(6) blood samples; (1) negative control (RNase free water); (2) positive control <i>L. hardjobovis</i> (strain 117123); (3)-(4) <i>Leptospira</i> sp. in blood of cattle; (5)-(6) <i>Leptospira</i> sp. in blood of goats; (7) positive control <i>L. hardjobovis</i> (strain 117123).	55

4.2	Direct detection of <i>Leptospira</i> sp. in urine from cattle using mPCR. (M) 100bp DNA ladder; (1) negative control (RNase free water); (2) positive control <i>L. hardjobovis</i> (strain 117123); (3) <i>Leptospira</i> sp. in urine of cattle.	57
4.3	Distribution of <i>Leptospira</i> sp. isolated from water and soil from livestock farms in Kelantan.	58
4.4	Six <i>Leptospira</i> sp. isolates from environmental samples identified by using mPCR. (M) 100bp DNA ladder; (1) negative control (RNase free water); (2) positive control <i>L. hardjobovis</i> (strain 117123); (3-6) <i>Leptospira</i> sp. isolates from environmental samples.	61
4.5	Evolutionary relationships of taxa based on 16S rRNA gene for <i>L. wolffii</i> isolates obtained from soil and water in Kelantan and designated with UPM strains.	63
5.1	Receiver Operating Characteristic (ROC) plots for three different serum dilutions (50, 100 and 200). The ROC curve was constructed from 160 serum samples tested using ELISA-Bejo-Iso9.	72
5.2	Receiver Operating Characteristic (ROC) plots for three different serum dilutions (50, 100 and 200). The ROC was constructed from 160 serum samples tested using ELISA-117123.	72

LIST OF ABBREVIATIONS

%	Percentage
5-AS	5-amino-salicylic acid
5-FU	5-fluorouracil
μM	Micromolar
μl	Microliter
ABTS	Azinobis-3-ethylbenzthiazole-sufonic-acid
AUC	Area under the curve
BLAST	Basic local alignment search tool
CI	Confidence interval
DNA	Deoxyribonucleic acid (s)
dNTP	Deoxynucleotide triphosphate
EDTA	ethylene-diamine-tetraacetic-acid
ELISA	Enzyme-linked immunosorbent assay
EMJH	Ellinghausen and McCullough modified by Johnson and Harris
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MAT	Microscopic agglutination test
mg	Miligram
MgCl_2	Magnesium chloride
ml	Mililiter
mM	Milimolar
mPCR	Multiplex polymerase chain reaction
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information

OD	Optical density
OPD	Ortho-phenylenediamine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ROC	Receiver operating characteristic
rpm	Revolutions per minute
TBE	Tris-borate-EDTA
TMB	Tetra-methylbenzidine
Tris-Cl	Tris-chloride



CHAPTER 1

INTRODUCTION

Leptospirosis is a zoonotic disease with a worldwide distribution and characterised by an acute febrile illness. It is caused by microorganisms of the genus *Leptospira* (Farr, 1995). This disease can infect humans and various species of animals (Koteeswaran, 2006). Rats are the reservoir for leptospires and transmit the infection to humans along with wild and domestic animals such as cattle, pigs, and dogs (Faine and Stallman, 1982; Ko, 1999; Vinetz, 2001). However, humans are known as 'dead-end' hosts whereby transmission of the bacteria between humans or human to animals is rare. Transmission of the bacteria can be directly between animal to human or indirect by from contaminated water or soil (Wynwood *et al.*, 2014).

The leptospiral infection causes flu-like episodes with frequent severe renal and hepatic damages leading to hemorrhages and jaundice in humans (Vijayachari *et al.*, 2008). In livestock, the disease causes abortions, stillbirths, weak newborns, drop in milk yield, mastitis, icterus, hemoglobinuria and conjunctivitis (Thiermann, 1984). Although most of the leptospiral infections are subclinical, virulent serovar may cause death and economic losses to livestock farmers (Ellis, 1984). Death is due to combination of kidney failure, liver failure as well as pulmonary hemorrhages (Bharti *et al.*, 2003). Certain leptospiral serovars are maintained in infected reservoir hosts either wild or domestic animals that serve as a potential source of infection in human and other animals (Jamshidi *et al.*, 2009).

Leptospirosis is common in tropical and subtropical regions. It is estimated that 0.1 to 1 per 100 000 people living in temperate climates are affected each year, with the number increasing to 10 or more per 100 000 people living in tropical climates (Leptospirosis | CDC, 2017). If there is an epidemic, the incidence can soar to 100 or more per 100 000 people. The disease is under-reported for many reasons, including difficulty in distinguishing clinical signs from a number of diseases and lack of appropriate diagnostic laboratory services (Leptospirosis | CDC, 2017). A report by World Health Organization (2015) revealed that the global burden of leptospirosis is 0.10–975 cases per 100,000 populations and case fatality in the region of 6.85% depending on the prevalent serovars, healthcare services and economic status of the population.

Although leptospirosis occurs worldwide, there are few risk factors that are associated with the disease. Leptospirosis is most common in urban slum areas, where there are inadequate sewage disposal and water treatment. It can also be an occupational hazard for those working outdoors or with animals

(Whitney *et al.*, 2009). This disease is also reported as recreational hazard for those participating in water-related activities. Epidemics are typically seen during flooding and changing environmental trends, with extreme weather patterns that may perpetuate these epidemics (WHO, 2017).

Human leptospirosis is prevalent in many countries (Levett, 2001; Pappas *et al.*, 2008) and is widespread in Malaysia (Bahaman *et al.*, 1988; Bahaman *et al.*, 1990; Bahaman *et al.*, 2002). According to Bahaman *et al.*, (1987), domestic animals play a role in the epidemiology of leptospirosis and represents a threat to public health. Since then, 37 leptospiral serovars have been isolated in Malaysia (Bahaman, 1996). Through the years, the number of deaths in human continues to increase significantly (Benacer *et al.*, 2016). In Malaysia, the humid environment favours the growth of pathogenic *Leptospira* sp. with tropical weather and flooding that occur frequently (Lim, 2011).

