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PROTEIN PROFILE AND ANTIGENICITY OF REPRESENTATIVE LEPTOSPIRAL SEROVARs REPORTED IN MALAYSIA AND EXPERIMENTAL \textit{LEPTOSPIRA INTERROGANS}

INFECTION IN DOGS

CHENG KIM SING

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INFECTION IN DOGS

By

CHENG KIM SING

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PROTEIN PROFILE AND ANTIGENICITY OF REPRESENTATIVE LEPTOSPIRAL SEROVARs REPORTED IN MALAYSIA AND EXPERIMENTAL LEPTOSPIRA INTERROGANS INFECTION IN DOGS

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March 2007

Chairman:  Professor Abdul Rani Bahaman, PhD

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Wide distribution of leptospires in the world has caused tremendous economic losses in agricultural sector due to decrease in animal production, quality of animal products and increased cost of treatments and also one of the public health concerns as this zoonosis has caused fatalities in human beings.

In the study, immunoprobing experiments with rabbit antisera against serovars canicola, icterohaemorrhagiae, hardjobovis, pomona and australis, band with molecular weight 137.6 kDa is unique in serovar canicola 35 kDa in icterohaemorrhagiae, 10.5 kDa and 71.4 kDa in hardjobovis, 48.1 kDa in pomona while 20.9 kDa and 25.0kDa in australis. These distinct bands could
have explained the selectiveness of different serovars on the target hosts, organs or perhaps tissues. In the experiment, *Leptospira interrogans* serovar *canicola* caused interstitial nephritis while serovar *icterohaemorrhagiae* caused liver damage in local stray dogs.

Two dimensional SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on both serovars *canicola* and *icterohaemorrhagiae* have revealed differences in the protein distributions. Three different protein spots sharing the same molecular weights at 42.1kDa, 126kDa and 136 kDa while at 60.9kDa, 65.2kDa and 89.6kDa, two proteins spots sharing the same molecular weight were detected in serovar *canicola*. Serovar *icterohaemorrhagiae* on the other hand has three protein spots at 31kDa, 36kDa and 45kDa while 5 protein spots at 32 kDa were detected.

Antibody titres peaked between 6 to 11 post inoculation day (P.I.D) with the highest titre at 1:1,600 in dogs infected with *Leptospira canicola* through intravenous route (Group 1). While dogs infected with serovar *icterohaemorrhagiae* through intravenous route had peak antibody titre between 9 to 11 (Group 2) P.I.D, with the highest titre at 1:1,600. Dogs infected with serovar *icterohaemorrhagiae* through oculonasal route (Group 3), the peak titre of antibody production was much delayed, at between 19 to 23 P.I.D but with much higher than the two previous tests (Group 1 and Group 2) at the highest titre reached was 1:3,200. The study also shows that
dogs once infected with leptospiral serovar, especially through oculonasal route, would shed the organism in its urine for a long period of time up till the end of study (nine months). This reflects the oculonasal route to be the actual route which dogs are most likely to be infected by leptospires in natural environment.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PROFIL PROTEIN DAN ANTIGENIK TERHADAP SEROVAR-SEROVAR LEPTOSPIRA CONTOH YANG DILAPORKAN DI MALAYSIA DAN JANGKITAN UJIKAJI LEPTOSPIRA INTERROGANS PADA ANJING

Oleh

CHENG KIM SING

Mac 2007

Pengerusi: Profesor Abdul Rani Bahaman, PhD

Fakulti: Perubatan Veterinar

Kadar taburan kuman leptospira yang tinggi di dunia ini telah mengakibatkan kerugian-kerugian yang amat tinggi di dalam sektor pertanian, kerana menyebabkan pengeluaran hasil yang semakin rendah, mutu kualiti yang semakin merosot dan kos rawatan yang tinggi serta ia juga merupakan salah satu kebimbangan kesihatan umum kerana berpotensi untuk menyebabkan kematian di kalangan manusia.

