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MOLECULAR DETECTION AND CHARACTERIZATION OF TICK-BORNE HEMOPATHOGENS IN STRAY DOGS IN MALAYSIA

KONTO MOHAMMED

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By

KONTO MOHAMMED

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

This thesis is dedicated to my beloved mother Late Hajiya Hadiza Mohammed, my father and teachers for their guidance, devotion and relentless patience.
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

MOLECULAR DETECTION AND CHARACTERIZATION OF TICK-BORNE HEMOPATHOGENS IN STRAY DOGS IN MALAYSIA

By

KONTO MOHAMMED

October 2016

Chairman : Associate Professor Malaika Watanabe, PhD
Faculty : Veterinary Medicine

Tick-borne hemopathogens like Babesia, Ehrlichia, Anaplasma and Hepatozoon species pose significant health threats to dogs worldwide. Southeast Asia is one of the few regions of the world with inadequate records concerning vector-borne diseases of dogs and cats, yet it happens to be among the most heavily populated with stray dogs and cats and a climate that is ideal for the tick vectors. In an attempt to achieve a comprehensive scenario for Malaysia, molecular screening and characterization of five major canine tick-borne hemopathogens (namely: Anaplasma platys, Babesia vogeli, Babesia gibsoni, Ehrlichia canis and Hepatozoon canis) was carried out.

West (Peninsular) Malaysia was divided into four quadrants; North, East, South and West Peninsular, while the East (Island) Malaysia was divided into North and South. Eight out of the 12 States of Malaysia were selected for this study which comprises: Penang, Kedah, Pahang, Selangor, Kuala Lumpur and Johor from West (Peninsular) Malaysia and Sarawak and Sabah from East (Island) Malaysia. A total of 436 stray dogs from 9 animal shelters were selected for this study comprising seven animal shelters from West Malaysia and two animal shelters from East Malaysia. Age-groups were stratified into young and adult, while the breeds were broadly classified into pedigree and local. Whole blood was collected from each dog and DNA extraction was carried out. All DNA samples were screened via standard PCR using family-specific primers that amplify the 360bp fragment of the 16S rRNA gene for Anaplasma and Ehrlichia and a genus specific primer that amplify the 350bp fragment of the 18S rRNA gene for Babesia and Hepatozoon. Amplicons obtained were extracted, purified and confirmed by sequencing. For characterization, DNA from the purified PCR product from each representative samples were cloned into a suitable bacterial cell (Escherichia coli). Relations between categorical outcomes were compared using the chi-square test and prevalence rates were calculated at 95% confidence interval. For the characterization study, sequences were aligned and edited using the BioEdit
ClustalW program. For comparing and analyzing the nucleotide sequences, the BLAST program was used. The phylogenetic trees were constructed using the Neighbor-Joining method.

This study confirmed the molecular prevalence of five tick-borne hemopatogens namely: *A. platys*, *B. vogeli*, *B. gibsoni*, *E. canis* and *H. canis* in Malaysia with *A. platys* being the most prevalent hemopathogen (36.5%), followed by *B. gibsoni* (27.6%), *B. vogeli* (22.5%), *E. canis* (18.8%) and *H. canis* (15.8%). Presence of co-infection was also established with a concurrent infection with *A. platys* and *Babesia* being the most prevalent (18.8%); followed by *Babesia* and *E. canis* (10.8%), *A. platys* and *E. canis* (8.4%), *H. canis* and *Babesia* (6.4%), *H. canis* and *E. canis* (4.6%) and *H. canis* and *A. platys* (2.7%). Triple infection combination of *Babesia-Anaplasma-Ehrlichia* predominated (61.3%, 19 dogs). However, local breeds showed a significantly (p > 0.05) higher infection rate than the pedigree breed for *H. canis*, *Babesia* and *A. platys* (p= 0.03, 0.001 and 0.04 respectively).

Spatial distribution based on individual pathogens per region showed that Northern East Malaysia (Sabah) had the highest prevalence for *H. canis* (37%); *B. vogeli* (35.7%) and *A. platys* (64.9%) predominated in West Peninsular, while *B. gibsoni* (76%) and *E. canis* (46%) predominated in Southern East Malaysia (Sarawak). Distribution of hemopathogens based on geographic locations in Malaysia revealed that Southern East Malaysia (Sarawak) had the highest prevalence for tick-borne hemopathogens in Malaysia.

Infectivity status to hemopathogens among stray dogs revealed that the Eastern Peninsular predominated for infectivity with at least one hemopathogen (35.4%), while the Southern East Malaysia (Sarawak) predominated for dogs infected with 2, 3, 4, and all the 5 pathogens under investigation with prevalence of 44%, 20%, 6% and 2% respectively.

This study reported for the first time the genetic diversity of tick-borne hemopathogens among stray dogs in Malaysia and indicated the presence of both indigenous and foreign genotypes in Malaysia. West Peninsular Malaysia showed the highest number of genotypes, followed by the Southern East Malaysia (Sarawak).

