



UNIVERSITI PUTRA MALAYSIA

***DEVELOPMENT OF A MULTIVALENT DNA VACCINE AGAINST
PATHOGENIC LEPTOSPIRAL INFECTION IN LABORATORY ANIMAL
MODEL***

GARBA BASHIRU

FPV 2018 5



**DEVELOPMENT OF A MULTIVALENT DNA VACCINE AGAINST
PATHOGENIC LEPTOSPIRAL INFECTION IN LABORATORY ANIMAL
MODEL**

By

GARBA BASHIRU

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

December 2017

COPYRIGHT

All material contained within the thesis, including without limitation text, logos, icons, photographs, and all other artwork, is copyright material 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 specifically wish to dedicate this dissertation work to my entire family. An unending feeling of gratitude to my parents, siblings, wife and children whose love and prayers kept me on and saw me through this most challenging part of my life.



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

DEVELOPMENT OF A MULTIVALENT DNA VACCINE AGAINST PATHOGENIC LEPTOSPIRAL INFECTION IN LABORATORY ANIMAL MODEL

By

GARBA BASHIRU

December 2017

Chairman : Professor Abdul Rani Bahaman, PhD
Faculty : Veterinary Medicine

Leptospirosis is neglected emerging zoonoses, occurring both in urban environments as well as rural regions worldwide. During occupational and recreational activities, humans that come in direct contact with infected animals or environments contaminated by the urine of reservoir animals are at a higher risk of infection. Prevention is basically through improved hygienic measures and rodent control which is very difficult to achieve particularly in developing countries. Development of an effective vaccine against leptospirosis remains a challenge. The heat-killed whole-cell vaccine has been seen to produce some undesirable side effects which include pain, nausea and fever, short term immunity and serovar restricted protection. Multi-epitope peptide DNA vaccines are effective against some viruses and they have recently been shown to have potential efficacy against some bacterial diseases including leptospire. They are also known mimic antigen processing and presentation during natural infection and can induce more potent immunoreaction than the whole protein vaccine. The aim of this study is to develop a multivalent DNA vaccine that can stimulate significant antibody production that will aid the control and prevention of leptospirosis using hamster model. Antigenic B cell epitopes from highly conserved leptospiral genes LipL32, LipL41, OmpL1, Loa22 and LigA were predicted using bioinformatics tools, assembled and linked using Gly-Ser spacer and chemically synthesized. The vaccine constructs were composed of the lipopolyscharide genes (LipL32, LipL41), the outer membrane porin and outer membrane-like protein (OmpL1, Loa22), the immunoglobulin-like

protein (LigA) and the final construct that is a combination of all the other constructs. The synthesized DNA was cloned in a pBudCE4.1 mammalian expression vector. The multivalent DNA(s) were expressed (*invitro*) and confirmed by indirect immunofluorescence antibody test. The indirect immunofluorescence test showed that the recombinant protein was expressed in CHO-K1 cells and it reacted with antibodies against the V5 and Myc epitope tags fused to the cloned expression plasmid vector at the 5' end of the constructs. To evaluate the efficacy of the DNA vaccines, 3-4 weeks old golden Syrian hamsters were immunized with 150µg of the vaccine in equal volume of incomplete Freund's adjuvant. Subsequently, all the hamster groups were challenged with *L. interrogans* Copenhageni Fiocruz strain except the control group. Analysis of humoral immune response by microscopic agglutination tests showed agglutinating antibodies production ($p < 0.05$) by the immunized hamsters and the antibodies were immunologically cross-reactive with a range of reference pathogenic leptospira strains including *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. santorasai* belonging to different serogroups. Similarly, all the vaccines were able to stimulate secretion of neutralizing antibodies that prevented the growth of a number of pathogenic leptospira species as indicated by the *invitro* growth inhibition test using a panel of 19 serovars. Histopathological evaluation of kidneys of challenged hamsters showed that the vaccine significantly reduced kidney colonization by the leptospire as indicated by the moderate to severe pathological lesions observed in the kidney tissues. In conclusion, the multi-epitope chimeric DNA vaccine proves to be a promising antigen for the prevention of renal colonization and urinary shedding of the bacteria, which is the major contributing factor for the persistence of the bacteria in susceptible animals as well as as the environment. It also showed great potential for stimulation of cross-reactive immunity against a broad range of pathogenic leptospira serovars.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**PEMBANGUNAN VAKSIN DNA MULTIVALEN TERHADAP JANGKITAN
LEPTOSPIRA ATAUPUN BAKTERIA KENCING TIKUS YANG
PATOGENIK PADA MODEL HAIWAN MAKMAL**

Oleh

GARBA BASHIRU

Disember 2017

Pengerusi : Profesor Abdul Rani Bahaman, PhD
Fakulti : Perubatan Veterinar

Leptospirosis merupakan penyakit zoonotik yang sering diabaikan, yang berlaku di kawasan persekitaran bandar dan luar bandar di seluruh dunia. Semasa aktiviti pekerjaan dan rekreasi, manusia yang bersentuhan secara langsung dengan haiwan yang dijangkiti atau dengan persekitaran yang dicemari disebabkan oleh takungan air kencing haiwan reservoir membawa risiko jangkitan yang lebih tinggi. Pencegahan pada asasnya adalah melalui langkah-langkah kebersihan dan pengawalan pembiakan tikus yang sangat sukar dicapai terutamanya di negara-negara yang sedang membangun. Pembangunan vaksin yang berkesan untuk leptospirosis tetap menjadi cabaran utama. Vaksin keseluruhan sel yang dibunuh dengan menggunakan suhu tinggi telah didapati memberikan beberapa kesan sampingan yang tidak diinginkan termasuk kesakitan, mual dan demam, imuniti jangka pendek dan perlindungan terhadap serovar. Vaksin DNA peptida yang mengandungi pelbagai epitop menunjukkan keberkesanannya terhadap sesetengah virus dan vaksin ini juga telah dikenalpasti mempunyai potensi terhadap beberapa penyakit yang disebabkan oleh bakteria termasuk jangkitan leptospirosis. Vaksin ini menunjukkan gaya pemprosesan antigen dan persembahannya seperti jangkitan semulajadi dan ini boleh menyebabkan lebih banyak imunoreaksi yang kuat yang boleh dirangsangkan berbanding dengan vaksin keseluruhan protein. Tujuan kajian ini adalah untuk membangunkan vaksin yang menyasarkan pelbagai DNA bakteria yang boleh merangsangkan sistem keimunan dan memastikan kawalan dan pencegahan leptospirosis menggunakan hamster sebagai model kajian.

Epitop sel antigenik B dari gen leptospira yang boleh didapati daripada LipL32, LipL41, OmpL1, Loa22 dan LigA telah diramalkan menggunakan kaedah bioinformatik, disusun dan dihubungkan menggunakan spacer Gly-Ser dan telah disintesis secara kimia. Ketiga-tiga pembentukan vaksin DNA "chimeric" terdiri daripada gen lipopolisakarid (LipL32, LipL41), membran luar porin dan membran luar protein (OmpL1, Loa22), immunoglobulin protein (LigA) dan pembinaan fasa akhir merupakan gabungan daripada semua gene yang disebutkan seperti di atas. DNA yang disintesis itu diklonkan dalam vektor ungkapan mamalia pBudCE4.1. DNA multivalent yang dinyatakan (*invitro*) telah disahkan dengan ujian antibodi immunofluoresensi secara tidak langsung. Ujian immunofluoresensi secara tidak langsung menunjukkan bahawa protein rekombinan yang dirangsangkan dalam sel CHO-K1 bertindak balas dengan antibodi terhadap tag V5 dan Myc epitop yang telah dicantumkan dengan vektor plasmid ekspresi klon. Penilaian keberkesanan vaksin DNA dijalankan dengan menggunakan hamsters Syria berumur 3-4 minggu, di mana ianya telah diimmunisasi dengan 150µg vaksin bersama dengan adjuvan Freund dengan kuantiti yang sama. Selapas itu, semua kumpulan hamster telah diinfeksi dengan strain *L. interrogans* Copenhageni Fiocruz kecuali kumpulan kawalan. Analisis tindak balas "humoral immuno" dicapai dengan ujian aglutinasi mikroskopik yang menunjukkan peningkatan dalam penghasilan antibodi ($p < 0.05$) daripada hamster yang telah diimmunisasi dan juga didapati antibodi tersebut boleh bertindak reaksi silang dengan pelbagai strain leptospira yang tergolong dalam serogroup yang berbeza. Selain itu, vaksin tersebut menimbulkan rembesan antibodi yang boleh meneutralkan dan menghalang pertumbuhan beberapa spesies leptospira patogen seperti yang ditunjukkan oleh ujian inhibisi pertumbuhan *invitro* menggunakan panel 19 serovar. Analisis histopatologi ginjal hamster yang telah diinfeksi menunjukkan bahawa vaksin tersebut boleh menambah baik sistem keimunan dalam pelbagai tahap seperti yang ditunjukkan oleh keputusan patologi yang sederhana sehingga parah yang diperhatikan di dalam tisu ginjal. Tambahan pula, vaksin ini juga dapat mengurangkan kolonisasi buah pinggang dengan ketara di kalangan hamster yang diinfeksi. Kesimpulannya, vaksin DNA pelbagai-epitop telah terbukti keberkesananya untuk menghalang perembesan bakteria pada ginjal dan air kencing, yang juga merupakan faktor utama bagi bakteria untuk hidup dalam haiwan yang mudah terjejas dengan pelbagai persekitaran. Ia juga menunjukkan potensi yang besar untuk merangsang imuniti silang balas terhadap pelbagai serovar leptospira patogen.

ACKNOWLEDGEMENTS

I begin by expressing my gratitude and adorations to **ALLAH (SWA)** for sparing my life and granting me the wisdom to achieve this great feat. May his blessings and mercies be upon his Holly prophet Muhammad (SAW), companions and the entire Muslim world. I will continue by thanking my supevisors, Professor Dato Dr. Abdul Rani Bahaman, Associate Professors, Abdul Rahim Mutalib, Siti Khairani Bejo and Zunita Zakaria, for their commitments, time and intellectual guidance. I am sincerely grateful for always giving me a listening ear and proffering solutions to my numerous challenges during the course of my studies.

I also wish to acknowledge the support of the Usmanu Danfodiyo University, Sokoto; for granting me the opportunity to embark on this programme. The enormous support and encouragements I also received from my faculty under the leadership of the Dean, Professor A.A. Magaji and Head of Department Professor O.O. Faleke and then Professor A.U. Junaidu are equally acknowledged.