In December 2014, Kelantan was hit by a massive flood following continuous heavy rainfall. During the flood, many reservoirs, carriers or maintenance host for leptospires died and contaminate the water. Humans and animals may contract leptospires from contaminated water or soil during or after the flood. During this period the incidence of leptospirosis in human in Kelantan was reported to be high (MOH, 2015). Altogether, 1229 leptospirosis cases in human were notified in Kelantan, before, during and after flood (Firdaus *et al.*, 2016). However, there is no information on leptospirosis in livestock in Kelantan following the massive flood. Therefore, a cross-sectional study was conducted in 10 districts of Kelantan after the flood to determine the status of leptospirosis in livestock and to identify the association between environmental contamination and hosts status. This study also highlighted the performance of in-house enzyme-linked immunosorbent assay (ELISA) in diagnosing leptospiral infection in animals.

1.1 Justification

This study was conducted to answer these research questions:

1. What is the prevalence of leptospiral infection in livestock in Kelantan following the flood.
2. How often does *Leptospira* sp. occur in the environment in Kelantan.

1.2 Objectives

Therefore the objectives of this study are:

1. To determine the bacteriological and serological prevalences of leptospiral infection in livestock in Kelantan following a massive flood.
2. To detect *Leptospira* sp. in livestock and environment in Kelantan.
3. To detect leptospiral infection in cattle in Kelantan using in-house immunoglobulin G (IgG) ELISA.

1.3 Hypotheses

The hypotheses of this study are:

1. The prevalence of leptospiral infection in livestock in Kelantan is high following the massive flood.
2. *Leptospira* sp. widely present in livestock and environment in Kelantan.
3. The performance of in-house IgG ELISA is good in terms of specificity and sensitivity.

REFERENCES

- Acha, P. & Szyfres, B. (2003). Leptospirosis. En: Acha P, Szyfres B (eds). Zoonosis y enfermedades transmisibles comunes al hombre y a los animales. *Pan American Health Organization OPS/OMS Washington, USA*, 175-186.
- Adler, B. & de la Peña Moctezuma, A. (2010). *Leptospira* and leptospirosis. *Veterinary Microbiology*, 140(3-4):287-296.
- Adler, B., Lo, M., Seemann, T., & Murray, G. (2011). Pathogenesis of leptospirosis: The influence of genomics. *Veterinary Microbiology*. 153(1-2):73-81.
- Agudelo-Florez, P., Londono A. F., Quiroz V. H., Angel, J. C., Moreno, N., Loaiza, E. T., Munoz, L. F. & Rodas, J. D. (2009). Prevalence of *Leptospira spp.* in urban rodents from a groceries trade center of Medellin, Colombia. *American Journal of Tropical Medicine and Hygiene*, 81:906–910.
- Ahmad, N., Anuar, N., Noormala, A., Salina A. A., Firdaus, H., Nurfarahain, K., Luqman, H., Sallehudin, I. & Muhamad, A. S. (2017). Rat and Leptospirosis Study in Tangkak, Johor, Malaysia 2014 and 2017. Conference Paper.
- Ahmad, S. N., Shah, S. & Ahmad, F., (2005). Laboratory Diagnosis of Leptospirosis. *Journal of Postgraduate Medicine*, 51:195-200.
- Ahmed, A., Grobush, M. P., Klatser, P. & Hartskeerl, R. A. (2012). Molecular approaches in the detection and characterization of *Leptospira*. *Journal of Bacteriology & Parasitology*, 3:133.
- Ahmed, S. A., Doblin, A. S., Musa, S., Hoe, C. H., Riadzi, M., Lau, K. L. & Tang, T. H. (2011). Rapid Diagnosis of Leptospirosis by Multiplex PCR. *Malaysian Journal of Medical Sciences*, 19(3):9-16.
- Alderman, K., Turner L. R. & Tong, S. (2012). Floods and human health. A systematic review *Environmental International*, 15(47):37-47.
- Amilasan, A. T., Ujiie, M. & Suzuki, M. (2012). Outbreak of Leptospirosis after flood, the Philippines, 2009. *Emerging Infectious Diseases*, 18(1): 91-94.
- Andre-Fontaine, G., Aviat, F. & Thorin, C. (2015). Waterborne Leptospirosis: Survival and Preservation of the Virulence of Pathogenic *Leptospira spp.* in Fresh Water. *Current Microbiology*, 71(1):136-42.

- Andre-Fontaine, G., Peslerbe, X. & Ganiere, J. P. (1992). Occupational Hazard of Unnoticed Leptospirosis in Water Ways Maintenance Staff. *European Journal of Epidemiology*, 8(2):228-232.
- Bahaman, A. R. & Ibrahim, A. (1988). A review of leptospirosis in Malaysia. *Veterinary Research Communications*. 12(2-3):179-189.
- Bahaman, A. R. (1996). Leptospirosis-A Model for Epidemiology, Diagnosis and Control of Infectious Diseases. *Universiti Pertanian Malaysia*, 1-15.
- Bahaman, A. R. (2016). Rats and Leptospirosis, A Unique Tropical Experience. *Malaysian Veterinary Journal*, 28(1):26-27.
- Bahaman, A. R., Ibrahim, A. & Adam, H. (1987). Serological prevalence of leptospiral infection in domestic animals in West Malaysia. *Epidemiology And Infection*. 99(2):379-392.
- Bahaman, A. R., Ibrahim, A., & Stallman, N. (1990). NOTES: *Leptospira interrogans* Serovar Unipertama Isolated in Malaysia. *International Journal Of Systematic Bacteriology*, 40(1):98-99.
- Bahaman, A. R., Ibrahim, A., Stallman, N. & Tinniswood, R. (1988). The bacteriological prevalence of leptospiral infection in cattle and buffaloes in West Malaysia. *Epidemiology And Infection*, 100(2):239-246.
- Bahaman, A. R., Khairani-Bejo, S., Yasin, M., Rohani, Hiu & Jessie. (2002). An acute and fatal case of human leptospirosis in Sabah, Malaysia. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, 10:17-19.
- Bal, A., Gravekamp, C., Hartskeerl, R. A., De Meza-Brewster, J., Korver, H. & Terpstra, W. J. (1994). Detection of leptospires in urine by PCR for early diagnosis of leptospirosis. *Journal of Clinical Microbiology*, 32:1894-1898.
- Benacer, D., Mohd, Z., Amran, F., Galloway, R. L. & Thong, K. L. (2013). Isolation and molecular characterization of *Leptospira interrogans* and *Leptospira borgpetersenii* isolates from the urban rat populations of Kuala Lumpur, Malaysia. *American Journal of Tropical Medicine and Hygiene*, 88:704–709.
- Benacer, D., Mohd-Zain, S. N., Sim, S. Z., Mohd-Khalid, K. N., Galloway, R. L., Souris, M. & Thong, K. L. (2016). Determination of *Leptospira borgpetersenii* serovar Javanica and *Leptospira interrogans* serovar Bataviae as the persistent *Leptospira* serovars circulating in the urban rat populations in Peninsular Malaysia. *Parasites & Vectors*, 9(117):1-11.
- Benacer, D., Thong, K. L., Min, N., Verasahib, K., Galloway, R. & Hartskeerl, R. A., *et al.* (2016). Epidemiology of human leptospirosis in Malaysia, 2004–2012. *Acta Tropica*, 157:162-168.