The findings from this study provided a comprehensive data on the etiologies of canine tick-borne hemopathogens, their distribution and genetic diversities in Malaysia. This study can also be used for future epidemiological studies and or intervention programme. However, the zoonotic aspects of this hemopathogens as well as the reservoirs of infection have not been looked into in this study; thus, there is the need to further expand our knowledge on that aspect.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENGESANAN MOLEKULAR DAN PENCIRIAN HEMOPATOGEN BAWAAN SENGKENIT PADA ANJING TERBIAR DI MALAYSIA

Oleh

KONTO MOHAMMED

Oktober 2016

Pengerusi : Profesor Madya Malaika Watanabe, PhD
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Beberapa patogen darah bawaan hama kecil contohnya sengkenit, seperti spesies Babesia, Ehrlichia, Anaplasma dan Hepatozoon telah menimbulkan ancaman kesihatan yang ketara kepada anjing seluruh dunia. Asia Tenggara adalah antara beberapa kawasan di dunia yang tidak mempunyai rekod mencukupi mengenai penyakit bawaan vektor bagi anjing dan kucing. Namun, kawasan ini adalah antara kawasan yang mempunyai taburan anjing dan kucing terbiar yang tinggi dan cuaca yang paling ideal untuk kelansungan hidup vektor seperti sengkenit. Dalam usaha untuk mengetahui senario yang lebih menyeluruh di Malaysia, saringan molekular dan penjujukan lima patogen darah bawaan sengkenit (seperti: Anaplasma platys, Babesia vogeli, Babesia gibsoni, Ehrlichia canis dan Hepatozoon canis) telah dijalankan.

dalam sel bakteria yang sesuai (Escherichia coli). Perhubungan antara hasil mutlak dibandingkan menggunakan ujian dan kelaziman kadar chi-square dikira pada 95% selang keyakinan. Untuk kajian pencirian, urutan jujukan telah disusun dan disunting menggunakan program BioEdit ClustalW. Bagi proses perbandingan dan analisa urutan jujukan, program BLAST telah digunakan. Pokok filogenetik dibangunkan menggunakan kaedah Neighbor-Joining.

Kajian ini mengesahkan prevalen molekular bagi 5 patogen darah bawaan sengkenit iaitu: A. platys, B. vogeli, B. gibsoni, E. canis dan H. canis di Malaysia dan A. platys (36.5%) mencatat prevalen tertinggi, diikuti B. gibsoni (27.6%), B. vogeli (22.5%), E. canis (18.8%) dan H. canis (15.8%). Kehadiran ko-infeksi antara A. platys dan Babesia juga dapat dirungkai dengan jumlah prevalen tinggi sebanyak 18.8%, diikuti ko-infeksi antara Babesia dan E. canis (10.8%), A. platys dan E. canis (8.4%), H. canis dan Babesia sp. (6.4%), H. canis dan E. canis (4.6%), dan H. canis dan A. platys (2.7%). Kombinasi 3 infeksi serentak antara Babesia-Anaplasma-Ehrlichia mendominasi (61.3%, n=9). Walau bagaimanapun, baka anjing tempatan menunjukkan kadar jangkitan yang ketara (p> 0.05) lebih tinggi daripada baka anjing asli bagi H. canis, Babesia dan A. platys (p = 0.03, 0.001 dan 0.04 ) masing-masing.

Distribusi spatial berdasarkan patogen individual mengikut setiap rantau menunjukkan bahawa bahagian Utara Malaysia Timur (Sabah) mempunyai prevalen tertinggi bagi H. canis (37%); B. vogeli (35.7%) dan A. platys (64.9%) mendominasi Barat Semenanjung, manakala B. gibsoni (76%) dan E. canis (46%) mendominasi Selatan Malaysia Timur (Sarawak). Distribusi patogen darah berdasarkan faktor lokasi geografi di Malaysia menunjukkan bahawa Selatan Malaysia Timur (Sarawak) mencatat prevalen tertinggi untuk patogen darah bawaan sengkenit di Malaysia.

Status jangkitan untuk patogen darah dalam kalangan anjing terbiar mendedahkan bahawa Timur Semenanjung mendominasi sekurang-kurangnya jangkitan satu patogen darah (35.4%), manakala Selatan Malaysia Timur (Sarawak) didominasi oleh anjing yang dijangkiti dengan 2, 3, 4, dan kesemua 5 patogen dengan prevalen sebanyak 44%, 20%, 6% dan 2% masing-masing.

Kajian ini adalah yang pertama melaporkan kepelbagaian genetik patogen darah dalam kalangan anjing terbiar di Malaysia dan menunjukkan kehadiran kedua-dua genotip asli dan asing di Malaysia. Barat Semenanjung Malaysia menunjukkan jumlah tertinggi genotip, diikuti oleh Selatan Malaysia Timur (Sarawak).

