UNIVERSITI PUTRA MALAYSIA

VIBRIOSIS VACCINE DEVELOPMENT: PATHOGENESIS, IMMUNOLOGICAL AND MOLECULAR CHARACTERIZATION OF VIBRIO ALGINOLYTICUS

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By

NOR AZIZAH BT. MOHD TAIB

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Medicine and Health Sciences Universiti Putra Malaysia

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To my late father and brother, to my dear mother, brother, sisters, brothers in-law, niece and nephews, thanks for the loves

Sam, Zila, Wan, Asma, Erina, Rina, Marina
F.R.A.N.C.E

Ramli
Thanks for everything

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairperson: **Mariana Nor bt. Shamsudin, Ph.D.**

Faculty: **Medicine and Health Sciences**

The aim of the research is to develop an effective vaccine against vibriosis. Vibriosis is a bacterial disease caused by *Vibrio spp* due to the intensive production activity of brackishwater ponds and cage-cultured fish. The pathogenicity study of *V. alginolyticus* was performed by challenging the juvenile seabass (10±0.75 g) with 5 different isolates of *V. alginolyticus* at different cell concentrations. All the isolates caused mortality to fish at a concentration as low as 0.2 optical density (6.28 X 10^3 CFU/ml). Ultrastructure changes observed by scanning and transmission electron microscopy, revealed the presence of *V. alginolyticus* in the gills, liver, muscle, spleen and kidney of infected fish. In addition, the *V. alginolyticus* cells were observed in the spleen. These pathological changes showed that *V. alginolyticus* was responsible for the death of the infected seabass.

The lipopolysaccharide (LPS) or endotoxin of the gram-negative bacteria was extracted from 5 *V. alginolyticus* isolates used in the pathogenicity study by

the Hot Phenol-Water method of Westphal and Jann, 1967. The lipopolysaccharide profiles were studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Three isolates were found to possess high molecular weight bands of LPS which ranged from 14.4 to 97.4 kDA. The other two isolates possessed only low molecular weight bands, ranging from 14.4 to 45 kDA, indicating that their LPS were not highly immunogenic. The mean agglutination titers were higher for sera of fish immunized with lipopolysaccharide (LPS) and formalin-killed cells (FKC) from isolates having high molecular weight bands of LPS compared sera from fish immunized with strains having low molecular weight bands of LPS. In the challenge study, fish vaccinated with LPS and FKC showed high survival rate and significantly higher ($p<0.05$) compared to unvaccinated control fish. The highest survival rate was seen from fish immunized with LPS and FKC from isolate 17 (70.8% - 88.3%), followed by fish immunized with LPS and FKC isolate 26 (65.7% - 86.15%) and fish immunized with LPS and FKC from isolate 78 (53.57% - 80.9%), respectively. Generally, fish immunized with LPS obtained the highest survival rate (80.9% - 90%) and significantly higher ($p<0.05$) compared to FKC via injection route (53.57% - 70.8%). Specifically, fish immunized with LPS having high molecular weight bands yielded significant protection against *V. alginolyticus.*

In addition to the immunogenicity study, molecular characterization of *V. alginolyticus* was also performed. From the random amplified polymorphic DNA (RAPD) studies, it was detected that different *V. alginolyticus* isolates gave different banding patterns. The dendrogram generated based on the DNA banding

patterns of 10 *V algınolyticus* isolates showed that the isolates were quite homogenous and 2 main groups were seen. The values of percent similarities of shared bands ranged from 29.79% to 98.53% between *V algınolyticus* isolates with a mean of 64.16%.

The presence of the LPS biosynthesis gene (*rfaZ* gene) in *V algınolyticus* isolates was screened by the dot-blot technique. All ten isolates showed positive dot-blot results. Furthermore, this gene of the size 1.2 kb was successfully amplified and isolated by polymerase chain reaction (PCR) and the results was confirmed by Southern blotting. The results also indicated that all isolates possessed the homologous DNA gene sequence of the *rfaZ* gene which was strongly conserved between *Escherichia coli K-12* and *Salmonella typhimurium*. The detection and successful isolation of this *rfaZ* gene in *V algınolyticus* gives an indication of the possibility of developing a subunit LPS vaccine via the recombinant DNA technology.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBANGUNAN VAKSIN VIBRIOSIS : PATOGENESIS, PENCIRIAN IMMUNOLOGI DAN MOLEKUL *VIBRIO ALGINOLYTICUS*

Oleh

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Fakulti: **Perubatan dan Sains Kesihatan**

Tujuan penyelidikan ini dijalankan adalah untuk membangunkan vaksin yang efektif terhadap vibriosis. Penyakit vibriosis adalah disebabkan oleh bakteria dari spesis *Vibrio* dan terjadi akibat dari aktiviti intensif penternakan ikan kolam air payau dan sangkar terapung. Kajian patogenisiti dijalankan dengan mendedahkan juvenil ikan siakap (10±0.35 g) pada 5 isolat *V. alginolyticus* dengan kepekatan sel yang berbeza. Kesemua isolat menyebabkan kematian ikan pada kepekatan sel serendah 0.2 OD (6.28 X 10^3 CFU/ml). Perubahan ultrastruktur yang dikesan menggunakan mikroskop pengimbas elektron (SEM) and mikroskop perpindahan elektron (TEM), menunjukkan kehadiran *V. alginolyticus* di dalam insang, hati, otot, limpa dan ginjal ikan yang dijangkiti. Sebagai tambahan, sel *V. alginolyticus* dikesan di dalam limpa. Kajian patologi ini menunjukkan bahawa *V. alginolyticus* adalah penyumbang kepada kematian ikan siakap yang dijangkitinya.