Di dalam kajian ini, pengesanan immuno dengan antisera-antisera arnab terhadap serovar-serovar canicola, icterohaemorrhagiae, hardjobovis, pomona serta australis telah dikaji dan didapati jalur protein dengan berat molekul 137.6 sangat unik bagi serovar canicola, 35 kDa bagi icterohaemorrhagiae, 10.5 kDa dan 71.4 kDa dalam hardjo –bovis, 48.1 kDa
dalam *pomona* dan akhir sekali 20.9 kDa serta 25.0 kDa dalam *australis*. Jalur-jalur yang nyata ini mungkin boleh menjelaskan tentang cirri-ciri pemilihan terhadap sasaran pembawa-pembawa, organ-organ serta tisu-tisu tertentu oleh pelbagai serovar. Di dalam kajian ini juga, didapati serovar *canicola* telah menyebabkan berlakunya nefritis interstisial (interstitial nephritis), manakala serovar *icterohaemorrhagiae* pula menyebabkan berlakunya kerosakan hati pada anjing-anjing liar yang dijangkitkan dengan kuman-kuman leptospira.

Elektroforesis gel SDS Poliakrilamida (SDS-PAGE) dua dimensi yang telah dijalankan terhadap kedua-dua serovar *canicola* dan *icterohaemorrhagiae* telah menunjukkan beberapa perbezaan dari segi taburan protin-protin. Tiga tompok protin yang berbeza tetapi mempunyai berat molekul yang sama telah dikesan pada berat molekul 42.1 kDa, 126 kDa dan 136 kDa manakala pada berat molekul 60.9 kDa, 65.2 kDa dan 89.6 kDa, dua tompok protin telah dikesan pada serovar *canicola*. Serovar *icterohaemorrhagiae* pula mempunyai tiga tompok protin pada kedudukan berat molekul pada 31 kDa, 36 kDa dan 45 kDa, manakala dikedudukan berat molekul 32 kDa, lima tompok protin dikesan.

Kemuncak antibodi telah dicapai dalam lingkungan enam hingga sebelas hari pasca-inokulasi dengan titer maksima pada 1:1,600 dalam anjing-anjing yang dijangkitkan dengan kuman *Leptospira canicola* melalui injeksi.
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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follow:

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Date: 9th August 2007
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at UPM or other institutions.

__________________
CHENG KIM SING

Date: 18\textsuperscript{th} July 2007
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### 3.4 Protein profiles of pathogenic serovars reported in Malaysia

Lanes: 5 and 16. Fermentas, U.S.A Protein marker (descending in size kDa; 166.0, 66.2, 45.0, 35.0 and 25.0), 1: *L. interrogans* serovar *canicola* strain Hond Utrecht IV, 2: *L. interrogans* serovar *pomona* strain Pomona, 3: *L. weili* serovar *celledoni* strain Celledoni, 4: *L. interrogans* serovar *pyrogenes* strain Salinem, 6: *L. interrogans* serovar *autumnalis* strain Akiyami A, 7: *L. interrogans* serovar *djasiman* strain Djasiman, 8: *L. interrogans* serovar *birkin* strain Birkin, 9: *L. interrogans* serovar *icterohaemorrhagiae* strain RGA, 10: *L. interrogans* serovar *smithii* strain Smith, 11: *L. interrogans* serovar *biggis* strain Biggs, 12: *L. interrogans* serovar *haemolytica* strain Marsh, 13: *L. interrogans* serovar *ricardi* strain Richardson, 14: *L. interrogans* serovar *wolffi* strain 3705, 15: *L. borgpetersenii* serovar *javanica* strain Veldrat Batavia. Type of staining used is silver staining.

### 3.5 Lipopolysaccharide (LPS) profiles of leptospiral serovars

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### 3.6 Two-dimensional SDS PAGE of *Leptospira interrogans* serovar *icterohaemorrhagiae*

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3.9 Crossreactivity between *Leptospira interrogans* serovar hardjoprajitno and *Leptospira interrogans* serovar smithii probed with rabbit hyperimmune serum against *Leptospira interrogans* serovar hardjoprajitno. Lane 1 *Leptospira interrogans* serovar hardjoprajitno, 2 *Leptospira interrogans* serovar smithii (soluble proteins), 3 *Leptospira interrogans* serovar smithii (cell debris extract) 4 Protein marker

3.10 Crossreactivity between *Leptospira interrogans* serovar hardjoprajitno and *Leptospira interrogans* serovar icterohaemorrhagiae probed with rabbit hyperimmune serum against *Leptospira interrogans* serovar icterohaemorrhagiae. Lane 1 *Leptospira interrogans* serovar hardjoprajitno, 2 *Leptospira interrogans* serovar icterohaemorrhagiae, 3 protein marker