Penemuan daripada kajian ini menyumbangkan data yang komprehensif mengenai etiologi patogen darah anjing bawaan sengkenit, distribusinya dan kepelbagaian genetiknya di Malaysia. Kajian ini juga boleh digunakan untuk kajian epidemiologi masa depan dan atau program intervensi. Walau bagaimanapun, aspek-aspek zoonotik patogen darah ini serta reservoir jangkitan masih belum diteliti dalam kajian ini; dengan itu, terdapat keperluan untuk mengembangkan lagi pengetahuan kita mengenai aspek tersebut.
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Finally, I would like to extend my profound gratitude to the School of Graduate Studies (SGS), Universiti Putra Malaysia for granting me three semesters partial scholarship under its International Graduate Research Scholarship Fund (IGRF) scheme to make my stay and study in Malaysia lively.
I certify that a Thesis Examination Committee has met on 14 October 2016 to conduct the final examination of Konto Mohammed on his thesis entitled "Molecular Detection and Characterization of Tick-Borne Hemopathogens in Stray Dogs in Malaysia" 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 Doctor of Philosophy.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>vi</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td>xx</td>
</tr>
<tr>
<td>LIST OF SYMBOLS AND ABBREVIATIONS</td>
<td>xxi</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Brief Overview</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Diseases induced and threat posed by tick-borne hemopathogens</td>
<td>1</td>
</tr>
<tr>
<td>1.3 Factors predisposing stray dogs to tick-borne diseases</td>
<td>2</td>
</tr>
<tr>
<td>1.4 Prevention and control of tick-borne diseases</td>
<td>2</td>
</tr>
<tr>
<td>1.5 Efforts toward tackling companion animal’s diseases in Malaysia</td>
<td>3</td>
</tr>
<tr>
<td>1.6 Justification of the study</td>
<td>3</td>
</tr>
<tr>
<td>1.7 Aim and objectives of the study</td>
<td>4</td>
</tr>
<tr>
<td>1.8 Research questions and Hypothesis</td>
<td>5</td>
</tr>
<tr>
<td>1.8.1 Research questions</td>
<td>5</td>
</tr>
<tr>
<td>1.8.2 Hypothesis</td>
<td>5</td>
</tr>
<tr>
<td>2 LITERATURE REVIEW</td>
<td>6</td>
</tr>
<tr>
<td>2.1 Dogs As Companion Animals</td>
<td>6</td>
</tr>
<tr>
<td>2.2 Canine tick-borne diseases</td>
<td>6</td>
</tr>
<tr>
<td>2.3 Tick-borne hemopathogens of dogs</td>
<td>7</td>
</tr>
<tr>
<td>2.3.1 Ehrlichia</td>
<td>7</td>
</tr>
<tr>
<td>2.3.2 Babesia species</td>
<td>16</td>
</tr>
<tr>
<td>2.3.3 Anaplasma species</td>
<td>23</td>
</tr>
<tr>
<td>2.3.4 Hepatozoon species</td>
<td>31</td>
</tr>
<tr>
<td>3 MOLECULAR DETECTION OF TICK-BORNE HEMOPATHOGENS IN STRAY DOGS IN MALAYSIA</td>
<td>38</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>38</td>
</tr>
<tr>
<td>3.2 Materials And Methods</td>
<td>39</td>
</tr>
<tr>
<td>3.2.1 Ethics statement</td>
<td>39</td>
</tr>
<tr>
<td>3.2.2 Sampling method</td>
<td>40</td>
</tr>
<tr>
<td>3.2.3 Study area</td>
<td>40</td>
</tr>
<tr>
<td>3.2.4 Sampling site inclusion criteria</td>
<td>41</td>
</tr>
<tr>
<td>3.2.5 Animal inclusion criteria</td>
<td>41</td>
</tr>
<tr>
<td>3.2.6 Collection and identification of ticks</td>
<td>42</td>
</tr>
<tr>
<td>3.2.7 Collection of blood samples</td>
<td>42</td>
</tr>
</tbody>
</table>
3.2.8 DNA extraction and PCR amplification
3.2.9 Sequence and similarity analysis
3.2.10 Statistical analysis
3.3 Results
3.4 Discussion
3.4.1 Conclusion

4 SPATIAL DISTRIBUTION OF TICK-BORNE HEMOPATHOGENS AMONG STRAY DOGS IN MALAYSIA
4.1 Introduction
4.2 Materials And Methods
4.2.1 Study area demarcation and Sampling distribution method
4.2.2 Statistical analysis
4.3 Results
4.4 Discussion
4.4.1 Conclusion

5 MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF Hepatozoon canis IN MALAYSIA
5.1 Introduction
5.1.1 Transmission occurs by ingestion of the whole vector containing the oocyst of the parasite
5.2 Materials And Methods
5.2.1 Blood sampling, DNA extraction and PCR amplification
5.2.2 Gel extraction and purification
5.2.3 Sub cloning
5.2.4 Transformation
5.2.5 Plasmid propagation
5.2.6 Plasmid extraction/purification
5.2.7 Sequencing and data analysis
5.3 Results
5.3.1 Phylogenetic analysis of Malaysian H. canis strain
5.3.2 Phylogenetic relationship with foreign genotypes on the databases
5.4 Discussion
5.4.1 Conclusion