- Benacer, D., Thong, K. L., Ooi, P. T., Souris, M., Lewis, J. W., Ahmed, A. A. & Mohd Z. (2017). Serological and molecular identification of *Leptospira* spp. in swine and stray dogs from Malaysia. *Tropical Biomedicine*, 34(1):89–97.
- Benacer, D., Woh, P. Y., Mohd, Z., Amran, F. & Thong, K. L. (2013). Pathogenic and saprophytic *Leptospira* species in water and soils from selected urban sites in peninsular Malaysia. *Microbes and Environment*, 28:135–140.
- Bercovich, Z., Taaijke, R. & Bokhout, B. A. (1990). Evaluation of an ELISA for the diagnosis of experimentally induced and naturally occurring *Leptospira hardjo* infections in cattle. *Veterinary Microbiology*, 21:255-262.
- Bharti, A. R., Nally, J. E., Ricaldi, J. N., Matthias, M. A., Diaz, M. M., Lovett, M. A., Levett, P. N., Gilman, R. H., Willig, M. R., Gotuzzo, E. & Vinetz, J. M. (2003). Leptospirosis: a zoonotic disease of global importance. *Lancet Infectious Diseases*, 3(12):757–771.
- Bolin, C. A., Zuerner, R. L. & Trueba, G. (1989). Comparison of three techniques to detect *Leptospira interrogans* serovar *hardjo* type *hardjo*-*bovis* in bovine urine. *American Journal of Veterinary Research*, 50:1001–1003.
- Bomfim, M., Barbosa-Stancioli, E. F. & Koury, M. C. (2008). Detection of pathogenic leptospires in urine from naturally infected cattle by nested PCR. *Veterinary Journal*, 178(2):251–256.
- Bourhy, P., Vray, M., & Picardeau, M. (2013). Evaluation of an in-house ELISA using the intermediate species *Leptospira fainei* for diagnosis of leptospirosis. *Journal of Medical Microbiology*, 62:822–827.
- Brenner, D. J., Kaufmann, A. F., Sulzer, K. R., Steigerwalt, A. G. & Rogers, F. C., *et al.* (1999) Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. *International Journal of Systematic and Evolutionary Microbiology*, 49:839-858.
- Broom, J. C. (1953). Leptospirosis in tropical countries. A review. *Transaction of the Royal Society of Tropical Medicine and Hygiene*, 47:273-291.
- Chadsuthi, S., Bicout, D., Wiratsudakul, A., Suwancharoen, D., Petkanchanapong, W., & Modchang, C., *et al.* (2017). Investigation on predominant *Leptospira* serovars and its distribution in humans and livestock in Thailand, 2010-2015. *PLoS Medicine Neglected Tropical Diseases*, 11(2).

- Cole, J. W., Sulzer, C. R. & Pursell, A. R. (1973). Improved microtechnique for the leptospiral agglutination test. *Applied Microbiology*, 25(6):976-980.
- Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., Stein, C., Abela-Ridder, B. & Ko, A. I. (2015). Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Neglected Tropical Diseases*, 9(9).
- Cousins, D. V., Robertson, G. M. & Hustas, L. (1985). The use of the enzyme-linked immunosorbent assay (ELISA) to detect the IgM and IgG antibody response to *Leptospira interrogans* serovars *hardjo*, *pomona* and *tarassovi* in cattle. *Veterinary Microbiology*, 10:439–450.
- Cullen, P. A., Haake, D. A. & Adler, B. (2004). Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiology Reviews*, 28:291–318.
- Cumberland, P., Everard, C. O. & Levett, P. N. (1999). Assessment of the efficacy of an IgM-ELISA and MAT in the diagnosis of acute leptospirosis. *American Journal of Tropical Medicine and Hygiene*, 61:731-734.
- Danaraj, T. J. (1950). Leptospirosis. Proceeding of the Alumni Association of King Edward V11 College of Medicine, Singapore. 3:326-339.
- DeLong, R., David, M., Daniel, L. & Clarke-Pearson. (1988). Comparing the Areas under Two or More Correlated Receiver Operating Characteristic Curves: A Nonparametric Approach. *International Biometric Society*, 44(3).
- Demerdash, Z. A., Diad, T. M., Aly, I. R., Mohamed, S. H. & Mahmoud, F. S. *et al.* (2011). Diagnostic efficacy of monoclonal antibody based sandwich enzyme linked immunosorbent assay (ELISA) for detection of *Fasciola gigantica* excretory/secretory antigens in both serum and stool. *BMC Parasites and Vectors*, 4:176.
- Duncan, C., Krafur, G., Podell, B., Baeten, L., LeVan, I., Charles, B., & Ehrhart, E. (2011). Leptospirosis and Tularaemia in Raccoons (*Procyon lotor*) of Larimer Country, Colorado. *Zoonoses And Public Health*, 59(1), 29-34.
- Dupouey, J., Faucher, B., Edouard, S., Richet, H., de Broucker, C., & Marié, J., *et al.* (2014). Epidemiological investigation of a human leptospirosis case reported in a suburban area near Marseille. *New Microbes And New Infections*, 2(3):82-83.
- El Jalii, I. M. (2008). Comparison between ELISA and the Microscopic Agglutination Test for the Diagnosis of Bovine Leptospirosis. *Pathologie Infectieuse communication, Revue Élev. Méd. vét. Pays trop.*, 61 (2):73-7.