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3.13 Crossreactivity of five anti leptospiral sera by using Leptospira borgpetersenii serovar hardjobovis as antigen. Lane 1 Protein marker, 2 Rabbit’s antiserum against Leptospira borgpetersenii serovar hardjobovis, 3 Rabbit’s antiserum against Leptospira interrogans serovar pomona, 4 Rabbit’s antiserum against Leptospira interrogans serovar canicola, 5 Rabbit’s antiserum against Leptospira interrogans serovar icterohaemorrhagiae, 6 Rabbit’s antiserum against Leptospira interrogans serovar australis

3.14 Crossreactivity of five anti leptospiral sera by using Leptospira interrogans serovar australis as antigen. Lane 1 Protein marker, 2 Rabbit’s antiserum against Leptospira borgpetersenii serovar hardjobovis, 3 Rabbit’s antiserum against Leptospira interrogans serovar australis, 4 Rabbit’s antiserum against Leptospira interrogans serovar icterohaemorrhagiae, 5 Rabbit’s antiserum against Leptospira interrogans serovar canicola, 6 Rabbit’s antiserum against Leptospira interrogans serovar pomona

3.15 Crossreactivity of five anti leptospiral sera by using serovars Leptospira interrogans serovar pomona as antigen. Lane 1 Protein marker, 2 Rabbit’s antiserum against Leptospira interrogans serovar pomona, 3 Rabbit’s antiserum against Leptospira interrogans serovar canicola, 4 Rabbit’s antiserum against Leptospira borgpetersenii serovar hardjobovis, 5 Rabbit’s antiserum against Leptospira interrogans serovar australis, 6 Rabbit’s antiserum against Leptospira interrogans serovar icterohaemorrhagiae

4.1 (a) and (b) Pictures of Leptospira interrogans serovar canicola and Leptospira interrogans serovar icterohaemorrhagiae, respectively (X 400)

4.2 Ethidium bromide stained 2% agarose gel showing no specific PCR products amplifield with G1/G2 in Group 1 urine samples prior to the injection. Individual dogs’ urine
samples, (Lane 1 to 3), Positive control (Lane 4), negative control (Lane 5) and 100 bp marker (Lane M)

4.3 Ethidium bromide stained 2% agarose gel showing specific PCR products amplified with G1/G2 in Group 1 blood samples at day 1, 2 and 3 post-injection. Positive control (Lane 1), negative control (Lane 2) and 100 bp marker (Lane 3). Blood samples from Group 4 (Lane 4-6). Individual dogs' urine samples at day 1, 2 and 3 (Lane 7 to 15)

4.4 Ethidium bromide stained 2% agarose gel showing specific PCR products amplified with G1/G2 in Group 1 urine samples at day 1, 2, 3 and 4 post-injection. Urine samples from Dog 1 (Lane 3-6), Dog 2 (Lane 7-10) and Dog 3 (Lane 11-14). Positive control (Lane 15), negative control (Lane 1) and 100 bp marker (Lane 2)

4.5 Ethidium bromide stained 2% agarose gel showing specific PCR products amplified with G1/G2 in Group 1 urine samples, post-injection. Urine samples from Dog 1 at day 69, 76, 83 and 90 (Lane 2-5), Dog 2 at day 69, 76, 83 and 90 (Lane 6-9) and Dog 3 at day 69, 76, 83 and 90 (Lane 10-13). 100 bp marker (Lane 1)

4.6 Ethidium bromide stained 2% agarose gel showing specific PCR products amplified with G1/G2 in Group 2 urine samples, post-injection. Urine samples from Dog 1 at day 69, 76, 83 and 90 (Lane 2-5), Dog 2 at day 69, 76, 83 and 90 (Lane 6-9) and Dog 3 at day 69, 76, 83 and 90 (Lane 10-13). 100 bp marker (Lane 1)

4.7 Ethidium bromide stained 2% agarose gel showing specific PCR products amplified with G1/G2 in Group 3 urine samples, post-injection. Urine samples from Dog 1 at day 225, 240, 255 and 270 (Lane 2-5), Dog 2 at day 225, 240, 255 and 270 (Lane 6-9) and Dog 3 at day 225, 240, 255 and 270 (Lane 10-13). 100 bp marker (Lane 1) and positive control (Lane 14)