6 MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF Anaplasma platys IN MALAYSIA
6.1 Introduction
6.2 Materials And Methods
6.2.1 Blood sampling, DNA extraction and PCR amplification
6.2.2 Sequencing and data analysis
6.3 Results

6.3.1 Molecular characterization of A. platys strains from Malaysia 96
6.3.2 Phylogenetic diversity of Malaysian A. platys strains from other geographically dispersed strains 98

6.4 Discussion

6.4.1 Conclusion 102

7 MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF Babesia vogeli AND Babesia gibsoni IN MALAYSIA BASED ON THE INTERNAL TRANSCRIBED SPACER GENE (ITS-2)

7.1 Introduction 104

7.2 Materials And Methods

7.2.1 Blood sampling, DNA extraction and PCR amplification 105
7.2.2 Gel extraction, purification, sub-cloning and plasmid extraction 106
7.2.3 Sequencing and data analysis 106

7.3 Results

7.3.1 Molecular characterization of Babesia strains from Malaysia 109
7.3.2 Phylogenetic relationships of Babesia strains from Malaysia with other geographically dispersed strains obtained from the GenBank 112

7.4 Discussion

7.4.1 Conclusion 117

8 MOLECULAR CHARACTERIZATION AND GENETIC DIVERSITY OF Ehrlichia canis IN MALAYSIA BASED ON THE TRP36 GENES

8.1 Introduction 118

8.2 Materials And Methods

8.2.1 Blood sampling, DNA extraction and PCR amplification 119
8.2.2 Gel extraction, purification and sub-cloning 121
8.2.3 Sequencing and data analysis 121

8.3 Results

8.3.1 Molecular characterization of E. canis strains from Malaysia 123
8.3.2 Genetic diversity of Malaysian E. canis TRP36 gene isolates from other geographically dispersed isolates 125

8.4 Discussion

8.4.1 Conclusion 136

9 SUMMARY, CONCLUSION, LIMITATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

9.1 Summary 137
9.1.1 Conceptual framework of the thesis revisited 137
9.1.2 Diagnostic implications 138
9.1.3 Molecular detection of tick-borne hemopathogens in stray dogs in Malaysia 139
9.1.4 Demographic distribution of tick-borne hemopathogens among stray dogs in Malaysia 139
9.1.5 Molecular characterization of the various hemopathogens from Malaysian stray dogs and their phylogenetic relationship with other geographically dispersed strains 142