- El Jalii, I. M., Bahaman, A. R., Mohd-Azmi, M. L. & Mutalib, A. R. (2000). Occurrence of human leptospirosis in Malaysia: A retrospective study. *Tropical Biomedicine*, 16:1-5.
- El Jalii, I. M., Bahaman, A. R., Mohd-Azmi, M. L. and Mutalib, A. R. (2002). Seroprevalence of human leptospirosis in representative population in Malaysia. *Tropical Biomedicine*, 19:97-101.
- Ellis, W. A. (1984). Bovine leptospirosis in the tropics: Prevalence, pathogenesis and control. *Preventive Veterinary Medicine*, 2(1-4):411-421.
- Ellis, W. A. (2015). Animal Leptospirosis. Current Topics in Microbiology and Immunology. *Springer*, 387:99-137.
- Engvall, E. & Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry*, 8(9):871-4.
- Eribo, B., Mingmongkolchai, S., Yan, T., Dubbs, P. & Nelson, K. (2012). Leptospire Genomic Diversity Revealed by Microarray-Based Comparative Genomic Hybridization. *Applied And Environmental Microbiology*, 78(9):3045-3050.
- Faine, S. B. & Stallman, N. (1982). Amended Descriptions of the Genus *Leptospira* Noguchi 1917 and the Species *L. interrogans* (Stimson 1907) Wenyon 1926 and *L. biflexa* (Wolbach and Binger 1914) Noguchi 1918. *International Journal Of Systematic Bacteriology*, 32(4):461-463.
- Faine, S. B., Adler, B., Bolin, C. & Perolat, P. (2000). *Leptospira* and Leptospirosis, Second Edition. *Mediscience Press, Melbourne, Australia*.
- Fajriyah, S. N., Udiyono, A. & Saraswati, L. D. (2017). Environmental and Risk Factors of Leptospirosis: A Spatial Analysis in Semarang City. *IOP Conference Series: Earth and Environmental Science*, 55(2017): 1-10.
- Farr, R. (1995). Leptospirosis. *Clinical Infectious Diseases*, 21(1):1-8.
- Fatla, A. W., Zajac, V., Wasinski, B., Sroka, J., Cisak, E., Sawczyn, A. & Dutkiewicz, J. (2014). Occurrence of *Leptospira* DNA in water and soil samples collected in eastern Poland. *Annals of Agricultural and Environmental Medicine*, 21(4): 730-732.
- Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39:783-791.
- Felzemburgh, R. D., Ribeiro, G. S., Costa, F., Reis, R. B., Hagan, J. E., Melendez, A. X. & *et al.* (2014). Prospective Study of Leptospirosis Transmission in an Urban Slum Community: Role of Poor Environment in

Repeated Exposures to the *Leptospira* Agent. *PLoS Neglected Tropical Diseases*, 8(5).

Firdaus, M. R., Jaafar, M. H., Hod, R. & Ahmad, N. (2016). Leptospirosis outbreak following the 2014 major flooding event in Kelantan, Malaysia- a spatial-temporal analysis. *International Society for Environmental Epidemiology*. Conference paper.

Fitzgerald, J. (2009). Availability of leptospirosis vaccine. *Veterinary Record*, 164(5):157-157.

Fletcher, W. (1928). Recent work on leptospirosis, tustsugamushi disease and tropical typhus in the Federal Malay States. *Transaction of the Royal Society of Tropical Medicine and Hygiene*, 21:265-288.

Fonseca, C., Teixeira, M., Romero, E. C., Tengan, F. M., da Silva, M. V. & Shikanai-Yasuda, M. A. (2006). Leptospiral DNA detection for the diagnosis of human leptospirosis. *Journal of Infections*, 52(1):15–22.

Fraga, T. R., Barbosa, A. S. & Isaac, L. (2011). Leptospirosis: Aspects of Innate Immunity, Immunopathogenesis and Immune Evasion From the Complement System. *Scandinavian Journal of Immunology*, 73(5):408-419.

Galton, M. M. (1962). Methods in the laboratory diagnosis of leptospirosis. *Annals of The New York Academy of Sciences*, 98(3):675-685.

Garba, B., Bahaman, A. R., Khairani-Bejo, S., Zunita, Z., Mutalib, A. R. (2017). Retrospective Study of Leptospirosis in Malaysia. *EcoHealth*, 14:389–398.

Goris, M. G., Leeflang, M. M., Boer, K. R., Goeijenbier, M., van Gorp, E. C., Wagenaar, J. F. & Hartskeerl, R. A. (2012). Establishment of valid laboratory case definition fo human leptospirosis. *Journal of Bacteriology & Parasitology*, 3(2):1-8.

Greiner, M., Pfeiffer, D., Smith, R. D. (2000). Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. *Preventive Veterinary Medicine*, 45(1-2):23-41.

Haake, D. A. & Levett, P. N. (2015). Leptospirosis in humans. *Current Topics in Microbiology and Immunology*, 387:65-97.

Haake, D. A., Chao, G., Zuerner, R. L., Barnett, J. K., Barnett, D., Mazel, M., Matsunaga, J., Levett, P. N. & Bolin, C. A. (2000). The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infection and Immunity*, 68:2276–2285.

