



**UNIVERSITI PUTRA MALAYSIA**

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

**NOR AZIZAH BT. MOHD TAIB**

**FPSK (M) 2001 1**

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

**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**

**August 2001**



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

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

By

**NOR AZIZAH BT. MOHD TAIB**

**August 2001**

**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 ( $6.28 \times 10^3$  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 alginolyticus* 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 alginolyticus* isolates with a mean of 64.16%.

The presence of the LPS biosynthesis gene (*rfaZ* gene) in *V alginolyticus* 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 alginolyticus* 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

**NOR AZIZAH BT. MOHD TAIB**

**Ogos 2001**

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

**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 ( $10 \pm 0.35$  g) pada 5 isolat *V. alginolyticus* dengan kepekatan sel yang berbeza. Kesemua isolat menyebabkan kematian ikan pada kepekatan sel serendah 0.2 OD ( $6.28 \times 10^3$  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 patogenesis 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 agglutinasia adalah lebih tinggi serum ikan yang diimunitasikan 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 diimunitasikan 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 diimunitasikan dengan LPS dan FKC dari isolat 17 (70.8% - 88.3%), diikuti dengan ikan yang diimunitasikan dengan LPS dan FKC dari isolat 26 (65.7% - 86.15%) dan ikan yang diimunitasikan dengan LPS (53.57% - 80.9%). Secara amnya, purata hidup ikan yang diimunitasikan dengan LPS adalah tertinggi (80.9% - 90%) dan menunjukkan perbezaan yang berkesan ( $p < 0.05$ ) dibandingkan dengan ikan yang diimunitasikan dengan FKC melalui teknik suntikan (53.57% - 70.8%). Secara spesifiknya, ikan yang diimunitasikan 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 diampikasi 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 *rfaZ* ini memberi arah kepada penemuan subunit vaksin melalui teknologi rekombinan DNA.



## ACKNOWLEDGEMENTS

In the name of Allah S W T , the most benevolent and merciful The author would like to express her gratitude to Him, the almighty for giving her the capability and patience to complete this project She would like to thank her supervisor, Dr Mariana Nor bt Shamsudin for the guidance, advice and support throughout her study She would also like to express her appreciation for the trust being given to her to complete this project The author would like to express her special thanks to Dr Rozita Rosh and Dr Fauziah Othman for being the co-supervisors and also for the ideas and suggestions For Encik Zanan Ahmad Ariffin, Encik Abdul Rahman Mohd Taib and Encik Ariff Mat, the author would like to express her appreciation for the technical assistance during the study The author would also like to take this opportunity to thank Professor Shariff Mohd Din, for providing the pure bacterial stock culture of *Vibrio alginolyticus* strains used in the study To her colleagues and friends, thank you for the support Not forgotten is the sincere appreciation to the UPM Electron Microscopy Unit Staff especially Mr Ho, Puan Faridah, Miss Suleka and Miss Azilah for their assistance in electron microscopy work Last but not least, the author would like to express her sincere appreciation to Assoc Prof Dr Jammal Ahmad Essa, the Dean, Faculty of Medicine and Health Science, Assoc Prof Dr Sabariah Abdul Rahman, the Deputy Dean (Research and Health Sciences) and Assoc Prof Dr Harcharan Singh Sidhu, the Head of Microbiology Unit, for their indirect support to her in completing the master's thesis



## TABLE OF CONTENTS

	<b>Page</b>
DEDICATION	ii
ABSTRACTS	iii
ABSTRAK	vi
ACKNOWLEDGEMENTS	ix
APPROVAL SHEET	xi
DECLARATION FORM	xii
LIST OF TABLES	xviii
LIST OF FIGURES	xix
LIST OF PLATES	xxiii
LIST OF ABBREVIATIONS	xxv

### CHAPTER

<b>1</b>	<b>INTRODUCTION</b>	<b>1</b>
<b>2</b>	<b>LITERATURE REVIEW</b>	<b>10</b>
	Vibriosis	10
	Aetiology	10
	Chemotherapy	14
	Immune Response and Protective Efficacies of a Bacterial Fish Vaccine	17
	<i>Vibrio alginolyticus</i>	23
	Bacterial Lipopolysaccharide	27
	Lipopolysaccharides (LPS) Structure	27
	Immunogenicity of LPS (Endotoxin)	30
	Analysis of LPS Profile	31
	LPS Genetics	33
	LPS Biosynthesis gene	34
	Polymerase Chain Reaction	36
	Asymmetry PCR	36
	Random Amplified Polymorphic DNA (RAPD)	40
	Nucleic Acid Hybridization	43
	Dot blot	44
	Southern blotting	46
<b>3</b>	<b>MATERIALS AND METHODS</b>	<b>48</b>
	Sources of bacteria	48
	Confirmation of <i>Vibrio alginolyticus</i> stock cultures	48
	Gram staining	48
	Growth on thiosulphate citrate bile salt sucrose (TCBS) agar	49
	Sensitivity to Vibriostat compound 0/129	49
	Pathogenesis of <i>Vibrio alginolyticus</i> on seabass	50
	Pathogenicity study	50