9.2 Conclusion 144
9.3 Limitations Of This Investigation 144
9.4 Recommendations For Future Research 145

REFERENCES 146
APPENDICES 178
BIODATA OF STUDENT 185
LIST OF PUBLICATIONS 186
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Prevalence of tick-borne hemopathogens of dogs in Malaysia using light microscopy</td>
</tr>
<tr>
<td>3.1</td>
<td>Primer sets used for PCR amplification</td>
</tr>
<tr>
<td>3.2</td>
<td>Thermal cycling parameters set for each primer set</td>
</tr>
<tr>
<td>3.3</td>
<td>Frequency distribution, independent t-test and One-way ANOVA to compare the mean difference of the number of ticks collected based on sex and age group from dogs during the study.</td>
</tr>
<tr>
<td>3.4</td>
<td>Multiple Comparisons test between the age groups using Dunnett T3</td>
</tr>
<tr>
<td>3.5</td>
<td>Frequency distribution table for the various hemopathogens infecting dogs in Malaysia</td>
</tr>
<tr>
<td>3.6</td>
<td>Frequency distribution table for the infectivity status of stray dogs to the various hemopathogens in Malaysia</td>
</tr>
<tr>
<td>3.7</td>
<td>Prevalence of tick-borne hemopathogens in Malaysia based on sex, age and breed.</td>
</tr>
<tr>
<td>4.1</td>
<td>Frequency distribution and prevalence (%) of tick-borne hemopathogens per region in Malaysia.</td>
</tr>
<tr>
<td>4.2</td>
<td>Frequency and spatial distribution of infectivity (%) status among stray dogs per region</td>
</tr>
<tr>
<td>4.3</td>
<td>Comparative regional prevalence of tick-borne hemopathogens in Malaysia using West Malaysia as reference group</td>
</tr>
<tr>
<td>5.1</td>
<td><em>Hepatozoon canis</em> genotypes obtained from this study and those obtained from GenBank for comparative studies.</td>
</tr>
<tr>
<td>5.2</td>
<td>Prevalence and demographic distribution of the various <em>Hepatozoon canis</em> genotypes identified from this study</td>
</tr>
<tr>
<td>5.3</td>
<td>Pairwise distance estimation between the 15 partial 18SrRNA sequences of <em>H. canis</em> from Malaysia</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td>6.1</td>
<td><em>A. platys</em> genotypes obtained from this study and those obtained from GenBank from other countries for comparative studies</td>
</tr>
<tr>
<td>6.2</td>
<td>Prevalence and demographic distribution of the various <em>A. platys</em> 16S rDNA genotypes identified from this study</td>
</tr>
<tr>
<td>6.3</td>
<td>Nucleotide polymorphisms within the 16S rDNA fragments of <em>A. platys</em> among Malaysian isolates</td>
</tr>
<tr>
<td>6.4</td>
<td>Nucleotide sequence differences among 16S rDNA from different strains of <em>A. platys</em></td>
</tr>
<tr>
<td>7.1</td>
<td><em>Babesia</em> sp. ITS-2 genotypes obtained from this study and those obtained from the GenBank from other countries for comparative studies</td>
</tr>
<tr>
<td>7.2</td>
<td>Prevalence and demographic distribution of the various <em>Babesia</em> sp. ITS-2 rDNA genotypes identified from this study</td>
</tr>
<tr>
<td>7.3</td>
<td>Pairwise distance estimation between 24 partial ITS-2 sequences of <em>Babesia</em> sp. from Malaysia and 8 other sequences obtained from the GenBank</td>
</tr>
<tr>
<td>8.1</td>
<td><em>E. canis</em> TRP36 genotypes obtained from this study and those obtained from GenBank from other countries for comparative studies</td>
</tr>
<tr>
<td>8.2</td>
<td>Demographic distribution of the various <em>E. canis</em> TRP 36 genotypes and number of tandem repeats identified from this study</td>
</tr>
<tr>
<td>8.3</td>
<td>Pairwise distance estimation between the 7 TRP 36 sequences of <em>E. canis</em> from Malaysia and with other 12 geographically dispersed strains obtained from the GenBank</td>
</tr>
<tr>
<td>8.4</td>
<td>Summary of different tandem repeat sequences identified in TRP36 gene from different <em>E. canis</em> strains</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Morula of <em>E. canis</em> inside monocyte</td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>Morula of <em>E. ewingii</em> inside neutrophil</td>
<td>9</td>
</tr>
<tr>
<td>2.3</td>
<td>Life cycle of <em>Ehrlichia canis</em></td>
<td>10</td>
</tr>
<tr>
<td>2.4</td>
<td>Photomicrograph of <em>Babesia canis</em></td>
<td>16</td>
</tr>
<tr>
<td>2.5</td>
<td>Photomicrograph of <em>Babesia gibsoni</em></td>
<td>17</td>
</tr>
<tr>
<td>2.6</td>
<td>Life cycle of <em>Babesia Sp.</em></td>
<td>19</td>
</tr>
<tr>
<td>2.7</td>
<td>Photomicrograph of <em>A. platys</em> inside platelet</td>
<td>24</td>
</tr>
<tr>
<td>2.8</td>
<td>Photomicrograph of <em>A. phagocytophilum</em> in neutrophil</td>
<td>24</td>
</tr>
<tr>
<td>2.9</td>
<td>Life cycle of canine Anaplasmosis</td>
<td>25</td>
</tr>
<tr>
<td>2.10</td>
<td><em>Hepatozoon canis</em> gamont inside the cell</td>
<td>32</td>
</tr>
<tr>
<td>2.11</td>
<td><em>Hepatozoon americanum</em> gamont inside the cell</td>
<td>33</td>
</tr>
<tr>
<td>2.12</td>
<td>Life cycle of <em>Hepatozoon canis</em></td>
<td>34</td>
</tr>
<tr>
<td>3.1</td>
<td>Map of Malaysia showing locations (in black diamond) where samples were taken</td>
<td>41</td>
</tr>
<tr>
<td>3.2</td>
<td>Prevalence of the various tick-borne hemopathogens in stray dogs in Malaysia</td>
<td>47</td>
</tr>
<tr>
<td>3.3</td>
<td><em>A. platys</em> positive band amplified at approximately 720bp fragment of 16S rRNA gene</td>
<td>48</td>
</tr>
<tr>
<td>3.4</td>
<td><em>B. gibsoni</em> positive bands amplified at 92bp fragment of the 18S rRNA gene</td>
<td>48</td>
</tr>
<tr>
<td>3.5</td>
<td><em>B. vogeli</em> positive bands amplified at approximately 455bp fragment of 18S rRNA gene</td>
<td>49</td>
</tr>
<tr>
<td>3.6</td>
<td><em>E. canis</em> positive band amplified at approximately 409bp fragment of 16S rRNA gene</td>
<td>49</td>
</tr>
<tr>
<td>3.7</td>
<td><em>H. canis</em> positive bands at approximately 665bp fragment of the 18S rRNA gene</td>
<td>50</td>
</tr>
</tbody>
</table>
3.8 Prevalence of *Babesia* sp. infection among stray dogs in Malaysia