- Haake, D. A., Dundoo, M., Cader, R., Kubak, B. M., Hartskeerl, R. A., Sejvar, J. J. & Ashford, D. A. (2002). Leptospirosis, water sports, and chemoprophylaxis. *Clinical Infectious Diseases*, 34:40–43.
- Hajikolaie, M. R., Ghorbanpour, M., Gharibi, D. & Abdollahpour, G. R. (2007). Serologic study on leptospiral infection in sheep in Ahvaz, Southern Iran. *Iranian Veterinary Research Journal*, 8:333-336.
- Hartman, E. G., Van, D., Houten, M., Frik, J. F. & Donk, J. A. (1984). Humoral immune response of dogs after vaccination against leptospirosis measured by an IgM- and IgG-specific ELISA. *Veterinary Immunology and Immunopathology*, 7:245–254.
- Hartman, E. G., Van, D., Ingh, T. & Rothuizen, J. (1986). Clinical, pathological and serological features of spontaneous canine leptospirosis. An evaluation of the IgM- and IgG-specific ELISA. *Veterinary Immunology and Immunopathology*, 13:261–271.
- Hartskeerl, R. A. & Smythe, L. D. (2015). The role of leptospirosis reference laboratories. *Current Topics in Microbiology and Immunobiology*, 387:273-88.
- Hartskeerl, R. A., Collares-Pereira, M. & Ellis, W. A. (2011). Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clinical Microbiology And Infection*, 17(4):494-501.
- Hartskeerl, R. A., *et al.* (2001). Manual International Course Laboratory Methods for Diagnosis of Leptospirosis, KIT Amsterdam.
- Hartskeerl, R. A., Smits, H. L., Korver, M., Goris, M. G. & Terpstra, W. J. (2002). International course on laboratory methods for the diagnosis of leptospirosis. 41-54.
- Hellstrom, J. S. & Marshall, R. B. (1978). Survival of *Leptospira interrogans* serovar Pomona in an acidic soil under simulated New Zealand field conditions. *Research in Veterinary Science*, 25:29–33.
- Honarmand, H. R., Abdollahpour, G., Eshraghi, S. S. (2010). Comparison of Two ELISA Methods for the Laboratory Diagnosis of Acute Leptospirosis. *Iranian Journal of Medical Sciences*, 35(2):116-121.
- Ismail, S., Wahab, N. Z., Badya, N., Rahman, N. I., Yeo, C. C., Latif, A. Z. & Haque, M. (2014). A Study on the Presence of Pathogenic *Leptospira spp.* in Environmental Water Samples Obtained from Selected Recreational Areas in Terengganu, Malaysia. *Research Journal of Pharmacy and Technology*, 7(10):1153-1157.

- Jamshidi, S., Akhavizadegan, M. A., Bokaie, S., Maazi, N. & Ghorban, A. A. (2009). Serologic study of feline leptospirosis in Tehran, Iran. *Iranian Journal of Microbiology*, 1(2):32-36.
- Jesse, L. H., Trish, E. P. & Benedikt, H. (2007). Evolvability as the proper focus of evolutionary developmental biology. *Evolution & Development*, 9(4):393–401.
- Johnson, R. C. & Faine, S. (1984). *Leptospira*. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology. *Williams & Wilkins, Baltimore*, 62–67.
- Johnson, R. C. & Gary, N. D. (1962). Nutrition Of *Leptospira Pomona*. I. A Chemically Defined Substitute for Rabbit Serum Ultrafiltrate. *Journal of Bacteriology*, 83(3):668-672.
- Johnson, R. C. & Harris, V. G. (1967). Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. *Journal of Microbiology*, 94(1):27-31.
- Johnson, R. C. & Rogers, P. (1964). 5-fluorouracil as a selective agent for growth of *Leptospiraceae*. *Journal of Bacteriology*, 87:422–426.
- Kawaguchi, L., Sengkeopraseuth, B., Tsuyuoka, R., Koizumi, N., Akashi, H., Vongphrachanh, P., Watanabe, H. & Aoyama, A. (2008). Seroprevalence of Leptospirosis and Risk Factor Analysis in Flood-prone Rural Areas in Lao PDR. *The American Journal of Tropical Medicine and Hygiene*, 78(6):957–961.
- Khairani-Bejo, S. & Oii, S. (2004). Rats: leptospirosis reservoir in Serdang Selangor residential area. *Journal of Animal and Veterinary Advances*, 3:66–69.
- Khairani-Bejo, S. & Bahaman, A. R. (1997). Isolation of leptospires from waters and soils in cattle farms. *The 9th Veterinary Association Malaysia Scientific Congress*, 41-42.
- Khairani-Bejo, S. (2001). Epidemiology of *Leptospira interrogans* serovar *hardjo* infection in cattle. PhD Thesis. Universiti Putra Malaysia, Serdang, Selangor, Malaysia.
- Khairani-Bejo, S., Bahaman, A. R., Zamri-Saad, M. & Mutalib, A. R. (2004). The survival of *Leptospira interrogans* Serovar Hardjo in the Malaysian environment. *Journal of Animal and Veterinary Advances*, 3:123-129.
- Khor, K. H., Tan, W. X., Lau, S. F., Roslan, M. A. & Radzi, R. (2016). Seroprevalence and molecular detection of leptospirosis from a dog shelter. *Tropical Biomedicine*, 33(2): 276–284.

- Kmety, E., Plesko, I., Bakoss, P. & Chorvath, B. (1966). Evaluation of methods for differentiating pathogenic and saprophytic leptospiral strains. *Annales des Sociétés Belges de Médecine Tropicale, de Parasitologie, et de Mycologie*, 46:111-122.
- Ko, A. (1999). Urban epidemic of severe leptospirosis in Brazil. *The Lancet Infectious Diseases*, 354(9185):820-825.
- Ko, A., Goarant, C. & Picardeau, M. (2009). *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nature Reviews Microbiology*, 7(10):736-747.
- Kossey-Vrain, C. (2004). Canine leptospirosis: a review. *Thesis Médecine Vétérinaire, ENVA*, 135:15.
- Koteeswaran, A. (2006). Seroprevalence of leptospirosis in man and animals in Tamilnadu. *Indian Journal Of Medical Microbiology*, 24(4):329.
- Kumar, S., Stecher, G. & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, 33:1870-1874.
- Laras, K., Van, C. B., Bounlu, K., Tien, N., Olson, J. G. & Thongchanh, S., et al. (2002). The importance of Leptospirosis in Southeast Asia. *The American Journal of Tropical Medicine and Hygiene*, 67(3):278–286.
- Lau, C. L., Smythe, L. D., Craig, S. B. & Weinstein, P. (2010). Climate change, flooding, urbanisation and leptospirosis: fuelling the fire? *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 104:631-638.
- Lau, S. F., Low, K. N., Khor, K. H. & Bahaman, A. R. (2016). Prevalence of leptospirosis in healthy dogs and dogs with kidney disease in Klang Valley, Malaysia. *Tropical Biomedicine*, 33(3):469–475.
- Lee, S. H., Kim, S., Park, S. C. & Kim, M. J. (2002). Cytotoxic activities of leptospira interrogans hemolysin SphH as a pore-forming protein on mammalian cells. *Infection and Immunity*, 70: 315-322.
- Leptospirosis | CDC. (2017). Cdc.gov. Retrieved 6 March 2017, from <https://www.cdc.gov/leptospirosis/>
- Levett, P. N. (1999). Leptospirosis: re-emerging or re-discovered disease?. *Journal Of Medical Microbiology*, 48(5):417-418.
- Levett, P. N. (2001). Leptospirosis. *Clinical Microbiology Reviews*, 14(2):296-326.

