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

MOHAMMAD SABRI BIN ABDUL RAHMAN

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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

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DEDICATION

I would like to dedicate this thesis to my mother, Sa’adiah 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 fulfilment of the requirement for the degree of Master of Veterinary Science

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MOHAMMAD SABRI ABDUL RAHMAN

March 2018

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

Leptospirosis caused by pathogenic leptospires 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 leptospires 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

DISTRIBUTI 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 diperoleh 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. k_meas/ 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 yang tidak didekah kan. Hasil ini menunjukkan potensi risiko yang berkaitan antara banjir dan leptospirosis. Satu *Leptospira* sp. isolat terasing dari ternakan yang terdedah kepada banjir berbanding yang tidak didekahkan. 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.


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 diperoleh, 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.
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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.

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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.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>5-AS</td>
<td>5-amino-salicylic acid</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>ABTS</td>
<td>Azinobis-3-ethylbenzthiazole-sulfonic-acid</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid(s)</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine-tetraacetic-acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMJH</td>
<td>Ellinghausen and McCullough modified by Johnson and Harris</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>MAT</td>
<td>Microscopic agglutination test</td>
</tr>
<tr>
<td>mg</td>
<td>Miligram</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Milimolar</td>
</tr>
<tr>
<td>mPCR</td>
<td>Multiplex polymerase chain reaction</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>OPD</td>
<td>Ortho-phenylenediamine</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-borate-EDTA</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetra-methylbenzidine</td>
</tr>
<tr>
<td>Tris-Cl</td>
<td>Tris-chloride</td>
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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 I 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 I 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


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).


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