3.9 Infectivity status based on number of hemopathogens per stray dog in Malaysia

3.10 Co-infectivity status for tick-borne hemopathogens among stray dogs in Malaysia

3.11 Prevalence and frequency distribution of number of triple infection among dogs in Malaysia

4.1 Map of Malaysia showing the six arbitrarily stratified zones representing the major geographical regions of the West (Peninsular) and East Malaysia separated by the South China Sea

4.2 Prevalence of tick-borne hemopathogens of stray dogs in the various zones in Malaysia

4.3 Spatial distribution of infectivity status among stray dogs per region

4.4 Infection statuses for at least one hemopathogen per stray dog per region in Malaysia.

5.1 Transmission of *H. canis* among dogs

5.2 *Hepatozoon canis* gamont in a Giemsa stained thin blood smear (x100)

5.3 *H. canis* positive bands at approximately 665bp fragment

5.4 Phylogenetic tree of 15 *Hepatozoon canis* partial 18S rRNA gene sequences obtained from stray dog blood samples collected in West and East Malaysia

5.5 Spatial distribution of the various *Hepatozoon canis* genotypes in Malaysia

5.6 Phylogenetic tree of the 18S rRNA gene sequences of 15 Malaysian *Hepatozoon canis* isolates identified in this study and 7 other isolates from other regions obtained from GenBank

6.1 *A. platys* positive band amplified at approximately 720bp fragment of the 16S rRNA gene
6.2 Phylogenetic tree of 15 *Anaplasma platys* partial 16S rDNA consensus sequences obtained from stray dog blood samples collected in Malaysia

6.3 Phylogenetic tree of the 16S rRNA gene sequences of 15 *Anaplasma platys* isolates identified in this study and 8 other isolates from other regions obtained from GenBank

7.1 Agarose gel showing the amplified 453bp region of Babesia sp. ITS-2 region

7.2 Phylogenetic tree of 24 Babesia sp. partial ITS-2 gene consensus sequences obtained from stray dog blood samples collected in Malaysia

7.3 Phylogenetic tree of the ITS-2 gene sequences of 24 Babesia sp. isolates identified in this study and 8 other isolates from other regions obtained from GenBank

8.1 *E. canis* 18S rDNA positive bands at approximately 420bp fragments

8.2 *E. canis* gp36 positive bands ranging between 800bp to 1000bp fragments

8.3 Alignment of the deduced amino acid sequences of *E. canis* TRP36 from Malaysia

8.4 Alignment of the deduced amino acid sequences of *E. canis* TRP36 from Malaysia and sequences from other geographical locations

8.5 Phylogenetic tree for *E. canis* TRP36 amino acid sequences from geographically dispersed *E. canis* strains

9.1 Shows the demographic distribution for the various tick-borne hemopathogens in Malaysia

9.2 Shows the demographic distribution of geno-groups (number of genotypes) for the various tick-borne hemopathogens in Malaysia
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td></td>
</tr>
<tr>
<td>A3</td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td></td>
</tr>
<tr>
<td>A5</td>
<td></td>
</tr>
<tr>
<td>A6</td>
<td></td>
</tr>
<tr>
<td>A7</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td></td>
</tr>
<tr>
<td>B5</td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td></td>
</tr>
<tr>
<td>B7</td>
<td></td>
</tr>
<tr>
<td>B8</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
</tbody>
</table>

A Sampling and other relevant photos 178
A1 Blood sampling at SPCA, Sabah 178
A2 Blood sampling at SPCA George Town, Pinang 178
A3 Blood sampling at Bentong sanctuary (SPCA), Pahang 179
A4 Sampling record book 179
A5 LB/ampillicin/IPTG/X-gal plates plated with transformation culture showing white colonies that indicate recombinant plasmids and blue colonies indicating non-recombinant plasmids. 180
A6 Sequence alignments using MEGA 6 180
A7 Multiple sequence alignment of the deduced amino acid sequences of gp36 of 7 E. canis isolates from different geographical locations of Malaysia 181
B Composition of buffers and solutions 182
B1 ITPG 182
B2 X-Gal (2ml) 182
B3 Luria Bertani (LB) medium (per liter) 182
B4 (Chapter 5, page 13) SOC medium (100ml) 182
B5 2M Mg$^{2+}$ 182
B6 2X Rapid Ligation Buffer, T4 DNA Ligase 182
B7 Ampicillin stock (50mg/ml) 183
B8 (Chapter 5, page 11) CaCl$_2$ 183
C Sample size estimation 183
# LIST OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>μm</td>
<td>Micro meter</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>Cm²</td>
<td>Square centimetre</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Deionized distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Brief Overview

Tick-borne hemopathogens are aetiological agents of a spectrum of emerging and re-emerging vector borne diseases of dogs that include Babesia, Theileria, Ehrlichia, Anaplasma and Hepatozoon species. These pathogens have been reported worldwide and exert impact in the tropics and subtropics (Watanabe et al., 2004; Matjila et al., 2008; Cardoso et al., 2010; Chomel, 2011; Chandrawathani et al., 2014) where they pose a significant health threat to dogs (Irwin and Jefferies, 2004; Mariana et al., 2011).