I would like to extend my appreciations to staff of the Bacteriology Lab, FPV, UPM, Mr. Azri Muhammad Roslan, Miss Krishnammah Kuppusamy and Rabiatuladawiyah Rosli as well as Mr. Rusdam of the Virology Lab for tolerating me and for their kindness and assistance during my lab work. I also wish to acknowledge the support and guidance of my colleagues for their care and words of encouragement, including assistance during my lab work. Although too many to mention, I will like to acknowledge the assistance and mentorship of Dr. Faruku Bande, Adamu Abdul Abubakar, Aliyu Mahmuda, Bashir Saidu, Nasiru Suleiman Yusuf Sani and Muhammad Bashir Bello Tambuwal. Your love and friendship can't be measured and I pray that ALLAH grant me the opportunity to repay you.

I will like to conclude by extending my most sincere love and gratitude to my family, my lovely wife Zainab Hamza Isah and kids Khalifa, Mimi and Ahmad for their patience and support during this most trying period of our lives. I salute your courage, resilience and tolerance. May ALLAH spare our lives to benefit from the results of these sacrifices.

Finally, I am grateful to my parents and siblings for their prayers, love and encouragement. I am grateful and may ALLAH reward you abundantly.

I certify that a Thesis Examination Committee has met on 8 December 2017 to conduct the final examination of Garba Bashiru on his thesis entitled "Development of a Multivalent DNA Vaccine Against Pathogenic Leptospiral Infection in Laboratory Animal Model" 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.

Members of the Thesis Examination Committee were as follows:

Abd. Wahid bin Haron, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Abdul Rahman bin Omar, PhD

Professor
Institute of Bioscience
Universiti Putra Malaysia
(Internal Examiner)

Siti Suri binti Arshad, PhD

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

Alan Murray, PhD

Professor
Massey University
New Zealand
(External Examiner)



NOR AINI AB. SHUKOR, PhD

Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 27 February 2018

This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Abdul Rani Bahaman, PhD

Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Abdul Rahim Mutalib, PhD

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

Siti Khairani Bejo, PhD

Associate 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 institutions;
- intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the 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: Garba Bashiru, GS 41839

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: Professor
Dr. Abdul Rani Bahaman

Signature: _____
Name of Member
of Supervisory Committee: Associate Professor
Dr. Abdul Rahim Mutalib

Signature: _____
Name of Member
of Supervisory Committee: Associate Professor
Dr. Siti Khairani Bejo

Signature: _____
Name of Member
of Supervisory Committee: Associate Professor
Dr. Zunita Zakaria

TABLE OF CONTENTS

	Page
ABSTRACT	i
ABSTRAK	iii
ACKNOWLEDGEMENTS	v
APPROVAL	vi
DECLARATION	viii
LIST OF TABLES	xiv
LIST OF FIGURES	xv
LIST OF ABBREVIATIONS	xviii
LIST OF APPENDICES	xx
CHAPTER	
1 INTRODUCTION	1
1.1 General overview	1
1.2 Problem statement	2
1.3 Significance of the study	3
1.4 Main aim of the research	5
2 LITERATURE REVIEW	6
2.1 Overview	6
2.2 Historical background	6
2.3 Causative agent	7
2.4 <i>In-vitro</i> cultivation	8
2.5 Molecular biology	8
2.6 Immunity	9
2.7 Epidemiology	9
2.8 Sources of infection	11
2.9 Transmission	11
2.10 Public health perspective of leptospirosis in Malaysia	12
2.11 Pathogenesis and virulence factors	13
2.12 Diagnosis	14
2.12.1 <i>Dark-field microscopy</i>	14
2.12.2 <i>Culture and isolation</i>	14
2.12.3 <i>Serology</i>	15
2.12.4 <i>Molecular approaches</i>	16
2.12.5 <i>Laboratory animal inoculation</i>	16
2.13 Leptospiral outer membrane OMP	16
2.13.1 <i>Lipopolysaccharide LPS</i>	17
2.13.2 <i>Outer membrane proteins OMPs</i>	17

2.13.3	<i>LipL32 , the major leptospira outer membrane Protein</i>	18
2.13.4	<i>Outer membrane lipoprotein LipL41</i>	18
2.13.5	<i>The porin, OmpL1</i>	19
2.13.6	<i>The OmpA-Like protein, Loa22</i>	19
2.13.7	<i>The immunoglobulin-like protein (Lig family)</i>	20
2.14	Vaccines against leptospirosis	21
2.14.1	<i>Live vaccine</i>	21
2.14.2	<i>Bacterins</i>	22
2.14.3	<i>Leptospiral lipopolysaccharide antigen vaccine</i>	22
2.14.4	<i>Recombinant sub-unit protein vaccine</i>	23
2.14.5	<i>Recombinant proteins vaccines</i>	23
2.14.6	<i>DNA vaccines</i>	24
2.15	Animal models for leptospirosis	25
3	BIOINFORMATICS AND IN-SILICO EPITOPE PREDICTION ANALYSIS OF HIGHLY CONSERVED PATHOGENIC LEPTOSPIRA GENES	26
3.1	Introduction	26
3.2	Materials and methods	26
3.2.1	Retrieval of nucleotide sequence	26
3.2.2	Multiple sequence alignment	27
3.2.3	Phylogenetic analysis	27
3.2.4	Physicochemical parameters	27
3.2.5	Linear B cell epitope prediction	28
3.2.6	Analysis of variability or conservation of epitopes	28
3.3	Results	28
3.3.1	Retrieval of nucleotide sequence	28
3.3.2	Multiple sequence alignment	29
3.3.3	Phylogenetic analysis	30
3.3.4	ProtParam results and physicochemical parameters	31
3.3.5	B cell epitope prediction and conservancy analysis	32
3.3.6	Predicted B cell epitopes for OmpL1 and Loa22 genes	36
3.4	Discussion	37
3.5	Conclusion	39
4	DESIGN AND EXPRESSION OF MULTI-EPITOPE DNA VACCINE BY INDIRECT IMMUNOFLUORESCENCE ANTIBODY TEST	40
4.1	Introduction	40
4.2	Materials and methods	41
4.2.1	Selection of the epitopes	42
4.2.2	Construction of multi-epitope containing plasmid	42
4.2.3	Confirmation of presence of insert in pUC57 vector	43

4.2.4	Cloning of multi-epitope gene(s) in pBudCE4.1 mammalian expression vector	43
4.2.4.1	Preparation Of Competent <i>E. Coli</i> Cells	44
4.2.4.2	Transformation And Propagation Of Pbudce4.1	45
4.2.5	Sub-cloning of multi-epitope genes in pBudCE4.1 expression vector	46
4.2.6	Confirmation of insert DNA in pBudCE4.1 eukaryotic expression vector	47
4.2.6.1	<i>Colony Pcr</i>	47
4.2.6.2	Restriction Enzyme Digestion	48
4.2.6.3	Sequence Analysis	48
4.2.7	CHO-K1 cell line and propagation	48
4.2.8	Transfection of CHO cells with plasmid DNA	49
4.2.9	Analysis of expression of plasmid by IIFT	49
4.3	Results	50
4.3.1	Transformation of Top10 <i>E. coli</i> cells	54
4.3.2	Confirmation of positive clones by colony PCR	54
4.3.3	Confirmation of insert DNA by restriction enzyme digestion	55
4.3.4	Confirmation of insert DNA by sequence analysis	57
4.3.5	Invitro expression of vaccine constructs	57
4.4	Discussion	59
5	EVALUATION OF THE IMMUNOGENICITY OF MULTI-EPI TOPE DNA VACCINE AGAINST LEPTOSPIRA USING HAMSTERS	63
5.1	Introduction	63
5.2	Materials and methods	64
5.2.1	Experimental animals	64
5.2.2	Challenge leptospira strain	65
5.2.3	Extraction and purification of plasmid DNA vaccine	65
5.2.4	Immunization of hamsters	66
5.2.5	Hamster Challenge with <i>L. interrogans Copenhageni</i> Fiocruz	66
5.2.6	Microscopic agglutination test MAT analysis	66
5.2.7	<i>In-vitro</i> leptospira growth inhibition test (IGIT)	67
5.2.8	Recovery of leptospire from kidneys by culture	68
5.2.9	Histopathological analysis	68
5.3	Statistical analyses	68
5.4	Results	69
5.4.1	Microscopic agglutination test (MAT)	69
5.4.2	Invitro leptospira growth inhibition test	70
5.4.3	Recovery of leptospire from kidneys by culture	71

5.4.4	Histopathological analysis	72
5.5	Discussion	73
5.6	Conclusion	76
6	GENERAL DISCUSSION AND RECOMMENDATIONS	77
	REFERENCES	79
	APPENDICES	100
	BIODATA OF STUDENT	112
	LIST OF PUBLICATIONS	113



LIST OF TABLES

Table		Page
3.1	Selected genes and their accession number	29
3.2	Physicochemical properties of protein sequences based on the ProtParam results.	32
3.3	Bepired predicted epitopes from the selected conserved genes	32
3.4	Predicted B cell epitopes for gene LipL32 of pathogenic leptospira species	33
3.5	Epitope conservancy analysis	34
3.6	Predicted B cell epitopes for gene LipL41 of pathogenic leptospira based on BepiPred prediction	35
3.7	Epitope conservancy analysis for LipL41 of pathogenic leptospira	36
3.8	Predicted B cell epitopes for Lig A gene	36
3.9	BepiPred predicted B cell epitopes for OmpL1 and Loa22 genes of pathogenic leptospira species	37
4.1	Restriction enzyme digestion protocol	46
4.2	Protocol for the amplification of insert genes based on colony PCR	47
4.3	Gene specific primers used for colony PCR for synthesized genes	47
5.1	Overall invitro growth inhibition test result showing neutralization antibody reaction deduced by absence of growth of leptospire after incubation	70
5.2	Recovery of leptospire from kidney samples of immunized and challenged hamsters	71

LIST OF FIGURES

Figure		Page
2.1	a. High resolution electron microscope view of leptospira showing spiral shape. www.leptospirosis.org . b. Leptospire under dark field microscope. www.austincc.edu	7
2.2	Number of leptospirosis cases by states in Malaysia (2013-2014 and 2014-2015) -MOH 2010	10
2.3	Outbreaks of leptospirosis in states of Malaysia	11
3.1	Multiple sequence alignment for N-terminal portion of LipL32 Multiple sequence alignment for N-terminal portion of LipL32 gene (haemolysis associated protein for <i>L. interrogans</i> , <i>L. borgpeterseni</i> , <i>L. kirschneri</i> , <i>L. kmetyi</i> , <i>L. noguchi</i> , <i>L. santorasai</i> , and <i>L. weilii</i>) showing high and low similarities between sequences aligned.	29
3.2	Phylogenetic tree for LipL32 gene using a neighbor joining method based on amino acid sequences. The figure shows all amino acid residue having 100% match with the nine commonly isolated leptospire.	31
3.3	Predicted B cell epitopes for gene LipL32 of pathogenic leptospira showing potentially antigenic epitopes (yellow peaks) above the threshold (red line).	34
3.4	Predicted B cell epitopes for gene LipL41 of pathogenic leptospira showing potentially antigenic epitopes (yellow peaks) above the threshold (red line).	35
4.1	Work flow for the construction of multiepitope DNA vaccine from the synthesized genes and evaluation by PCR, RE digestion and sequencing	41
4.2	Map of pBudCE4.1 vector showing different features of the vector including the multiple cloning site, restriction enzymes, origin of replication, antibiotic resistance gene and epitope tags	44

