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

GARBA BASHIRU

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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

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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

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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 leptospires. 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 leptospires 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 diingini termasuk kesakitan, mual dan demam, imuniti jangka pendek dan perlindungan terhad serovar. Vaksin DNA peptida yang mengandungi pelbagai epitop menunjukkan keberkesananya terhadap sesetengah virus dan vaksin ini juga telah dikenalpasti mempunyai potensi terhadap beberapa penyakit yang disebabkan oleh bakteria termasuk jangkitan leptospirosis. Vaksin ini menujukkan gaya pemprosesan antigen dan persembahanya 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 mutivalent yang dinyatakan (*invitro*) telah disahkan dengan ujian antibodi immunofluoresensi secara tidak langsung. Ujian imunofluoresensi 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 diimunisasi 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 diimunisasi 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.

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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:

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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

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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	Ethyline diamine teraacetic acid
EF-1α	Human elongation factor 1α
IEDB	Immune epitope data base
IGIT	Invitro growth inhibition test
IL	Interleukin
FBS	Fetal bovine serum
FITC	Fluorescence isothiocyanate
g	Gram
GFP	Green fluorescent protein
LPS	Lipopolysacharide
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

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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; Sapian 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 rsik of infection follwing environmental contamination with urine or body fluids of reservoir wild and ferral 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 preodminatly whole cell killed bacterins can not protect against serovars not included in the vaccine preprations. 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 privided 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 immunecompoenents. 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 urdened 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).

Leptospires 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 leptospires (Koizumi & Watanabe, 2005). Immunization with outer membrane protein OmpL1, which is a transmembrane protein conserved among pathogenic leptospires 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 echnique.
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

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