Lipopolisakarida (LPS) atau endotoksin bagi 5 isolat *V. alginolyticus* yang digunakan dalam ujian patogenisiti diestrak dengan menggunakan kaedah "Hot Phenol-Water" (Westphal dan Jann, 1967). Profil LPS dikaji menggunakan teknik elektroporesis gel dodecyl sulphate polyacrylamide (SDS-PAGE). Tiga dari isolat tersebut mengandungi LPS yang mempunyai berat molekul yang tinggi, pada julat 14.4 hingga 97.4 kDA. Dua isolat berikutnya mengandungi LPS yang mempunyai berat molekul yang rendah iaitu 14.4 hingga 45 kDA, yang mana menunjukkan bahawa LPS isolat tersebut kurang immunogenik. Min titer agglutinasi adalah lebih tinggi serum ikan yang diimunisasikan dengan lipopolisakarida (LPS) dan sel yang dimatikan dengan formalin (FKC) dari isolat yang mengandungi LPS yang mempunyai berat molekul yang tinggi dibandingkan dengan serum ikan yang diimunisasikan dengan isolat yang mengandungi LPS yang mempunyai berat molekul yang rendah. Dalam ujian pendedahan ikan pada isolat yang virulen, peratus hidup ikan yang diberi vaksin LPS dan FKC adalah tinggi dan menunjukkan perbezaan yang berkesan ($p<0.05$) berbanding ikan kawalan yang tidak menerima rawatan vaksin. Peratus hidup yang tertinggi didapati dari ikan yang diimunisasikan dengan LPS dan FKC dari isolat 17 (70.8% - 88.3%), diikuti dengan ikan yang diimunisasikan dengan LPS dan FKC dari isolat 26 (65.7% - 86.15%) dan ikan yang diimunisasikan dengan LPS (53.57% - 80.9%). Secara amnya, purata hidup ikan yang diimunisasikan dengan LPS adalah tertinggi (80.9% - 90%) dan menunjukkan perbezaan yang berkesan ($p<0.05$) dibandingkan dengan ikan yang diimunisasikan dengan FKC melalui teknik suntikan (53.57% - 70.8%). Secara spesifiknya, ikan yang diimunisasikan dengan LPS yang mempunyai berat molekul yang tinggi memberi perlindungan yang signifikan terhadap *V. alginolyticus*.

Selain dari ujian immunogenisiti, pencirian molekul *V. alginolyticus* telah dijalankan. Dari analisa DNA polimorfik menggunakan primer rawak (RAPD), didapati setiap isolat *V. alginolyticus* memberikan jalur DNA yang berlainan. Dendrogram yang dihasilkan berdasarkan kepada corak jalur DNA menunjukkan semua isolat adalah berdekatan dari segi genetik dan terbahagi kepada 2 kumpulan yang utama. Julat nilai peratus persamaan berdasarkan perkongsian jalur adalah 29.79 hingga 98.53% dengan nilai min 64.16%.

Kehadiran gen biosintesis LPS (*rfaZ*) pada isolat-isolat *V. alginolyticus* dikesan melalui teknik "dot blot". Kesemua 10 isolat yang dikaji menunjukkan keputusan yang positif. Selanjutnya, gen ini pada kedudukan 1.2 kb telah berjaya diamplikasi dan dipencil melalui teknik tindakbalas berantai polymerase (PCR) dan disahkan menggunakan teknik "Southern blotting". Keputusan ini menunjukkan kesemua isolat mempunyai jujukan DNA yang sama dengan gen *rfaZ* di mana gen tersebut adalah kuat terpulihara di antara *Escherichia coli* K-12 dan *Salmonella typhimurium.* Pengesanan dan kejayaan pemencilan gen r*faZ* ini memberi arah kepada penemuan subunit vaksin melalui teknologi rekombinan DNA.

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LIST OF ABBREVIATIONS

˚C	degree celcius
µg/ml	microgram per milliliter
µm	micron
ASW	artificial sea water
ATCC	American Type Culture Collection
bp	basepair
$CaSO_4$	Calcium sulfate
CFU/ml	colony-forming units per milliliter
Corp.	Corporation
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
FKC	formalin-killed cells
g	gram
H_2O	water
HCl	hydrochloric acid
IgG	immunoglobulin G
IgM	immunoglobulin M
IMM	immersion
Inc.	incorporated
IP	intraperitoneal
kb	kilobase
KCl	Potassium chloride
kDa	kilo Dalton
LB	Luria-Bertanii