4.8 Group 1 dogs anti-leptospiral antibodies against *Leptospira interrogans* serovar *canicola* in MAT
4.9 Group 2 dogs anti-leptospiral antibodies against *Leptospira interrogans* serovar *icterohaemorrhagiae* in MAT

4.10 Group 3 dogs anti-leptospiral antibodies against *Leptospira interrogans* serovar *icterohaemorrhagiae* in MAT

4.11 Dog’s kidney with white focal areas consistent with accumulation of lymphocytes in the interstitial tissues. Cortex was pale

4.12 Immunoperoxidase staining of dog’s kidney; positive. Leptospires presence in tubular epithelial cells (arrow A) and in the infiltrate (arrow B). Presence of lymphocytes in interstitial tissue indicating non-suppurative interstitial nephritis consistent with leptospirosis

4.13 H and E staining of dog’s kidney: tubule necrosis and lymphocytes infiltration in interstitial tissues indicating non-suppurative interstitial nephritis consistent with leptospirosis. X 20

4.14 Jaundice observed due to the retention of bile in canaliculi

4.15 Immunoperoxidase staining of dog’s liver: positive of leptospires presence, necrotic. Clumping of nuclei chromatin showing pyknosis (evidence of necrosis) found in Kupffer cells surrounded or accumulation of lymphocytes. X100

4.16 H and E staining of Dog’s liver: necrosis and degeneration of hepatocytes, Kupffer cell. Retention of bile in canaliculi was observed (arrows). Normal areas were indicated as N.  X 20

4.17 Dog liver, normal

4.18 PCR on kidney and liver of infected dogs and control. Lane 1 100 bp DNA marker; 2 infected kidney, 3 infected liver (Group 1); 4 infected kidney, 5 infected liver (Group 2); 6 infected kidney, 7 infected liver (Group 3); 8 non-infected kidney, 9 non-infected liver (Group 4); 10 positive control and 11 negative control
LIST OF ABBREVIATIONS

% percentage
x g gravity
°C Degree Celsius
2D PAGE Two-Dimensional-Polyacrylamide Gel Electrophoresis
5-FU 5-Fluorouracil
μg microgramme
μl microliter
μm micrometer
bp base pair
BRENDA Bacterial Restriction Endonuclease
BSA Bovine Serum Albumin
cm centimeter
CSF Fluid
DBKL Dewan Bandaraya Kuala Lumpur
ddH₂O double distilled water
DNA Deoxyribonucleic acid
DTT Dithiothreitol
EDTA ethylenediamine tetraacetic acid
ELISA Enzyme-linked Immunosorbent Assay
H & E haematoxylin and eosin
HCl hydrochloric acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>HIS</td>
<td>hyperimmune serum</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilized pH gradient</td>
</tr>
<tr>
<td>JS</td>
<td>Johnson and Seiter</td>
</tr>
<tr>
<td>KCl</td>
<td>kalium chloride</td>
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<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>LipL</td>
<td>Lipoprotein</td>
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<td>Lipopolysaccharide</td>
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<tr>
<td>mA</td>
<td>milliampere</td>
</tr>
<tr>
<td>MAT</td>
<td>microscopic agglutination test</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>N.C</td>
<td>nitrocellulose</td>
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<td>Natrium chloride</td>
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<tr>
<td>OMP</td>
<td>outer membrane protein</td>
</tr>
<tr>
<td>P</td>
<td>pico</td>
</tr>
<tr>
<td>P.I.D</td>
<td>post inoculation day</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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</tbody>
</table>
PBS-T  phosphate buffered saline with Tween
PCR  polymerase chain reaction
PFGE  pulsed field gel electrophoresis
pH  hydrogen ion exponent
PVDF  polyvinylidene difluoride
rpm  revolution per minute
SDS-PAGE  Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoreis
spp.  species
T.B  transfer buffer
TBE  Tris-borate-EDTA electrophoresis buffer
TEMED  N,N,N',N'-tetramethylethylenediamine
TMB  3,3',5,5'-tetramethylbenzidine
Tris-HCl  Tris (hydroxymethyl) aminomethane hydrochloride
U.K.  United Kingdom
v/v  volume per volume
W.H.O.  World Health Organization
w/v  weight per volume