- Levett, P. N. (2003). *Leptospira* and *Leptonema*. In : Manual of Clinical Microbiology. American Society for Microbiology Press 8th edition, 929-936.
- Levett, P. N., Haake, D. A., Mandell, G. L., Bennett, J. E. & Dolin, R. (2010). Principles and practice of infectious diseases: *Leptospira species* (leptospirosis). Churchill Livingstone Elsevier; Philadelphia, 3059–3065.
- Levett, P. N., Morey, R. E., Galloway, R. L. & Steigerwalt, A. G. (2006). *Leptospira broomii* sp. nov., isolated from humans with leptospirosis. *International Journal of Systemic Evolutionary Microbiology*, 56:671–673.
- Lilenbaum, W., Varges, R., Brandao, F. Z., Cortez, A., de Souza, S. O. & Brandao, P. E., *et al.* (2008). Detection of *Leptospira spp.* in semen and vaginal fluids of goats and sheep by polymerase chain reaction. *Theriogenology*, 69(7):837–842.
- Lim, V. K. (2011). Leptospirosis: a re-emerging infection. *Malaysian Journal Pathology*, 33(1):1–5
- Loan, H. K., Van, C. N., Takhampunya, R., Kiet, B. T., Campbell, J., Them, L. N., Bryant, J. E., Tippayachai, B., Van, H. N., Morand, S., Be, H. V. & Carrique-Mas, J. J. (2015). How important are rats as vectors of leptospirosis in the mekong delta of Vietnam? *Vector-Borne and Zoonotic Diseases*, 15:56–64.
- Mansour, G. F., Sarshad, A., Fallah, M. S., Pourhabibi, A. & Pourhabibi, K. (2005). Leptospirosis in Guilan, a northern province of Iran: assessment of the clinical presentation of 74 cases. *Medical Science Monitor*, 11:219-23.
- Matthias, M. A., Ricaldi, J. N., Cespedes, M., Diaz, M. M., Galloway, R. L., Saito, M., Steigerwalt, A. G., Patra, K. P. & Ore, C. V. (2009). *Leptospira licerasiae* sp. nov. In List of New Names and New Combinations Previously Effectively, but not Validly, Published, Validation List no. 125. *International Journal of Systematic and Evolutionary Microbiology*, 59:1–2.
- Mendoza, R. (2010). Leptospirosis in the Tropics: When Prevention Doesn't Easily Sell as a Ton of Cure. *American Journal Of Economics And Business Administration*, 2(3):307-316.
- Merien, F., Amouriaux, P., Perolat, P., Baranton, G. & Saint Girons, I. (1992). Polymerase chain reaction for detection of *Leptospira spp.* in clinical samples. *Journal of Clinical Microbiology*, 30:2219-2224.
- MOH (2015). Epidemiology and Current Situation of Leptospirosis in Malaysia. Dr. Zainudin AW Persidangan Kesihatan Persekitaran Pihak Berkuasa Tempatan 2015 8-9 September 2015, WP Labuan.

- Mohammed, H., Nozha, C., Hakim, K., Abdelaziz, F. & Reikia, B. (2011). *Leptospira*: Morphology, Classification and Pathogenesis. *Journal of Bacteriology Parasitology*, 2:120.
- Monahan, A., Miller, I., & Nally, J. (2009). Leptospirosis: risks during recreational activities. *Journal Of Applied Microbiology*, 107(3):707-716.
- Morey, R. E., Galloway, R. L., Bragg, S. L., Steigerwalt, A. G. & Mayer, L. W., *et al.* (2006). Species-specific identification of *Leptospiraceae* by 16S rRNA gene sequencing. *Journal of Clinical Microbiology*, 44:3510-3516.
- Musso, D., and Scola, B. (2013). Laboratory diagnosis of leptospirosis: a challenge. *Journal of Microbiology, Immunology and Infections*, 46(4):245-52.
- Mwachui, M. A., Crump, L., Hartskeerl, R., Zinsstag, J. & Hattendorf, J. (2015). Environmental and Behavioural Determinants of Leptospirosis Transmission: A Systematic Review. *PLoS Neglected Tropical Diseases*, 9(9):1-15.
- Natarajaseenivasan, K., Vijayachari, P., Sharma, S., Sugunan, A. P., Selvin, J. & Sehgal, S. C. (2008). Serodiagnosis of severe leptospirosis: Evaluation of ELISA based on recombinant OmpL1 or LipL41 antigens of *Leptospira interrogans* serovar *autumnalis*. *Annals of Tropical Medicine and Parasitology*, 102:699-708.
- Nei, M. & Kumar, S. (2000). Molecular Evolution and Phylogenetics. *Oxford University Press, New York*, 77(1):117120.
- Noor, A. M., Ahmad, N. & Mahiyuddin, W. R. (2015). The Optimization of Igm In-House ELISA for the Laboratory Diagnosis of Melioidosis in Malaysia. *International Journal of Pathology and Clinical Research*, 1:1.
- Noubade, R., Krishnamurthy, G., Murag, S., Venkatesha, M. & Krishnappa, G. (2002). Differentiation of pathogenic and saprophytic leptospires by polymerase chain reaction. *Indian Journal of Medical Microbiology*, 20(1):33-36.
- OIE (2005). Leptospirosis. Center for Food Security and Public Health, College of Veterinary Medicine, Iowa State University. <http://www.cfsph.iastate.edu>. Accessed 29 January 2017.
- OIE (2014). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. <http://www.oie.int/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals/>. Accessed 29 January 2017.
- Oliveira, M. A., Leal, E. A., C. M. A., Filho, J. C., Dias, R. S. & Serufo, J. C. (2017). Human leptospirosis: occurrence of serovars of *Leptospira spp.* in

the state of Minas Gerais, Brazil, from 2008 to 2012. *Brazilian Journal of Microbiology*, 48:483-488.