4.3	Schematic diagram of pBudCE4.1 plasmid without any insert showing the multiple cloning site and unique enzyme cut sites	51
4.4	Schematic diagram showing insilico cloning of LipDNA-pBudCE4.101 and LigDNA-pBudCE4.1-03 (green fragment) fused upstream of the V5 epitope tag under the control of the EF1 α promoter	52
4.5	Schematic diagram showing insilico cloning of OmpDNA-pBudCE4.121 and CompDNA-pBudCE4.1-04 (green fragment) fused upstream of the cMyc epitope tag under the control of the CMV promoter	53
4.6	Linear map of segment of the plasmid vector showing V5 and cMyc epitope tags region where [a] fusion of LipDNA01 and LigDNA03 and [b] fusion of OmpDNA03 and CompDNA04 genes under the control of EF1 α and CMV promoters respectively	54
4.7	Showing bands for the screening of successfully cloned genes by colony PCR with positive bands against the 1kb DNA marker. Lane 1 DNA ladder; lane 2-4 LipDNA01 (520bp); lane 6&7 OmpDNA02 (609bp); lane 9 CompDNA04 (1299bp) and lane 15-19 LigDNA03 (236bp)	55
4.8	Gel electrophoresis of double digested genes. Digested genes from pUC 57 cloning vector lane 1 is 1kb DNA ladder, lane 2-5 LiPDNA01 size 520bp; 7-10 OmpDNA02 size 630bp; lane 12-15 is LigDNA03 size 236bp and 17-19 is CompDNA04 size 1299bp.	55
4.9	Gel electrophoresis gel-extracted fragment of the genes for cloning and sequencing. Lane 1 is 1kb ladder, lane 2 LiPDNA01 size 520bp, lane 3 OmpDNA02 size 630bp, lane 4 is LigDNA03 size 236bp lane 5 is CompDNA04 size 1299bp and lane 6 is pBudCE plasmid vector size 4595 to be used for ligation of the gene	56

4.10	Gel electrophoresis of double digested genes. Confirmation of bands from digested genes after subcloning in BudCE4.1 vector. Lane 1 is 1kb ladder, lane 2 & 3 is LipDNA01 size 520bp, lane 5 & 6 is OmpDNA02 size 630bp, lane 8 & 9 is LigDNA03 size 236bp and lane 11 & 12 is CompDNA04 size 1299bp	56
4.11	Demonstration of expression of plasmid DNA constructs in CHO-K1 cells at 48 hours incubation after transfection with vaccine constructs as well as untransfected control plasmid after counter staining with DAPI	58
5.1	Agglutinating leptospiral antibody titre after hamster immunization with plasmid DNA vaccines and controls. (2=LipDNA01; 3=OmpDNA02; 4=LigDNA03; 5=CompDNA04; 6=pBudCE4.1; 7=PBS and 8=Copenhageni-positive control).	69
5.2	Mean score of histopathology of kidney tissues after challenge with pathogenic leptospira indicating normal, mild, moderate and severe lesions. The red bar indicate severe lesions with the control groups (PBS and plasmid) showing high score, while the green bar indicates normal kidney indicating better protection by vaccinated groups	72
5.3	Histopathology of kidney tissues indicating normal, mild, moderate and severe lesions. Photomicrographs of kidney sections	73

LIST OF ABBREVIATIONS

AA	Amino acids
ATCC	American type culture collection
A-T	Adenine-thymine
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
CO ₂	Carbondioxide
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamine tetraacetic acid
EF-1 α	Human elongation factor 1 α
IEDB	Immune epitope data base
IGIT	In vitro growth inhibition test
IL	Interleukin
FBS	Fetal bovine serum
FITC	Fluorescence isothiocyanate
g	Gram
GFP	Green fluorescent protein
LPS	Lipopolysaccharide
MAT	Microscopic agglutination test
Mg	Milligram
mM	Milli molar
mls	Millilitres

MUSCLE	Multiple sequence comparison by log-expectation
NCBI	Nationaal center for biotechnology information
NEB	New England Biolabs
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
LPS	Lypopolysaccharide



LIST OF APPENDICES

Appendix		Page
1	Full length sequences of synthesized genes	100
2	Amino acid sequences of synthesized genes	102
3	BLAST analysis based on immunodominant LipL32 gene	104
4	Sequencing results for gel purified genes	105
5	IACUC approval letter	109
6	Raw kidney results for histopathological analysis after challenge and sacrifice	110

CHAPTER 1

INTRODUCTION

1.1 General overview

Leptospirosis is a zoonoses affecting a wide range of mammals including humans with significant public health implications (Haake & Levett 2015; Levett, 2001). The disease has a worldwide distribution, with varying severity depending on the infecting serovar, economic status and prevailing environmental condition (Vinetz, 2004). It is caused by pathogenic *Leptospira* species with over 200 serovars worldwide (Levett, 2001). Humans and susceptible animals acquire the infection following direct exposure to infected urine or body fluids of reservoir animals or through indirect contact with the environment contaminated with urine of rodents and other reservoir animals (Villanueva et al., 2010). The infection route is usually through mucosal and skin lesions (through scratches and cuts) as well as via intact skin penetration following prolonged stay in a wet or damp soil (Adler & de la Peña, 2010). The clinical presentation of human leptospirosis ranges from asymptomatic, mild self-limiting illness followed by fatality rate that may be as high as 20% in immune-compromised individuals (Levett, 2001).

Leptospirosis is recognized as an important public health problem due to increasing incidence of the disease and its occurrence in epidemic proportions in both developing and developed countries. Since its initial demonstration by Weil, sporadic outbreaks have occurred throughout the world with fatal outcomes (Adler, 2015a; Haake & Levett, 2015). In the past century, several epidemics of leptospirosis have been reported worldwide. In Malaysia, leptospirosis is endemic in most of the states in the Malaysian Peninsular and the Borneo Island where a number of outbreaks have been reported after flooding caused by heavy seasonal rainfall or following recreational activities involving water (Thayaparan et al., 2013; Sopian et al., 2012; Lau et al., 2010a; Pappas et al., 2008). Epidemiological investigations indicate that infection is usually associated with some occupational groups such as farmers, sewage workers, veterinarians, and animal handlers and most of the serovars circulating in Malaysia reside in rodent reservoirs, hence they serve as the principal source of both human and animal leptospirosis infection (Rafizah et al., 2013; Sulong et al., 2011; Ridzlan et al., 2010).

Like other regions of the world, there is dearth of information on the overall incidence of leptospirosis in the Asia pacific region, principally due to under reporting and lack of laboratory diagnosis of the disease (Levett, 2001). Control measures should be geared towards rodents control, since they are recognized as the most important reservoir in the transmission of the disease, improvement in sanitation and hygienic conditions as well as prophylactic and chemotherapeutic medical and veterinary interventions (Victoriano et al., 2009; Bharti et al., 2003) among others.

1.2 Problem statement

Leptospirosis is an important neglected emerging zoonoses, occurring both in urban environments as well as rural regions worldwide (Cook et al., 2016;). During occupational and recreational activities, humans that come in direct contact with infected animals or contaminated environment particularly water are at a higher risk of infection (Wynwood et al., 2014 Victoriano et al., 2009). Similarly, susceptible animals are also at high risk of infection following environmental contamination with urine or body fluids of reservoir wild and feral animals (Ellis 2015). Prevention in both cases is basically through improved hygienic measures and rodent control which is very difficult to implement particularly in developing countries (Dellagostin & Grassmann, 2011).

Current available vaccine for both medical and veterinary applications which are predominantly whole cell killed bacterins can not protect against serovars not included in the vaccine preparations. Despite the diverse serovar distribution, there is an obvious lack of cross reactivity among antigenically distinct serovars which further limit the ability of available vaccines to provide heterologous protection. Unfortunately, majority of the recombinant vaccines are still at experimental phase. However, for recombinant protein vaccines, the heightened immune response provided is usually short lived. Hence, development of an effective vaccine against leptospirosis is still lacking.

In the recent past, human bacterin vaccines have been seen to produce some undesirable side effects which include pain, nausea, fever, short term immunity and serovar restricted protection (Dellagostin et al., 2017; Adler, 2015a). On the other hand, commercially available polyvalent vaccines for animal use is also associated with short term protection and potential for reversion to virulent form (Vijayachari et al., 2015). Eventhough killed whole cell vaccines (Vax-Spiral®) have been used in several countries like Japan (Koizumi & Watanabe, 2005), France (Rodriguez-Gonzalez et al., 2004) and

China (Faisal et al., 2008) among humans with varying degrees of success, lack of international reports has affected the acceptance of these agents as suitable for vaccination against leptospirosis (Dellagostin & Grassmann, 2011). Similarly, several of the commercially available vaccines for dogs (Nobivac® L4), swine (Lepto-Eryvac®) and bovine (LeptoShield®) species can not prevent transmission of the disease and can only protect against two or three serovars (Sonada et al., 2017). However short term, serovar specific protection remains a major issue, hence the need for an annual booster to achieve protective immunity (Faine et al., 1999).

These draw backs as well as availability of bacterins only in high risk populations, highlights the need for development of new vaccines for the prevention of leptospirosis (Dellagostin & Grassmann, 2011). Furthermore, leptospiruria resulting from renal infection or reproductive failure may still arise among vaccinated animals (Bolin et al., 1989) and since urinary shedding is an important cause of leptospirosis in humans and non-vaccinated animals (Michna, 1970), improvement of existing vaccines is required. Additionally, safe and effective vaccines for humans are not available (Adler, 2015a; Wang et al., 2007; Koizumi & Watanabe, 2005; Zuerner et al., 1991).

DNA vaccine is viewed as a suitable alternative due to its ability to induce a long lasting immune response that involves both the humoral and cell mediated immunecomponents. Previously reported DNA vaccines based on full length LipL45 gene couldn't provide sufficient immune response due to lack of cross reaction and antigenicity of the gene (Vijayachari et al., 2015). In addition, the body may be over ordered expressing the entire gene, while only a fraction of the gene may be antigenic. Reverse vaccinology an also be employed to rationally design multivalent vaccines containing only antigenic fractions of pathogenic leptospira genes with potential to induce antibodies against multiple serovars. In this regard, the golden Syrian hamster is the most desired model due to their susceptibility to acute infection with different serovars and the reproducibility of the results (Haake, 2006).