1.2 Diseases induced and threat posed by tick-borne hemopathogens

A wide variety of tick-borne pathogens have been reported to infect dogs in Malaysia, notable among them are protozoa, bacteria and rickettsiae species that cause diseases such as babesiosis, theileriosis, hepatozoonosis, anaplasmosis, ehrlichiosis and rickettsiosis (Rajamanickam, 1985; Watanabe 2012; Nazari et al., 2013; Mokhtar 2013, Chandrawathani, 2014).

Tick and other vector-borne diseases pose an increasingly big threat to dogs and other animals in Malaysia. The recent increase in pet ownership, a high influx of tourists and other exotic pet varieties into the country necessitates for an urgent control measures due to the health and economic impact associated with these pathogens.

International standards for animal health and hygiene for the quality control of pets and other animals’ disease prevention and control are essential to sustain the Malaysian expanding economy. The large populations of stray dogs and the increasing popularity of pet ownership in Southeast Asian countries including Malaysia coupled with the ideal environment for the sustainability of vectors helps in transmission of vector-borne disease to susceptible hosts (Irwin and Jefferies, 2004).

In similitude with other developing countries, stray dogs remain a serious public health issue. Priority for controlling stray animal population includes an effective, sustainable and practical approach, but this has always been a problem due to the limitation in financial support and lack of public awareness. With the overpopulation of the stray animals, animal shelters are normally operating with insufficient funding, staffs, and expertise in managing the shelters.

1
1.3 Factors predisposing stray dogs to tick-borne diseases

Increased vector population densities due to ecological changes is one of the most important factor that leads to the emergence of new or undiscovered diseases and the resurgence of the old quiescent ones. Additionally, unrestricted movements of infected animals from one location to another have also led to the spread of vector-borne pathogens from an endemic region to non-endemic areas (Wilson, 1995; Gratz, 1999; Irwin and Jefferies, 2004; Watanabe, 2012).

Climate, immune status, availability of vertebrate animals and vector populations’ densities, and abundance of suitable reservoir hosts, among other factors, exerts influence on disease transmission and establishment (Irwin and Jefferies, 2004; Salman, 2012). However, availability of suitable tick populations is the most important factors that determine the establishment of infection in a new environment (Emmons, 1988).

The three most important factors that moderate the transmission and infection intensity of tick-borne diseases worldwide are climate change, changes in land use or ecological influence and movements of animals through importation of exotic and wildlife species, migratory birds and movement of domestic animals (Gratz, 1999; Robson and Allen, 2000; Gubler et al., 2001; Cumming and Van-Vuuren, 2006; Ergönül, 2006; Nijhof et al., 2007; Tack et al., 2010; Molin et al., 2011; Salman 2012; Madder and Pascucci, 2012; Low et al., 2014). These factors influence the prevalence and proliferation of both the ticks and the pathogens they transmit by moderating the survival and fecundity rate of the ticks, time of the year and level of tick activity; specifically the blood sucking rate and time taken for a complete lifecycle of the tick-borne pathogens within the ticks (Gratz, 1999; Salman, 2012).

Interactions between these three factors are important for forecasting how the prevalence and distribution of ticks and tick-borne diseases may appear in an area. Risk assessments should focus on looking for combinations of factors that may directly or indirectly affect these three factors. A risk assessment module can be proposed based on these factors and a framework designed for this purpose could be used to screen for the emergence of unexpected disease events (Gale et al., 2009).

1.4 Prevention and control of tick-borne diseases

Good quality and proper prevention and control measures can best be achieved by an accurate disease diagnosis method. Newer disease diagnosis method like polymerase chain reaction (PCR) are more accurate and reliable than other conventional techniques like Serology and microscopy that are less accurate especially when there is a case of low level infection and lack of morphological distinguishing features during microscopy and in a case of
cross-reactivity and latent infection as in the case of serology, making detection and identification to specie and sub-specie levels very difficult.

With the advent of newer molecular diagnostic techniques, all anomalies and difficulties associated with accurate pathogen detection and identification of tick-borne hemopathogens were solved; and many new species of pathogens were discovered and many were re-classified.

1.5 Efforts toward tackling companion animal’s diseases in Malaysia

The current joint efforts by the Division of Veterinary Services Malaysia and various non-governmental organisations (NGO’s) in providing veterinary services for companion animals have yielded tremendous results by creating awareness among animal owners, preventing cruelty to animals by establishing animal welfare facilities, especially in rural areas, and infrastructure for diagnostic support (such as regional veterinary pathology laboratories) in small animal practices. Limited surveillance for the accumulation of information on canine and feline diseases has been put in place in each region. The pet owners on their part have also started to maintain high standards of hygiene in the cities. In recent years, researchers in the field of Veterinary and Animal Sciences have achieved tremendously in their research findings towards providing other specialist in the field with updated and relevant information.