- Ooteman, M. C., Vago, A. R. & Koury, M. C. (2006). Evaluation of MAT, IgM ELISA and PCR methods for the diagnosis of human leptospirosis. *Journal of Microbiology Methods*, 65(2):247–257.
- Pappas, G., Papadimitriou, P., Siozopoulou, V., Christou, L., & Akritidis, N. (2008). The globalization of leptospirosis: worldwide incidence trends. *International Journal Of Infectious Diseases*, 12(4):351-357.
- Pettersson, E., Lundeberg, J. & Ahmadian, A. (2009). Generations of sequencing technologies. *Genomics*, 93 (2):105–11.
- Phumoon, T. (2001). Epidemiology of canine leptospirosis in Kuala Lumpur and Selangor. Master Thesis. Universiti Putra Malaysia, Serdang, Selangor, Malaysia.
- Plank, R. & Dean, D. (2000). Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira spp.* in humans. *Microbes And Infection*, 2(10):1265-1276.
- Puah, S. M., Puthucheary, S. D. & Chua, K. H. (2013). Potential Immunogenic Polypeptides of *Burkholderia pseudomallei* by Shotgun Expression Library and Evaluation of Their Efficacy for Serodiagnosis of Melioidosis. *International Journal of Medical Sciences*, 10:539-547.
- Pui, C. F., Bilung, L. M., Apun, K. & Su'ut, L. (2017a). Diversity of *Leptospira spp.* in Rats and Environment from Urban Areas of Sarawak, Malaysia. *Journal of Tropical Medicine*, 2017:1-8.
- Pui, C. F., Bilung, L. M., Su'ut, L. & Apun, K. (2015). Prevalence of *Leptospira* Species in Environment Soil and Water from National Parks in Sarawak, Malaysia. *Borneo Journal Resource Science and Technology*, 5(1):59-57.
- Pui, C. F., Bilung, L. M., Su'ut, L., Chong, Y. L. & Apun, K. (2017b). Detection of *Leptospira spp.* in Selected National Service Training Centres and Paddy Fields of Sarawak, Malaysia using Polymerase Chain Reaction Technique. *Tropical Agriculture Science*, 40 (1):99-110.
- Radl, C., Müller, M., Revilla-Fernandez, S., Karner-Zuser, S., de Martin, A., Schauer, U., Karner, F., Stanek, G., Balcke, P., Hallas, A., Frank, H., Fürnschliel, A., Erhart, F. & Allerberger, F. (2010). Outbreak of leptospirosis among triathlon participants in Langau, Austria, 2010. *Wiener klinische Wochenschrift*, 123(23-24):751-5.
- Ribotta, M. J., Higgins, R., Gottschalk, M. & Lallier, R. (2000). Development of an indirect enzyme-linked immunosorbent assay for the detection of

- leptospiral antibodies in dogs. *Canadian Journal of Veterinary Research*, 64:32–37.
- 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*, 27:632–638
- Robinson, C. R. & Kennedy, H. F. (1956). An investigation into clinical and laboratory features of outbreak of 31 cases of anicteric leptospirosis. *Journal of the Royal Army Medical Corps*, 102:196-206.
- Rodríguez, A. G., Yoandra, R. J., Niurka, B. S., Yolanda, V. A., Juan, F. N. & Marta, G. G. (2005). Evaluation of the Vaccine Potentiality of 2 Strains From *Leptospira interrogans* Serogroup Ballum. *Revista Cubana de Medicina Tropical*, 57(1):63-64.
- Romero, E. C., Billerbeck, A., Lando, V. S., Camargo, E. D., Souza, C. C. & Yasuda, P. H. (1998). Detection of *Leptospira* DNA in patients with aseptic meningitis by PCR. *Journal of Clinical Microbiology*, 36:1453-1455.
- Saitou, N. & Nei, M., (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4:406-425.
- Samir, A., Soliman, R., El-Hariri, M., Abdel-Moein, K. & Hatem, M. E. (2015). Leptospirosis in animals and human contacts in Egypt: broad range surveillance. *Revista da Sociedade Brasileira de Medicina Tropical*, 48(3):272-277.
- Samsi, N. S., Zainol, A., Darus, A., Zainun, Z., Wang, C. S., Zainal, Z. & Hashim, A. (2013). Serodiagnosis of Leptospirosis in Domestic Animals and Humans. *Malaysian Journal of Veterinary Research*, 4(2):21-26.
- Schrader, C., Schielke, A., Ellerbroek, L. & Johne, R. (2012). PCR inhibitors – occurrence, properties and removal. *Journal of Applied Microbiology*, 1014-1026.
- Shang, E. S., Exner, M. M., Summers, T. A., Martinich, C., Champion, C. I., Hancock, R. E. & Haake, D. A. (1995). The rare outer membrane protein, OmpL1, of pathogenic *Leptospira* species is a heat-modifiable porin. *Infection and immunity*, 63(8):3174-81.
- Shimizu, T., Matsusaka, E., Takayanagi, K., Masuzawa, T. & Iwamoto, Y. (1987). Biological activities of lipopolysaccharide-like substance (LLS) extracted from *Leptospira interrogans* serovar *canicola* strain Moulton. *Microbiology and Immunology*, 31:727-735.
- Slack, A. T. (2010). Leptospirosis. *Australian Family Physician*, 39(7):495-8.