1.3 Significance of the study

Leptospirosis is endemic in Malaysia and it is gaining popularity due to several incidents that have resulted in human deaths (Thayaparan et al., 2013; El Jalii & Bahaman, 2004). In 2011, about 186 cases were reported in Sarawak with 13 mortalities in addition to the "Eco-challenge" outbreak in Sabah in 2000. The increase in the incidence of leptospirosis may be

connected with urbanization, where humans are seen encroaching and occupying wild territories exposing them to infectious agents.

Most of the commercially available vaccines for human and veterinary application (monovalent and polyvalent), are not effective at controlling clinical disease and preventing mortality and in most cases are not capable of preventing urinary shedding following recovery especially in animals which otherwise is an important factor in reducing the spread of this zoonotic disease (Rawlins et al., 2014; Zuerner et al., 2011).

Leptospire have evolved ways to escape the body's immune defence system with the speed at which they translocate through the cell monolayers into the blood stream and subsequent dissemination to multiple organs, hence the challenge for development of a safe and effective leptospirosis vaccine (Guerreiro et al., 2001). Hence, efforts has therefore shifted to identifying antigenic determinants like as the surface exposed antigens (Guerreiro et al., 2001; Haake et al., 2000) and bioinformatics analysis of the leptospira genome sequence to identify vaccine candidates.

The protein LipL32, is the major outer membrane protein that is conserved among the pathogenic leptospira specie, it is also the most abundant antigen found in the leptosiral total protein profile (Picardeau et al., 2008; Haake et al., 2000; Zuerner et al., 1991). A protein extract containing LipL32 confers immunogenic protection to Gerbils challenged with leptospira. However, a high percentage survival was recorded when LipL32 was administered as a DNA vaccine to Gerbils. Moreover, the surface protein LipL32 has also been identified as a target during natural infection (Wang et al., 2007).

Leptospiral immunoglobulin-like proteins; LigA, LigB and LigC are also present in pathogenic leptospira specie. The proteins serves as virulence determinants and and they have been reported to confer protection to mice against lethal challenge with leptospire (Koizumi & Watanabe, 2005). Immunization with outer membrane protein OmpL1, which is a transmembrane protein conserved among pathogenic leptospire and also detected in natural infection confer immunity to hamsters challenged with virulent *L. kirshneri* in combination with LipL41 (Lin et al., 2011; Haake et al., 1999). Survival in animals vaccinated with both proteins was 75% compared with 25% survival in the control group. Even though, vaccination with OmpL1 alone did not afford significant protection at 28 days after challenge, it clearly possess the potential as a candidates for vaccine development (Haake et al., 1999).

1.4 Main aim of the research

The main aim of the research is to develop a multivalent DNA vaccine for potential application in the prevention of leptospirosis in humans and animals.

The specific objectives of the study are:

1. To determine the immunogenic epitopes from pathogenic leptospira species and serovars using *in-silico* bioinformatics approach,
2. To construct multi-epitope plasmid DNA and analyse its *invitro* expression using cell culture technique.
3. To evaluate the immunogenicity of multi-epitope DNA vaccine in a hamster model.

Hypothesis of the study

H_A : the multivalent DNA vaccine can stimulate antibody production against pathogenic leptospiral serovars.

H_o : the multivalent DNA vaccine cannot produce antibodies against broad pathogenic leptospiral serovars

REFERENCES

- Adler, B. (2015a). History of leptospirosis and leptospira. *Current Topics in Microbiology and Immunology*, 387, 1–9. http://doi.org/10.1007/978-3-662-45059-8_1
- Adler, B. (2015b). Vaccines against leptospirosis. *Current Topics in Microbiology and Immunology*, 387, 251–72. http://doi.org/10.1007/978-3-662-45059-8_10
- Adler, B., & de la Peña Moctezuma, A. (2010b). Leptospira and leptospirosis. *Veterinary Microbiology*, 140(3–4), 287–96.
- Ahmad, S. N., Shah, S., & Ahmad, F. M. (2005). Laboratory diagnosis of leptospirosis. *Journal of Postgraduate Medicine*, 51(3), 195–200.
- Bahaman, A. R., & Ibrahim, A. L. (1988). A review of leptospirosis in Malaysia. *Veterinary Research Communications*, 12(2–3), 179–189.
- Bahaman, A. R., Ibrahim, A. L., & Adam, H. (1987). Serological prevalence of leptospiral infection in domestic animals in West Malaysia. *Epidemiology and Infection*, 99(2), 379–92.
- Bahaman, A. R., Ibrahim, A. L., Stallman, N. D., & Tinniswood, R. D. (1988). The bacteriological prevalence of leptospiral infection in cattle and buffaloes in West Malaysia. *Epidemiology and Infection*, 100(2), 239–46.
- Baril, C., Herrmann, J. L., Richaud, C., Margarita, D., & Girons, I. S. (1992). Scattering of the rRNA genes on the physical map of the circular chromosome of *Leptospira interrogans* serovar icterohaemorrhagiae. *Journal of Bacteriology*, 174(23), 7566–71.
- Benacer, D., Mohd Zain, S. N., 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. *The American Journal of Tropical Medicine and Hygiene*, 88(4), 704–9.
- Benacer, D., Woh, P. Y., Mohd Zain, S. N., Amran, F., & Thong, K. L. (2013). Pathogenic and saprophytic *Leptospira* species in water and soils from selected urban sites in peninsular Malaysia. *Microbes and Environments / JSME*, 28(1), 135–40.

- Bharti, A. R., Nally, J. E., Ricaldi, J. N., Matthias, M. A., Diaz, M. M., Lovett, M. A & Vinetz, J. M. (2003). Leptospirosis: a zoonotic disease of global importance. *The Lancet Infectious Diseases*, 3(12), 757-771.
- Blythe, M. J., & Flower, D. R. (2005). Benchmarking B cell epitope prediction: underperformance of existing methods. *Protein Science : A Publication of the Protein Society*, 14(1), 246-8.
- Bolin, C. A., Zuerner, R. L., & Trueba, G. (1989). Effect of vaccination with a pentavalent leptospiral vaccine containing *Leptospira interrogans* serovar hardjo type hardjo-bovis on type hardjo-bovis infection of cattle. *American Journal of Veterinary Research*, 50(12), 2004-8.
- Branger, C., Chatrenet, B., Gauvrit, A., Aviat, F., Aubert, A., Bach, J. M., & André-Fontaine, G. (2005). Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. *Infection and Immunity*, 73(7), 4062-9.
- Branger, C., Sonrier, C., Chatrenet, B., Klonjowski, B., Ruvoen-Clouet, N., Aubert, A & Eloit, M. (2001). Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. *Infection and Immunity*, 69(11), 6831-8.
- Bui, H.-H., Sidney, J., Li, W., Fusseder, N., & Sette, A. (2007). Development of an epitope conservancy analysis tool to facilitate the design of epitope-based diagnostics and vaccines. *BMC Bioinformatics*, 8(1), 361.
- Bulach, D. M., Kalambaheti, T., de la Peña-Moctezuma, A., & Adler, B. (2000). Lipopolysaccharide biosynthesis in *Leptospira*. *Journal of Molecular Microbiology and Biotechnology*, 2(4), 375-80.
- Castiblanco-Valencia, M. M., Fraga, T. R., Silva, L. B. da, Monaris, D., Abreu, P. A. E., Strobel, S & Barbosa, A. S. (2012). Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. *The Journal of Infectious Diseases*, 205(6), 995-1004.
- Cerqueira, G. M., McBride, A. J. A., Picardeau, M., Ribeiro, S. G., Moreira, A. N., Morel, V & Dellagostin, O. A. (2009). Distribution of the leptospiral immunoglobulin-like (lig) genes in pathogenic *Leptospira* species and application of ligB to typing leptospiral isolates. *Journal of Medical Microbiology*, 58(Pt 9), 1173-81.

- Chang, M.-Y., Cheng, Y.-C., Hsu, S.-H., Ma, T.-L., Chou, L.-F., Hsu, H.-H & Yang, C.-W. (2016). Leptospiral outer membrane protein LipL32 induces inflammation and kidney injury in zebrafish larvae. *Scientific Reports*, 6, 27838.
- Chang, Y.-F., Chen, C.-S., Palaniappan, R. U. M., He, H., McDonough, S. P., Barr, S. C & Chang, C.-F. (2007a). Immunogenicity of the recombinant leptospiral putative outer membrane proteins as vaccine candidates. *Vaccine*, 25(48), 8190-8197.
- Chang, Y.-F., Chen, C.-S., Palaniappan, R. U. M., He, H., McDonough, S. P., Barr, S. C & Chang, C.-F. (2007b). Immunogenicity of the recombinant leptospiral putative outer membrane proteins as vaccine candidates. *Vaccine*, 25(48), 8190-7.
- Chiaromonte, F., Miller, W., & Bouhassira, E. E. (2003). Gene length and proximity to neighbors affect genome-wide expression levels. *Genome Research*, 13(12), 2602-8.
- Chung, B. K.-S., & Lee, D.-Y. (2012). Computational codon optimization of synthetic gene for protein expression. *BMC Systems Biology*, 6(1), 134.
- Cook, E. A. J., de Glanville, W. A., Thomas, L. F., Kariuki, S., Bronsvoort, B. M. de C., Fèvre, E. M & Fèvre, E. M. (2016). Risk factors for leptospirosis seropositivity in slaughterhouse workers in western Kenya. *Occupational and Environmental Medicine*, oemed-2016-103895.
- Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S & Ko, A. I. (2015a). Global Morbidity and Mortality of Leptospirosis: A Systematic Review. *PLoS Neglected Tropical Diseases*, 9(9), e0003898.
- Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S & Ko, A. I. (2015b). Global Morbidity and Mortality of Leptospirosis: A Systematic Review. *PLoS Neglected Tropical Diseases*, 9(9), e0003898.
- Cullen, P. A., Haake, D. A., & Adler, B. (2004). Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiology Reviews*, 28(3), 291-318.
- de Nardi Júnior, G., Genovez, M. E., Ribeiro, M. G., Castro, V., & Jorge, A. M. (2010). An in vitro growth inhibition test for measuring the potency of *Leptospira* spp. Sejroe group vaccine in buffaloes. *Biologicals*, 38(4), 474-478.