Modern diagnostic tests and epidemiological surveys suitable for diagnosis of infections have been going on to differentiate between a new and previous infections, and or diagnosis in individuals with high antibody levels living in endemic areas. To overcome these limitations, and those associated with traditional pathogen-detection systems such as microscopy, highly sensitive DNA-based methods have been developed. Unfortunately, this new technology is not cost effective and will take many years before it is readily available in all localities. However, it is now apparent from the studies in South East Asia which have applied these molecular tools to companion animal blood or to their arthropod parasites that there are intriguing, and maybe even surprising, discoveries to be made soon.

1.6 Justification of the study

Southeast Asia is one of the few regions of the world with inadequate records concerning diseases of pet animals, yet they happen to be among the most heavily populated with stray dogs and cats (Irwin and Jefferies, 2004). The combination of the tropical climate, large population of stray dogs and cats, and an increase in pet ownership in the region provides favorable conditions for the survival and maintenance of both the tick vector and the pathogens that they harbor. The stray animals not only serve as reservoirs of infection to other animals but also increase the risk of zoonotic disease transmission. Basic
demographics for pet populations are scarce to non-existent in most countries of the region (Peters and Pasvol, 2002; Batson, 2008).

Despite the large stray dog population and an ever increasing pet population in Malaysia, there is limited published data on the molecular prevalence, to non concerning their epidemiology and phylogenetics of canine vector-borne diseases. Since early 80s, researchers have been striving to report the prevalence of ticks and tick-borne pathogens of dogs in Malaysia and its neighboring countries (Rajamanickan et al., 1985, Inokuma et al., 2003, Irwin and Jefferies, 2004, Rahman et al., 2010, Mariana et al., 2011, Nazari et al., 2013; Mokhtar et al., 2013, Chandrawathani et al., 2014). However, there is no adequate, specific and detailed information regarding the prevalence, spatial distribution and phylogenetic analyses of tick-borne hemopathogens of dogs in the country. Therefore, the needs to further investigate, using more sensitive techniques to determine the prevalence, spatial distribution and phylogenetics of these pathogens infecting dogs in Malaysia.

1. Early reports suggested that tick-borne transmitted infections associated with protozoa, rickettsia and bacteria are prevalent in Southeast Asia including Malaysia (Rajamanickan et al., 1985, Suksawat et al., 2001a; Irwin and Jefferies, 2004, Rahman et al., 2010, Mariana et al., 2011, Chandrawathani et al., 2014). Therefore, for further studies are essential for us to obtain a clear picture of their diversity and distribution.

2. Currently, there is a paucity of information regarding the molecular prevalence, spatial distribution and phylogenetic of the various tick-borne hemopathogens of dogs in Malaysia.

3. The potential risk posed by the increasing number of stray dogs in the country and the pathogens that they harbor requires investigation.

1.7 Aim and objectives of the study

**Aim:** The aim of this study was to determine the prevalence and spatial distribution as well as to carry out molecular characterization of the various tick-borne hemopathogens of stray dogs in Malaysia to provide a more accurate picture of the current situation of these etiological agents of disease in the country.

**Objectives:** The objectives of this study are to:

1. Determine the molecular prevalence of common tick-borne hemopathogens of stray dogs in Malaysia.
2. Determine the spatial distribution of the various hemopathogens in Malaysia.
3. Further characterize the hemopathogens identified during the course of the study by polymerase chain reaction.
1.8 Research questions and Hypothesis

1.8.1 Research questions

1. What are the prevalences of the common tick-borne pathogens of stray dogs in Malaysia?
2. What is their distribution pattern among region, gender, age and breeds?
3. What are the genetic similarities of the Malaysian strains of the hemopathogens identified from this study from other geographically dispersed strains?

1.8.2 Hypothesis

H₁ = Prevalence of tick-borne hemopathogens among stray dogs in Malaysia is high at α ≤ 0.05 (H₀ = µprev > µ₀).

H₁ = Prevalence of tick-borne hemopathogens among stray dogs in Malaysia is low at α ≤ 0.05 (Hₐ = µprev < µ₀).

H₂ = There is equal distribution of canine tick-borne hemopathogens among region, gender, age and breeds in Malaysia at α ≤ 0.05 (H₀ = µdistribution (West, male, young, locals) = µdistribution (East, female, adult, pedigree)).

H₂ = There is no equal distribution of canine tick-borne hemopathogens among region, gender, age and breeds in Malaysia at α ≤ 0.05 (Hₐ = µdistribution (West, male, young, locals) ≠ µdistribution (East, female, adult, pedigree)).

H₃ = The Malasian isolates for the various hemopathogens are genetically similar to other geographically dispersed strains at α ≤ 0.05 (H₀ = µMalaysian strain = µother strains).

H₃ = The Malasian isolates for the various hemopathogens are not genetically similar to other geographically dispersed strains at α ≤ 0.05 (H₀ = µMalaysian strain ≠ µother strains).
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