- Slack, A. T., Kalambaheti, T., Symonds, M. L., Dohnt, M. F., Galloway, R. L., Steigerwalt, A. G., Chaicumpa, W., Bunyaraksyotin, G., Craig, S., Harrower, B. J. & Smythe, L. D. (2008). *Leptospira wolffii* sp. nov., isolated from a human with suspected leptospirosis in Thailand. *International Journal of Systematic and Evolutionary Microbiology*, 58(10):2305-2308.
- Slack, A. T., Khairani-Bejo, S., Symonds, M., Dohnt, M., Galloway, R., & Steigerwalt, A., *et al.* (2009). *Leptospira kmetyi* sp. nov., isolated from an environmental source in Malaysia. *International Journal of Systematic and Evolutionary Microbiology*, 59(4):705-708.
- Smythe, L. D., Adler, B., Hartskeerl, R. A., Galloway, R. L., Turenne, C. Y. & Levett, P. N. (2013). Classification of *Leptospira* genomospecies 1, 3, 4 and 5 as *Leptospira alstonii* sp. nov., *Leptospira vanthielii* sp. nov., *Leptospira terpstrae* sp. nov. and *Leptospira yanagawae* sp. nov., respectively. *International Journal of Systematic and Evolutionary Microbiology*, 63:1859–1862.
- Smythe, L. D., Smith, I. L., Smith, G. A., Dohnt, M. F., Symonds, M. L., Barnett, L. J. & McKay, D. B. (2002). A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. *BMC Infectious Diseases*, 2:13.
- Souza, V., Brant, J. L., Arsky, M. L. & Araújo, W. N. (2010). Evaluation of the Brazilian National Epidemiological Surveillance System for Leptospirosis-Brazil, 2007. *Cadernos de Saúde Coletiva*, 18:115-117.
- Spickler, A. R. & Leedom, K. R. (2013). Leptospirosis. Retrieved at <http://www.cfsph.iastate.edu/DiseaseInfo/factsheets.php>.
- Suwanpakdee, S., Kaewkungwal, J., White, L. J., Asensio, N., Ratanakorn, P., Singhasivanon, P., Day, P. J. & Pun-Ngum, W. (2015). Spatio-temporal patterns of leptospirosis in Thailand: is flooding a risk factor? *Epidemiology and Infection*, 143:2106–2115.
- Tamura, K., Nei, M. & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences (USA)*, 101:11030-11035.
- Tan, S. K., Suleiman, A. B. & Jeyaindran, S. (1986). 16 cases of acute renal failure due to leptospirosis. *Medical Journal of Malaysia*, 41(2):152-155.
- Tan, X. T., Amran, F., Cheong, K. C., & Ahmad, N. (2014). In-house ELISA screening using a locally-isolated *Leptospira* in Malaysia: determination of its cut-off points. *BMC Infectious Diseases*, 14(1):1-5.
- Tansuphasiri, U., Chanchai, T., Duangporn, P. & Charnchudhi, C. (2006). Duplex PCR-hybridization Based Detection of Pathogenic *Leptospira* in Environmental Water Samples Obtained From Endemic Areas in

Northeast Region of Thailand. *Southeast Asian Journal of Tropical Medicine Public Health*, 37(4):729-741.

Thiermann, A. B. (1984). Isolation of leptospire in diagnosis in leptospirosis. *Modern Veterinary Practice*, 65:758-759.

Trueba, G., Zapata, S., Madrid, K., Cullen, P. & Haake, D. (2004). Cell aggregation: a mechanism of pathogenic *Leptospira* to survive in fresh water. *International Microbiology*, 7:35–40.

Ungku Omar, A. (1967). Veterinary public health with particular reference to Malaysia. *Kajian Veterinar*, 1:54-62.

Vadivelu, J., Puthuchery, S. D., Gendeh, G. S. & Parasakhti, N. (1995). Serodiagnosis of Melioidosis in Malaysia. *Singapore Medicine Journal*, 36:299-302.

Valones, M. A., Guimarães, R. L., Brandão, L. A., de Souza, P. R., de Albuquerque T. C., & Crovela, S. (2009). Principles and applications of polymerase chain reaction in medical diagnostic fields: a review. *Brazilian Journal of Microbiology*, 40(1):1–11.

Victoriano, A., Smythe, L. D. & Gloriani-Barzaga, N. (2009). Leptospirosis in the Asia Pacific region. *BMC Infectious Diseases*, 9:147–156.

Vijayachari, P., Sugunan, A., & Shriram, A. (2008). Leptospirosis: an emerging global public health problem. *Journal Of Biosciences*, 33(4):557-569.

Vinetz, J. (2001). Leptospirosis. *Current Opinion In Infectious Diseases*, 14(5):527-538.

Wan-Yussof, W. N. (2015). Flood in Kelantan in 2014: An Introspection. Report from Centre for Strategic Studies, the State of Kelantan, Malaysia.

Ward, M. P. (2002). Seasonality of canine leptospirosis in the United States and Canada and its association with rainfall. *Preventive Veterinary Medicine*, 56:203–13.

Whitney, E., Ailes, E., Myers, L., Saliki, J. & Berkelman, R. (2009). Prevalence of and risk factors for serum antibodies against *Leptospira* serovars in US veterinarians. *Journal Of The American Veterinary Medical Association*, 234(7):938-944.

WHO (2011). Leptospirosis: an emerging public health problem. Weekly epidemiological record. 86:6 pp 45–52. Retrieved from <http://www.who.int/wer/2011/wer8606/en>

- WHO (2015). Zoonoses : Leptospirosis Burden Epidemiology Reference Group (LERG). pp 1-7. Retrived from <http://www.who.int/zoonoses/diseases/lerg/en/index2.html>
- WHO (2017). Zoonoses : Leptospirosis Burden Epidemiology Reference Group (LERG). pp 1-7. Retrived from <http://www.who.int/zoonoses/diseases/lerg/en/index2.html>
- Woo, T. H., Patel, B. K., Smythe L. D., Symonds, M. L., Norris, M. A. & Dohnt, M. F. (1997). Identification of Pathogenic *Leptospira* Genospecies by Continuous Monitoring of Fluorogenic Hybridization Probes During Rapid-Cycle PCR. *Journal of Clinical Microbiology*, 35 (12), 3140-3146.
- Wynwood, S. J., Graham, G. C., Weier, S. L., Collet, T. A., McKay, D. B. & Craig, S. B. (2014). Leptospirosis from water sources. *Pathogens and Global Health*, 108(7):1-5.
- Yang, C. W., Wu, M. S., Pan, M. J., Hsieh, W. J., Vandewalle, A. & Huang, C. C. (2002). The *Leptospira* outer membrane protein LipL32 induces tubulointerstitial nephritis-mediated gene expression in mouse proximal tubule cells. *Journal of American Society Nephrology*, 13:2037–2045.
- Zakeri, S., Khorami, N., Ganji, Z. F., Sepahian, N., Malmasi, A. A., Gouya, M. M. & Djadid, N. D. (2010). *Leptospira wolffii*, a potential new pathogenic *Leptospira* species detected in human, sheep and dog. *Infections, Genetics and Evolution*, 10(2010):273-277.