- De Vries, S. G., Visser, B. J., Nagel, I. M., Goris, M. G. A., Hartskeerl, R. A., & Grobusch, M. P. (2014). Leptospirosis in Sub-Saharan Africa: a systematic review. *International Journal of Infectious Diseases : IJID : Official Publication of the International Society for Infectious Diseases*, 28C, 47–64.
- Dellagostin, O., & Grassmann, A. (2011). Recombinant vaccines against leptospirosis. *Vaccines*, 7(11), 1215–1224.
- Dellagostin, O., Grassmann, A., Rizzi, C., Schuch, R., Jorge, S., Oliveira, T & Hartwig, D. (2017). Reverse Vaccinology: An Approach for Identifying Leptospiral Vaccine Candidates. *International Journal of Molecular Sciences*, 18(1), 158.
- Depla, E., Van der Aa, A., Livingston, B. D., Crimi, C., Allosery, K., De Brabandere, V & Meheus, L. (2008). Rational Design of a Multiepitope Vaccine Encoding T-Lymphocyte Epitopes for Treatment of Chronic Hepatitis B Virus Infections. *Journal of Virology*, 82(1), 435–450.
- Desvars, A., Cardinale, E., & Michault, A. (2011). Animal leptospirosis in small tropical areas. *Epidemiology and Infection*, 139(2), 167–88.
- Deveson Lucas, D. S., Lo, M., Bulach, D. M., Quinsey, N. S., Murray, G. L., Allen, A., & Adler, B. (2014). Recombinant LipL32 stimulates interferon-gamma production in cattle vaccinated with a monovalent *Leptospira borgpetersenii* serovar Hardjo subtype Hardjobovis vaccine. *Veterinary Microbiology*, 169(3–4), 163–70.
- Dezhbord, M., Esmaelizad, M., Khaki, P., Fotohi, F., & Zarehparvar Moghaddam, A. (2014). Molecular identification of the ompL1 gene within *Leptospira interrogans* standard serovars. *Journal of Infection in Developing Countries*, 8(6), 688–93.
- Dhama, K., Mahendran, M., Gupta, P. K., & Rai, A. (2008). DNA vaccines and their applications in veterinary practice: current perspectives. *Veterinary Research Communications*, 32(5), 341–56.
- Dickson, K. S., Burns, C. M., & Richardson, J. P. (2000). Determination of the Free-Energy Change for Repair of a DNA Phosphodiester Bond. *Journal of Biological Chemistry*, 275(21), 15828–15831.
- Ding, J., Qian, W., Liu, Q., & Liu, Q. (2012). Multi-epitope recombinant vaccine induces immunoprotection against mixed infection of *Eimeria* spp. *Parasitology Research*, 110(6), 2297–2306.

- Dong, H., Hu, Y., Xue, F., Sun, D., Ojcius, D. M., Mao, Y., & Yan, J. (2008). Characterization of the ompL1 gene of pathogenic *Leptospira* species in China and cross-immunogenicity of the OmpL1 protein. *BMC Microbiology*, 8, 223.
- Donnelly, J. J., Wahren, B., & Liu, M. A. (2005). DNA vaccines: progress and challenges. *Journal of Immunology (Baltimore, Md. : 1950)*, 175(2), 633-9.
- Doytchinova, I. A., & Flower, D. R. (2007). VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinformatics*, 8, 4.
- Duan, G., & Walther, D. (2015). The roles of post-translational modifications in the context of protein interaction networks. *PLoS Computational Biology*, 11(2), e1004049.
- Dupouey, J., Faucher, B., Edouard, S., Richet, H., Kodjo, A., Drancourt, M., & Davoust, B. (2014). Human leptospirosis: an emerging risk in Europe? *Comparative Immunology, Microbiology and Infectious Diseases*, 37(2), 77-83.
- Edgar, R. C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics*, 5, 113.
- El Jalii, I. M., & Bahaman, A. R. (2004). A review of human leptospirosis in Malaysia. *Tropical Biomedicine*, 21(2), 113-9.
- Ellis, W. A. (1994). Leptospirosis as a cause of reproductive failure. *The Veterinary Clinics of North America. Food Animal Practice*, 10(3), 463-78.
- Ellis, W. A. (2015). Animal Leptospirosis. *Current Topics in Microbiology and Immunology*. 387-99: -137.
- Escalona, E., Sáez, D., & Oñate, A. (2017). Immunogenicity of a Multi-Epitope DNA Vaccine Encoding Epitopes from Cu-Zn Superoxide Dismutase and Open Reading Frames of *Brucella abortus* in Mice. *Frontiers in Immunology*, 8, 125.
- Evangelista, K. V., & Coburn, J. (2010). *Leptospira* as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. *Future Microbiology*, 5(9), 1413-25.
- Faine SB, Adler B, Bolin C, P. (1999). *Leptospira and leptospirosis*. Melbourne, Australia: MediSci. jourlib.org. Retrieved December 5, 2014, from <http://www.jourlib.org/references/4579736>

- Faisal, S. M., Yan, W., Chen, C.-S., Palaniappan, R. U. M., McDonough, S. P., & Chang, Y.-F. (2008a). Evaluation of protective immunity of *Leptospira* immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. *Vaccine*, 26(2), 277–87.
- Faisal, S. M., Yan, W., Chen, C. S., Palaniappan, R. U. M., McDonough, S. P., & Chang, Y. F. (2008b). Evaluation of protective immunity of *Leptospira* immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. *Vaccine*, 26(2), 277–287.
- Fernandes, L. G., Siqueira, G. H., Teixeira, A. R. F., Silva, L. P., Figueredo, J. M., Cosate, M. R., & Nascimento, A. L. T. O. (2016). *Leptospira* spp.: Novel insights into host–pathogen interactions. *Veterinary Immunology and Immunopathology*, 176, 50–57.
- Forster, K. M., Hartwig, D. D., Oliveira, T. L., Bacelo, K. L., Schuch, R., Amaral, M. G., & Dellagostin, O. A. (2015). DNA prime-protein boost based vaccination with a conserved region of leptospiral immunoglobulin-like A and B proteins enhances protection against leptospirosis. *Memórias Do Instituto Oswaldo Cruz*, 110(8), 989–995.
- Forster, K. M., Hartwig, D. D., Seixas, F. K., Bacelo, K. L., Amaral, M., Hartleben, C. P., & Dellagostin, O. A. (2013). A conserved region of leptospiral immunoglobulin-like A and B proteins as a DNA vaccine elicits a prophylactic immune response against leptospirosis. *Clinical and Vaccine Immunology : CVI*, 20(5), 725–31.
- Fouts, D. E., Matthias, M. A., Adhikarla, H., Adler, B., Amorim-Santos, L., Berg, D. E., & Vinetz, J. M. (2016). What Makes a Bacterial Species Pathogenic?: Comparative Genomic Analysis of the Genus *Leptospira*. *PLOS Neglected Tropical Diseases*, 10(2), e0004403.
- 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), 408–19.
- Fraga, T. R., Isaac, L., & Barbosa, A. S. (2016). Complement Evasion by Pathogenic *Leptospira*. *Frontiers in Immunology*, 7, 623.
- Gamberini, M., Gómez, R. M., Atzingen, M. V., Martins, E. A. L., Vasconcellos, S. A., Romero, E. C., & Nascimento, A. L. T. O. (2005). Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. *FEMS Microbiology Letters*, 244(2), 305–313.

- Garba, B., Bahaman, A. R., Khairani-Bejo, S., Zakaria, Z., & Mutalib, A. R. (2017). Retrospective Study of Leptospirosis in Malaysia. *EcoHealth*, 14(2).
- Gershoni, J. M., Roitburd-Berman, A., Siman-Tov, D. D., Tarnovitski Freund, N., & Weiss, Y. (2007). Epitope mapping: the first step in developing epitope-based vaccines. *BioDrugs: Clinical Immunotherapeutics, Biopharmaceuticals and Gene Therapy*, 21(3), 145–56.
- Grassmann, A. A., Félix, S. R., dos Santos, C. X., Amaral, M. G., Seixas Neto, A. C. P., Fagundes, M. Q., & Dellagostin, O. A. (2012). Protection against lethal leptospirosis after vaccination with LipL32 coupled or coadministered with the B subunit of Escherichia coli heat-labile enterotoxin. *Clinical and Vaccine Immunology: CVI*, 19(5), 740–5.
- Grassmann, A. A., Kremer, F. S., dos Santos, J. C., Souza, J. D., Pinto, L. da S., & McBride, A. J. A. (2017). Discovery of Novel Leptospirosis Vaccine Candidates Using Reverse and Structural Vaccinology. *Frontiers in Immunology*, 8, 463.
- Grassmann, A. A., Souza, J. D., & McBride, A. J. A. (2017). A Universal Vaccine against Leptospirosis: Are We Going in the Right Direction? *Frontiers in Immunology*, 8, 256.
- Guernier, V., Lagadec, E., Cordonin, C., Le Minter, G., Gomard, Y., Pagès, F., & Dellagi, K. (2016). Human Leptospirosis on Reunion Island, Indian Ocean: Are Rodents the (Only) Ones to Blame? *PLoS Neglected Tropical Diseases*, 10(6), e0004733.
- Guerra, M. A. (2013). Leptospirosis: public health perspectives. *Biologicals: Journal of the International Association of Biological Standardization*, 41(5), 295–7.
- Guerreiro, H., Croda, J., Flannery, B., Mazel, M., Matsunaga, J., Galvão Reis, M., & Haake, D. A. (2001b). Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. *Infection and Immunity*, 69(8), 4958–68. 1
- Haake, D. A. (2006). Hamster model of leptospirosis. *Current Protocols in Microbiology*, Chapter 12, Unit 12E.2.

- Haake, D. A., Champion, C. I., Martinich, C., Shang, E. S., Blanco, D. R., Miller, J. N., & Lovett, M. A. (1993). Molecular cloning and sequence analysis of the gene encoding OmpL1, a transmembrane outer membrane protein of pathogenic *Leptospira* spp. *Journal of Bacteriology*, 175(13), 4225–4234.
- Haake, D. A., Chao, G., Zuerner, R. L., Barnett, J. K., Barnett, D., Mazel, M., & Bolin, C. A. (2000). The Leptospiral Major Outer Membrane Protein LipL32 Is a Lipoprotein Expressed during Mammalian Infection. *Infection and Immunity*, 68(4), 2276–2285.
- Haake, D. A., Chao, G., Zuerner, R. L., Barnett, J. K., Barnett, D., Mazel, M., & Bolin, C. A. (2000). The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infection and Immunity*, 68(4), 2276–85.
- Haake, D. A., & Levett, P. N. (2015). Leptospirosis in humans. *Current Topics in Microbiology and Immunology*, 387, 65–97.
- Haake, D. A., Mazel, M. K., McCoy, A. M., Milward, F., Chao, G., Matsunaga, J., & Wagar, E. A. (1999). Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. *Infection and Immunity*, 67(12), 6572–6582.
- Haake, D. A., Mazel, M. K., McCoy, A. M., Milward, F., Chao, G., Matsunaga, J., & Wagar, E. A. (1999). Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. *Infection and Immunity*, 67(12), 6572–82.
- Haake, D. A., & Zückert, W. R. (2015). The leptospiral outer membrane. *Current Topics in Microbiology and Immunology*, 387, 187–221.
- 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: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, 17(4), 494–501.
- Hartwig, D. D., Oliveira, T. L., Seixas, F. K., Forster, K. M., Rizzi, C., Hartleben, C. P., & Dellagostin, O. A. (2010). High yield expression of leptospirosis vaccine candidates LigA and LipL32 in the methylotrophic yeast *Pichia pastoris*. *Microbial Cell Factories*, 9, 98.

- He, H. J., Wang, W. Y., Wu, Z. D., Lv, Z. Y., Li, J., & Tan, L. Z. (2008). Protection of guinea pigs against *Leptospira interrogans* serovar Lai by LipL21 DNA vaccine. *Cellular & Molecular Immunology*, 5(5), 385–391.
- Hsieh, W.-J., & Pan, M.-J. (2004). Identification *Leptospira santarosai* serovar shermani specific sequences by suppression subtractive hybridization. *FEMS Microbiology Letters*, 235(1).
- Hu, W., Lin, X., & Yan, J. (2014). *Leptospira* and leptospirosis in China. *Current Opinion in Infectious Diseases*, 27(5), 432–6.
- Humphryes, P. C., Weeks, M. E., AbuOun, M., Thomson, G., Núñez, A., & Coldham, N. G. (2014). Vaccination with leptospiral outer membrane lipoprotein LipL32 reduces kidney invasion of *Leptospira interrogans* serovar canicola in hamsters. *Clinical and Vaccine Immunology: CVI*, 21(4), 546–51.
- Jost, B. H., Adler, B., & Faine, S. (1989). Experimental immunisation of hamsters with lipopolysaccharide antigens of *Leptospira interrogans*. *Journal of Medical Microbiology*, 29(2), 115–20.
- Khairani-Bejo S; Ooi, S. S. Bahaman, A. R. (2004). Rats: Leptospirosis Reservoir in Serdang Selangor Residential Area. *Journal of Animal and Veterinary Advances*.3 (2) 66-69.
- Khodaverdi Darian, E., Forghanifard, M. M., Moradi Bidhendi, S., Chang, Y.-F., Yahaghi, E., Esmaelizad, M., & Khaki, P. (2013). Cloning and Sequence Analysis of LipL32, a Surface-Exposed Lipoprotein of Pathogenic *Leptospira* Spp. *Iranian Red Crescent Medical Journal*, 15(11), e8793.
- King, A. M., Bartpho, T., Sermswan, R. W., Bulach, D. M., Eshghi, A., Picardeau, M., & Murray, G. L. (2013). Leptospiral outer membrane protein LipL41 is not essential for acute leptospirosis but requires a small chaperone protein, lep, for stable expression. *Infection and Immunity*, 81(8), 2768–76.
- Ko, A. I., Goarant, C., & Picardeau, M. (2009). *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nature Reviews. Microbiology*, 7(10), 736–47.

- Ko, H.-J., Ko, S.-Y., Kim, Y.-J., Lee, E.-G., Cho, S.-N., & Kang, C.-Y. (2005). Optimization of codon usage enhances the immunogenicity of a DNA vaccine encoding mycobacterial antigen Ag85B. *Infection and Immunity*, 73(9), 5666–74.
- Koebnik, R., Locher, K. P., & Van Gelder, P. (2000). Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Molecular Microbiology*, 37(2), 239–253.
- Koizumi, N., & Watanabe, H. (2005). Leptospirosis vaccines: past, present, and future. *Journal of Postgraduate Medicine*, 51(3), 210–4.
- Krawiec, S., & Riley, M. (1990). Organization of the bacterial chromosome. *Microbiological Reviews*, 54(4), 502–39.
- Kung, S. H., Retchless, A. C., Kwan, J. Y., & Almeida, R. P. P. (2013). Effects of DNA Size on Transformation and Recombination Efficiencies in *Xylella fastidiosa*. *Applied and Environmental Microbiology*, 79(5), 1712–1717.
- Kunjantarachot, A., Yan, W., McDonough, S. P., Prapong, S., Theeragool, G., & Chang, Y.-F. (2014). Immunogenicity of *Leptospira interrogans* Outer Membrane Vesicles in a Hamster Model. *Journal of Vaccines & Vaccination*, 5(4).
- Lanza, A. M., Curran, K. A., Rey, L. G., & Alper, H. S. (2014). A condition-specific codon optimization approach for improved heterologous gene expression in *Saccharomyces cerevisiae*. *BMC Systems Biology*, 8(1), 33.
- Larsen, J. E. P., Lund, O., & Nielsen, M. (2006). Improved method for predicting linear B-cell epitopes. *Immunome Research*, 2, 2.
- 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(10), 631–8.
- Lau, C., Smythe, L., & Weinstein, P. (2010). Leptospirosis: an emerging disease in travellers. *Travel Medicine and Infectious Disease*, 8(1), 33–9.
- Ledergerber, C., & Dessimoz, C. (2011). Base-calling for next-generation sequencing platforms. *Briefings in Bioinformatics*, 12(5), 489–97.

- Lehmann, J. S., Fouts, D. E., Haft, D. H., Cannella, A. P., Ricaldi, J. N., Brinkac, L., & Matthias, M. A. (2013). Pathogenomic inference of virulence-associated genes in *Leptospira interrogans*. *PLoS Neglected Tropical Diseases*, 7(10), e2468.
- Levett, P. N. (2001). Leptospirosis. *Clinical Microbiology Reviews*, 14(2), p. 296–326.
- Levett, P. N. (2015). Systematics of leptospiraceae. *Current Topics in Microbiology and Immunology*, 387, 11–20.
- Li, S., Wang, D., Zhang, C., Wei, X., Tian, K., Li, X., & Yan, J. (2013). Source tracking of human leptospirosis: serotyping and genotyping of *Leptospira* isolated from rodents in the epidemic area of Guizhou province, China. *BMC Microbiology*, 13, 75.
- Lin, X., Sun, A., Ruan, P., Zhang, Z., & Yan, J. (2011). Characterization of conserved combined T and B cell epitopes in *Leptospira interrogans* major outer membrane proteins OmpL1 and LipL41. *BMC Microbiology*, 11(1), 21.
- Lin, X., Zhao, J., Qian, J., Mao, Y., Pan, J., Li, L., & Yan, J. (2010). Identification of immunodominant B- and T-cell combined epitopes in outer membrane lipoproteins LipL32 and LipL21 of *Leptospira interrogans*. *Clinical and Vaccine Immunology : CVI*, 17(5), 778–83.
- Lotterberger, J., Guerrero, S. A., Tonarelli, G. G., Frank, R., Tarabla, H., & Vanasco, N. B. (2009). Epitope mapping of pathogenic *Leptospira* LipL32. *Letters in Applied Microbiology*, 49(5), 641–645.
- Maneewatch, S., Tapchaisri, P., Sakolvaree, Y., Klaysing, B., Tongtawe, P., Chaisri, U., & Chaicumpa, W. (2007). OmpL1 DNA vaccine cross-protects against heterologous *Leptospira* spp. challenge. *Asian Pacific Journal of Allergy and Immunology*, 25(1), 75–82.
- Mariya, R., Chaudhary, P., Kumar, A. A., Thangapandian, E., Amutha, R., & Srivastava, S. K. (2006). Evaluation of a recombinant LipL41 antigen of *Leptospira interrogans* serovar Canicola in ELISA for serodiagnosis of bovine Leptospirosis. *Comparative Immunology, Microbiology and Infectious Diseases*, 29(5), 269–277.

- Martínez, R. (2010). vax-SPIRAL®: Cuban antileptospirosis vaccines for humans: Clinical and field assays and impact of the vaccine on the disease after 11 years of application in Cuba. *International Journal of Infectious Diseases*, 14, e448.
- Martínez, R., Pérez, A., Quiñones, M. del C., Cruz, R., Alvarez, A., Armesto, M., & Fernández, N. (2004). [Efficacy and safety of a vaccine against human leptospirosis in Cuba]. *Revista Panamericana de Salud Pública = Pan American Journal of Public Health*, 15(4), 249-55.
- Masuzawa, T., Nakamura, R., Shimizu, T., Iwamoto, Y., Morita, T., & Yanagihara, Y. (1989). Immunological characteristics of the glycolipid antigen of *Leptospira interrogans* serovar lai. *Infection and Immunity*, 57(8), 2502-6.
- Matsunaga, J., Barocchi, M. A., Croda, J., Young, T. A., Sanchez, Y., Siqueira, I., & Ko, A. I. (2003). Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. *Molecular Microbiology*, 49(4), 929-45.
- Matsuo, K., Isogai, E., & Araki, Y. (2000). Control of immunologically crossreactive leptospiral infection by administration of lipopolysaccharides from a nonpathogenic strain of *Leptospira biflexa*. *Microbiology and Immunology*, 44(11), 887-90.
- Matthias, M. A., Ricaldi, J. N., Cespedes, M., Diaz, M. M., Galloway, R. L., Saito, M., & Vinetz, J. M. (2008). Human leptospirosis caused by a new, antigenically unique *Leptospira* associated with a *Rattus* species reservoir in the Peruvian Amazon. *PLoS Neglected Tropical Diseases*, 2(4), e213.
- McBride, A. J. A., Cerqueira, G. M., Suchard, M. A., Moreira, A. N., Zuerner, R. L., Reis, M. G & Dellagostin, O. A. (2009). Genetic diversity of the Leptospiral immunoglobulin-like (Lig) genes in pathogenic *Leptospira* spp. *Infection, Genetics and Evolution: Journal of Molecular Epidemiology and Evolutionary Genetics in Infectious Diseases*, 9(2), 196-205.
- McBride, A. J., Athanazio, D. A., Reis, M. G., Ko, A. I., Faisal, S. M., Yan, W., & Chang, Y. F. (2005). Evaluation of protective immunity of *Leptospira* immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. *Current Opinion in Infectious Diseases*, 26(5), 277-287.
- McBride, A. J., Athanazio, D. a, Reis, M. G., & Ko, A. I. (2005). Leptospirosis. *Current Opinion in Infectious Diseases*, 18(5), 376-386.

- Meenambigai Timiri Varadarajan, Ravikumar Gopalakrishnan, Balakrishnan Govindan, A., & Rajendiran, and K. K. (2015). Virulence Gene Loa22 – The Molecular Diagnostic Beacon of Canine Leptospirosis. *International Journal of Chemical, Environmental & Biological Scienc (IJCEBS)* , Volume 3(Issue 1).
- Michna, S. W. (1970). Leptospirosis. *The Veterinary Record*, 86(17), 484–96.
- Mohamed-Hassan, S. N., Bahaman, A. R., Mutalib, A. R., & Khairani-Bejo, S. (2010). Serological prevalence of leptospiral infection in wild rats at the National Service Training Centres in Kelantan and Terengganu. *Tropical Biomedicine*, 27(1), 30–2.
- Mor, G. (1998). Plasmid DNA: a new era in vaccinology. *Biochemical Pharmacology*, 55(8), 1151–3.
- Moreno, S., López-Fuertes, L., Vila-Coro, A. J., Sack, F., Smith, C. A., König, S. A., & Timón, M. (2004). DNA immunisation with minimalistic expression constructs. *Vaccine*, 22(13–14), 1709–1716.
- Mortimer, R. B. (2005). Leptospirosis in a caver returned from Sarawak, Malaysia. *Wilderness & Environmental Medicine*, 16(3), 129–31.
- Murray, G. L. (2013). The lipoprotein LipL32, an enigma of leptospiral biology. *Veterinary Microbiology*, 162(2–4), 305–14.
- Murray, G. L. (2015b). The molecular basis of leptospiral pathogenesis. *Current Topics in Microbiology and Immunology*, 387, 139–85.
- Muthiah, B., Kumar, S., & . R. (2015). Characterization, Comparison Study of Outer Membrane Protein OMPL1 of Pathogenic Leptospira Species for Disease Diagnosis. *Research Journal of Microbiology*, 10(2), 54–65.
- Narita, M., Fujitani, S., Haake, D. A., & Paterson, D. L. (2005). Leptospirosis after recreational exposure to water in the Yaeyama islands, Japan. *The American Journal of Tropical Medicine and Hygiene*, 73(4), 652–6.
- Nascimento, A. L. T. O., Ko, A. I., Martins, E. A. L., Monteiro-Vitorello, C. B., Ho, P. L., Haake, D. A., & Van Sluys, M. A. (2004). Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *Journal of Bacteriology*, 186(7), 2164–72.

- Ohse, M., Takahashi, K., Kadowaki, Y., & Kusaoke, H. (1995). Effects of plasmid DNA sizes and several other factors on transformation of *Bacillus subtilis* ISW1214 with plasmid DNA by electroporation. *Bioscience, Biotechnology, and Biochemistry*, 59(8), 1433–7.
- Oliveira, T. L., Bacelo, K. L., Schuch, R. A., Seixas, F. K., Collares, T., Rodrigues, O. E., & Hartwig, D. D. (2016). Immune response in hamsters immunised with a recombinant fragment of LigA from *Leptospira interrogans*, associated with carrier molecules. *Mem Inst Oswaldo Cruz Rio de Janeiro*, 111(11), 712–716.
- Oliveira, T. L., Grassmann, A. A., Schuch, R. A., Seixas Neto, A. C. P., Mendonça, M., Hartwig, D. D., & Dellagostin, O. A. (2015). Evaluation of the *Leptospira interrogans* Outer Membrane Protein OmpL37 as a Vaccine Candidate. *PloS One*, 10(11), e0142821.
- Overview of Leptospirosis: Leptospirosis: Merck Veterinary Manual. (n.d.). Retrieved November 24, 2014, from http://www.merckmanuals.com/vet/generalized_conditions/leptospirosis/overview_of_leptospirosis.html
- Palaniappan, R. U. M., Chang, Y.-F., Hassan, F., McDonough, S. P., Pough, M., Barr, S. C., & Roe, B. (2004). Expression of leptospiral immunoglobulin-like protein by *Leptospira interrogans* and evaluation of its diagnostic potential in a kinetic ELISA. *Journal of Medical Microbiology*, 53(10), 975–984.
- Palaniappan, R. U. M., Ramanujam, S., & Chang, Y.-F. (2007). Leptospirosis: pathogenesis, immunity, and diagnosis. *Current Opinion in Infectious Diseases*, 20(3), 284–92.
- Pappas, G., Papadimitriou, P., Siozopoulou, V., Christou, L., & Akritidis, N. (2008). The globalization of leptospirosis: worldwide incidence trends. *International Journal of Infectious Diseases : IJID : Official Publication of the International Society for Infectious Diseases*, 12(4), 351–7.
- Paster, B. J., Dewhirst, F. E., Weisburg, W. G., Tordoff, L. A., Fraser, G. J., Hespell, R. B., ... Woese, C. R. (1991). Phylogenetic analysis of the spirochetes. *Journal of Bacteriology*, 173(19), 6101–9.
- Patra, K. P., Choudhury, B., Matthias, M. M., Baga, S., Bandyopadhyay, K., & Vinetz, J. M. (2015). Comparative analysis of lipopolysaccharides of pathogenic and intermediately pathogenic *Leptospira* species. *BMC Microbiology*, 15(1), 244.

- Pfeifer, G. P., You, Y.-H., & Besaratinia, A. (2005). Mutations induced by ultraviolet light. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 571(1), 19–31.
- Picardeau, M. (2013). Diagnosis and epidemiology of leptospirosis. *Médecine et Maladies Infectieuses*, 43(1), 1–9.
- Picardeau, M., Bertherat, E., Jancloues, M., Skouloudis, A. N., Durski, K., & Hartskeerl, R. A. (2014). Rapid tests for diagnosis of leptospirosis: current tools and emerging technologies. *Diagnostic Microbiology and Infectious Disease*, 78(1), 1–8.
- Picardeau, M., Bulach, D. M., Bouchier, C., Zuerner, R. L., Zidane, N., Wilson, P. J., & Adler, B. (2008). Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PloS One*, 3(2), e1607.
- Pinne, M., & Haake, D. A. (2013). LipL32 Is a Subsurface Lipoprotein of *Leptospira interrogans*: presentation of new data and reevaluation of previous studies. *PloS One*, 8(1), e51025.
- Pishraft Sabet, L., Taheri, T., Memarnejadian, A., Mokhtari Azad, T., Asgari, F., Rahimnia, R., & Samimi Rad, K. (2014). Immunogenicity of Multi-Epitope DNA and Peptide Vaccine Candidates Based on Core, E2, NS3 and NS5B HCV Epitopes in BALB/c Mice. *Hepatitis Monthly*, 14(10), e22215.
- Rafizah, A. A. N., Aziah, B. D., Azwany, Y. N., Imran, M. K., Rusli, A. M., Nazri, S. M., & Zaliha, I. (2013). Risk factors of leptospirosis among febrile hospital admissions in northeastern Malaysia. *Preventive Medicine*, 57 Suppl, S11-3.
- Ramasamy Victor, A. A. (2014). Phylogenetic Characterization and Threading Based-Epitope Mapping of Leptospiral Outer Membrane Lipoprotein LipL41. *Journal of Proteomics & Bioinformatics*, 7(8).
- Rastogi, R. P., Richa, Kumar, A., Tyagi, M. B., Sinha, R. P., Rastogi, R. P., & Sinha. (2010). Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. *Journal of Nucleic Acids*, 2010, 592980.
- Rawlins, J., Portanova, A., Zuckerman, I., Loftis, A., Ceccato, P., Willingham, A. L., & Verma, A. (2014). Molecular detection of leptospiral DNA in environmental water on St. Kitts. *International Journal of Environmental Research and Public Health*, 11(8), 7953–60.

- Reis, R. B., Ribeiro, G. S., Felzemburgh, R. D. M., Santana, F. S., Mohr, S., Melendez, A. X. T. O., & Ko, A. I. (2008). Impact of environment and social gradient on *Leptospira* infection in urban slums. *PLoS Neglected Tropical Diseases*, 2(4), e228.
- Ridzlan, F. R., Bahaman, A. R., Khairani-Bejo, S., & Mutalib, A. R. (2010). Detection of pathogenic *Leptospira* from selected environment in Kelantan and Terengganu, Malaysia. *Tropical Biomedicine*, 27(3), 632–8.
- Ristow, P., Bourhy, P., da Cruz McBride, F. W., Figueira, C. P., Huerre, M., Ave, P., & Picardeau, M. (2007). The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathogens*, 3(7), e97.
- Rodriguez-Gonzalez, I., Fillonneau, C., Blanchet, B., Suard, I., Catilina, P., & Andre-Fontaine, G. (2004). [Efficacy of Spirolept vaccine against human leptospirosis as estimated by passive protection of laboratory rodents]. *Médecine et Maladies Infectieuses*, 34(5), 196–200.
- Ssamsudeen .N.S., S., S., S., T.Z.M.T., J., S.M., N., & O., M. (2015, January 18). Seroprevalence of Leptospiral Antibodies And Knowledge, Attitude And Practices of Leptospirosis to Non High Risk Group In Selangor. *International Journal of Public Health and Clinical Sciences*.
- Saito, M., Y A M Villanueva, S., Masuzawa, T., Yanagihara, Y., & Yoshida, S. (2014). [Leptospirosis now-the centennial of the discovery of Weil's disease pathogen]. *Nihon Saikingaku Zasshi. Japanese Journal of Bacteriology*, 69(4), 589–600.
- Sambrook J, R. D. and I. N. M. cloning: A. laboratory manual. 3rd edition 2001. (n.d.). Molecular cloning: a laboratory manual | Clc. Retrieved November 2, 2014, from <http://library.wur.nl/WebQuery/clc/1627558>
- Samsudin, S., Masri, S. N., Jamaluddin, T. Z. M. T., Saudi, S. N. S., Ariffin, U. K. M., Amran, F., & Osman, M. (2015). Seroprevalence of Leptospiral Antibodies among Healthy Municipal Service Workers in Selangor. *Advances in Public Health*, 2015.
- Sapian, M., Khair, M. T., How, S. H., Rajalingam, R., Sahhir, K., Norazah, A., & Jamalludin, A. R. (2012). Outbreak of melioidosis and leptospirosis co-infection following a rescue operation. *The Medical Journal of Malaysia*, 67(3), 293–7.

- Sarkar, J., Chopra, A., Katageri, B., Raj, H., & Goel, A. (2012). Leptospirosis: a re-emerging infection. *Asian Pacific Journal of Tropical Medicine*, 5(6), 500–2.
- Schreiber, P., Martin, V., Najbar, W., & Sanquer, A. (n.d.). Prevention of a severe disease by a *Leptospira* vaccination with a multivalent vaccine. *Recomedvet.com*.
- Seixas, F. K., da Silva, É. F., Hartwig, D. D., Cerqueira, G. M., Amaral, M., Fagundes, M. Q., & Dellagostin, O. A. (2007). Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. *Vaccine*, 26(1), 88–95.
- Sejvar, J., Bancroft, E., Winthrop, K., Bettinger, J., Bajani, M., Bragg, S., & Rosenstein, N. (2003). Leptospirosis in “Eco-Challenge” athletes, Malaysian Borneo, 2000. *Emerging Infectious Diseases*, 9(6), 702–7.
- Sette, A., & Fikes, J. (2003). Epitope-based vaccines: An update on epitope identification, vaccine design and delivery. *Current Opinion in Immunology*, 15(4), 461–470.
- Sette, A., Livingston, B., McKinney, D., Appella, E., Fikes, J., Sidney, J., & Chesnut, R. (2001). The development of multi-epitope vaccines: epitope identification, vaccine design and clinical evaluation. *Biologicals : Journal of the International Association of Biological Standardization*, 29(3–4), 271–6.
- Shafei, M. N., & Sulong, M. R. (2012). Seroprevalance of leptospirosis among Town Service workers in Northeastern state of Malaysia. *International Journal of Collaborative Research on Internal Medicine and Public Health*, 4(4), 395–403.
- Shang, E. S., Summers, T. A., & Haake, D. A. (1996). Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic *Leptospira* species. *Infection and Immunity*, 64(6), 2322–30.
- Sharma, S., Vijayachari, P., Sugunan, A. P., Natarajaseenivasan, K., & Sehgal, S. C. (2006). Seroprevalence of leptospirosis among high-risk population of Andaman Islands, India. *The American Journal of Tropical Medicine and Hygiene*, 74(2), 278–83.

- Silva, E. F., Medeiros, M. A., McBride, A. J. A., Matsunaga, J., Esteves, G. S., Ramos, J. G. R., & Ko, A. I. (2007). The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. *Vaccine*, 25(33), 6277–86.
- Singh, H., Ansari, H. R., & Raghava, G. P. S. (2013). Improved method for linear B-cell epitope prediction using antigen's primary sequence. *PloS One*, 8(5), e62216.
- Siva, T., Ian, R., Fairuz, A., Lela, S., & Mohd Tajuddin, A. (2013, March 26). Serological Prevalence of Leptospiral Infection in Wildlife in Sarawak, Malaysia. *Borneo Journal Resource Science Technology*. *Borneo Journal Resource Science Technology*.
- Sonada, R. B., Azevedo, S. S. de, Soto, F. R. M., Costa, D. F. da, Morais, Z. M. de, Souza, G. O. de, & Vasconcellos, S. A. (2017). Efficacy of leptospiral commercial vaccines on the protection against an autochthonous strain recovered in Brazil. *Brazilian Journal of Microbiology*.
- Sonrier, C., Branger, C., Michel, V., Ruvoën-Clouet, N., Ganière, J. P., & André-Fontaine, G. (2000). Evidence of cross-protection within *Leptospira interrogans* in an experimental model. *Vaccine*, 19(1), 86–94.
- Soria-Guerra, R. E., Nieto-Gomez, R., Govea-Alonso, D. O., & Rosales-Mendoza, S. (2014). An overview of bioinformatics tools for epitope prediction: implications on vaccine development. *Journal of Biomedical Informatics*.
- Srikram, A., Zhang, K., Bartpho, T., Lo, M., Hoke, D. E., Sermswan, R. W., & Murray, G. L. (2011). Cross-protective immunity against leptospirosis elicited by a live, attenuated lipopolysaccharide mutant. *The Journal of Infectious Diseases*, 203(6), 870–9.
- Strynadka, N. C. J., Luo, Y., Frey, E. A., Pfuetzner, R. A., Creagh, A. L., Knoechel, D. G., & Finlay, B. B. (2000). Crystal structure of enteropathogenic *Escherichia coli* intimin-receptor complex. *Nature*, 405(6790), 1073–1077.
- Sulong, Mohd Rahim; Shafei, Mohd Nazri; Yaacob, Nor Azwany; Hassan, Habsah; Daud, Aziah; Mohamad, Wan Mohd Zahiruddin Wan; Ismail, Zaliha; Abdullah, M. R. (2011). Risk Factors Associated with Leptospirosis among Town Service Workers. *International Medical Journal*, 18:(2), 83.

- Swords, W. E. (2003). Chemical Transformation of *E. coli*. In *E. coli Plasmid Vectors* (pp. 49–54). New Jersey: Humana Press.
- Tereza Cristina, M. M., & Silva, T. C. C. (2015). Pathogenesis of Leptospirosis: Important Issues. *Journal of Medical Microbiology & Diagnosis*, 4(1).
- Terpstra, W. J. (2006). Historical perspectives in leptospirosis. *Indian Journal of Medical Microbiology*, 24(4), 316–20.
- Thayaparan, S., Robertson, I. D., Fairuz, A., Suut, L., & Abdullah, M. T. (2013). Leptospirosis, an emerging zoonotic disease in Malaysia. *The Malaysian Journal of Pathology*, 35(2), 123–32.
- Thompson, J. D., Plewniak, F., & Poch, O. (1999). A comprehensive comparison of multiple sequence alignment programs. *Nucleic Acids Research*, 27(13), 2682–90.
- Torgerson, P. R., Hagan, J. E., Costa, F., Calcagno, J., Kane, M., Martinez-Silveira, M. S., & Abela-Ridder, B. (2015). Global Burden of Leptospirosis: Estimated in Terms of Disability Adjusted Life Years. *PLoS Neglected Tropical Diseases*, 9(10), e0004122.
- Trinh, R., Gurbaxani, B., Morrison, S. L., & Seyfzadeh, M. (2004). Optimization of codon pair use within the (GGGGS)₃ linker sequence results in enhanced protein expression. *Molecular Immunology*, 40(10), 717–722.
- 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: The Official Journal of the Spanish Society for Microbiology*, 7(1), 35–40.
- Atzingen, V. M. (2012). Evaluation of Immunoprotective Activity of Six Leptospiral Proteins in the Hamster Model of Leptospirosis. *The Open Microbiology Journal*.
- Victoriano, A. F. B., Smythe, L. D., Gloriani-Barzaga, N., Cavinta, L. L., Kasai, T., Limpakarnjanarat, K., & Adler, B. (2009). Leptospirosis in the Asia Pacific region. *BMC Infectious Diseases*, 9, 147.
- Vieira, M. L., Pimenta, D. C., de Moraes, Z. M., Vasconcellos, S. A., & Nascimento, A. L. T. O. (2009). Proteome analysis of *Leptospira interrogans* virulent strain. *The Open Microbiology Journal*, 3, 69–74.

- Vijayachari, P., Sugunan, A. P., & Shriram, A. N. (2008). Leptospirosis: an emerging global public health problem. *Journal of Biosciences*, 33(4), 557–569.
- Vijayachari, P., Vedhagiri, K., Mallilankaraman, K., Mathur, P. P., Sardesai, N. Y., Weiner, D. B., & Muthumani, K. (2015). Immunogenicity of a novel enhanced consensus DNA vaccine encoding the leptospiral protein LipL45. *Human Vaccines & Immunotherapeutics*, 11(8), 1945–53.
- Villanueva, S. Y. A. M., Ezoe, H., Baterna, R. A., Yanagihara, Y., Muto, M., Koizumi, N., & Yoshida, S. (2010). Serologic and molecular studies of *Leptospira* and leptospirosis among rats in the Philippines. *The American Journal of Tropical Medicine and Hygiene*, 82(5), 889–98.
- Vinetz, J. M. (2004). Leptospirosis is everywhere, just have to know what to look for. But how? *Swiss Medical Weekly*, 134(23–24), 331–2.
- Vinh, T. U., Shi, M. H., Adler, B., & Faine, S. (1989). Characterization and taxonomic significance of lipopolysaccharides of *Leptospira interrogans* serovar hardjo. *Journal of General Microbiology*, 135(10), 2663–73.
- Wang, Z., Jin, L., & Węgrzyn, A. (2007). Leptospirosis vaccines. *Microbial Cell Factories*, 6, 39.
- Wang, Z., Jin, L., Węgrzyn, A., Kong, L., Chen, X., Sun, D., & Cheville, N. (2007). Leptospirosis vaccines. *Microbial Cell Factories*, 6(1), 39.
- Wasiński, B., & Dutkiewicz, J. (2013). Leptospirosis: Current risk factors connected with human activity and the environment. *Annals of Agricultural and Environmental Medicine : AAEM*, 20(2), 239–44.
- Werts, C., Tapping, R. I., Mathison, J. C., Chuang, T. H., Kravchenko, V., Saint Girons, I., & Ulevitch, R. J. (2001). Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nature Immunology*, 2(4), 346–52.
- 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), 334–8.
- Xiong, A.-S., Peng, R.-H., Zhuang, J., Gao, F., Li, Y., Cheng, Z.-M., & Yao, Q.-H. (2008). Chemical gene synthesis: strategies, softwares, error corrections, and applications. *FEMS Microbiology Reviews*, 32(3), 522–540.

- Yan, W., Faisal, S. M., McDonough, S. P., Chang, C.-F., Pan, M.-J., Akey, B., & Chang, Y.-F. (2010). Identification and characterization of OmpA-like proteins as novel vaccine candidates for Leptospirosis. *Vaccine*, 28(11), 2277-83.
- Yan, W., Faisal, S. M., McDonough, S. P., Divers, T. J., Barr, S. C., Chang, C.-F., & Chang, Y.-F. (2009). Immunogenicity and protective efficacy of recombinant *Leptospira* immunoglobulin-like protein B (rLigB) in a hamster challenge model. *Microbes and Infection / Institut Pasteur*, 11(2), 230-7.
- Yan, Y., Chen, Y., Liou, W., Ding, J., Chen, J., Zhang, J., & Xiao, Y. (2003). An evaluation of the serological and epidemiological effects of the outer envelope vaccine to leptospira. *Journal of the Chinese Medical Association : JCMA*, 66(4), 224-30.
- Yu, K., Liu, C., Kim, B.-G., & Lee, D.-Y. (2014). Synthetic fusion protein design and applications. *Biotechnology Advances*, 33(1), 155-164.
- Zariri, A., & van der Ley, P. (2015). Biosynthetically engineered lipopolysaccharide as vaccine adjuvant. *Expert Review of Vaccines*, 14(6), 861-76.
- Zhao, C., Sun, Y., Zhao, Y., Wang, S., Yu, T., Du, F., & Luo, E. (2012). Immunogenicity of a multi-epitope DNA vaccine against hantavirus. *Human Vaccines & Immunotherapeutics*, 8(2), 208-15.
- Zuerner, R., Haake, D., Adler, B., & Segers, R. (2000). Technological advances in the molecular biology of *Leptospira*. *Journal of Molecular Microbiology and Biotechnology*, 2(4), 455-462.
- Zuerner, R. L. (1991). Physical map of chromosomal and plasmid DNA comprising the genome of *Leptospira interrogans*. *Nucleic Acids Research*, 19(18), 4857-60.
- Zuerner, R. L., Alt, D. P., Palmer, M. V, Thacker, T. C., & Olsen, S. C. (2011). A *Leptospira borgpetersenii* serovar Hardjo vaccine induces a Th1 response, activates NK cells, and reduces renal colonization. *Clinical and Vaccine Immunology : CVI*, 18(4), 684-91.
- Zuerner, R. L., Knudtson, W., Bolin, C. A., & Trueba, G. (1991). Characterization of outer membrane and secreted proteins of *Leptospira interrogans* serovar pomona. *Microbial Pathogenesis*, 10(4), 311-22.