Experimental Fish	50
Isolates	50
Preparation of bacteria with different optical densities	51
Exposure of fish to bacteria	52
Pathological studies	53
Scanning Electron Microscopy	53
Transmission Electron Microscopy	54
Immunological characterization of <i>Vibrio alginolyticus</i>	55
Propagation of bacteria	55
Antigens Preparation	57
LPS Extraction	57
Preparation of dialysis tube for LPS extraction	57
Phenol-water extraction	57
Purification of LPS	58
Preparation of formalin-killed particulate bacterial antigen	61
SDS-PAGE Electrophoresis	61
Silver staining	63
Immunogenicity study	64
Antiserum Sampling	64
Fish and Fish Handling	64
Isolates	65
Injection vaccination	65
Immersion vaccination	66
Determination of Antibody Titer	67
Agglutination Titer	68
Passive Hemagglutination Assay (PHA)	68
Preparation of Sheep Red Blood Cells stock solution.	69
Protective efficacy	69
Challenge study	69
Statistical analysis	70
Molecular characterization of <i>V. alginolyticus</i>	70
Propagation of bacteria	70
Total DNA extraction	71
DNA quantitation	72
Agarose gel electrophoresis	73
Random Amplified Polymorphic DNA (RAPD)	75
RAPD Amplification	75
RAPD Analysis	76
Dot blot	77
Immobilization of DNA on the nylon membrane	77
Prehybridization	78
Hybridization	78
Detection of the nucleic acids with Chemiluminescent Detection Kit	78
Blocking Step	79
Streptavidin incubation	79
Washing 2 times with Wash Solution 1	79



	Biotinylated Alkaline Phosphatase incubation	79
	Washing 1 time with Blocking Solution	80
	Washing 2 times with Wash Solution II	80
	Detection of DNA	80
	Development of X-ray film	81
	Amplification and isolation of <i>rfaZ</i> gene in <i>V. alginolyticus</i> with Asymmetry PCR.	82
	Detection of <i>rfaZ</i> gene detected in <i>V. alginolyticus</i> isolates using gene probe technique (Southern hybridization)	83
	Gel preparation	83
	Membrane preparation	83
	Capillary transfer	84
	Fixing the DNA on the membrane	85
	Prehybridization	85
	Preparation of the probe	85
	Hybridization	86
	Detection of DNA with chemiluminescent Kit	86
	Development of X-ray film	87
<b>4</b>	<b>RESULTS</b>	88
	Confirmation of <i>V. alginolyticus</i> stock cultures	88
	Pathogenesis of <i>Vibrio alginolyticus</i> on seabass	90
	Pathogenicity study	90
	Pathological study	92
	Clinical signs and gross lesions	92
	Scanning electron microscopy	94
	Transmission electron microscopy	94
	Lipopolysaccharides (LPS) banding profiles	103
	Immunogenicity study	105
	Agglutinating antibody	105
	Protective Efficacy	107
	Molecular characterization of <i>V. alginolyticus</i>	110
	Total genomic DNA extraction	110
	RAPD analysis	112
	RAPD banding patterns	112
	Dendrogram	120
	Genetic distance and percentage (%) of similarities	121
	Dot blot	122
	Isolation and detection of <i>rfaZ</i> gene in <i>V. alginolyticus</i> isolates	123
<b>5</b>	<b>DISCUSSION</b>	125
	Characterization test for confirmation of <i>V. alginolyticus</i> pure stock culture isolated from vibriosis infected seabass	125
	Growth on thiosulphate citrate bile-salt sucrose agar (TCBS) with 50% artificial sea water (ASW)	125
	Sensitivity to Vibriostatic Compound 0/129	125
	Gram-staining	126

Pathogenesis of <i>V. alginolyticus</i> on seabass	126
Pathogenicity study	126
Pathological studies	130
Lipopolysaccharide (LPS) profile analysis	138
Immunogenicity study	141
Agglutinating antibody titer	141
Protective Efficacies of <i>V. alginolyticus</i> antigens	143
Molecular Characterization of <i>V. alginolyticus</i>	148
Total genomic DNA extraction	148
Genotyping of <i>V. alginolyticus</i> strains using random amplified polymorphic DNA	149
Genetic distance and percentage (%)of similarities	152
Screening of <i>rfaZ</i> gene by Dot Blot Technique	154
Amplification and isolation of <i>rfaZ</i> gene	156
Detection of <i>rfaZ</i> gene using probe method	158
<b>6 CONCLUSION</b>	<b>160</b>
<b>REFERENCES</b>	<b>163</b>
<b>APPENDICES</b>	<b>184</b>
<b>VITA</b>	<b>198</b>



## LIST OF TABLES

		<b>Page</b>
1	Primers used in RAPD amplification	75
2	The concentration and purity values of the DNA of <i>V. alginolyticus</i> isolates.	111
3	Value of genetic distances and percentage of similarity between 10 <i>V. alginolyticus</i> isolates based on RAPD data.	122



## LIST OF FIGURES

		<b>Page</b>
1	Electron micrograph of <i>V. alginolyticus</i> grown in liquid medium showing the sheathed polar flagellum (arrow). Shadowed preparation (X13,000) (Golten and Scheffer, 1975).	24
2	Diagram showing the lipopolysaccharide (LPS) layer in the outer membrane of gram-negative bacterium (Thomas <i>et al.</i> , 1997)	28
3	Structure of a typical gram-negative lipopolysaccharide (LPS, endotoxin). Lipid A is inserted into the bacterial outer membrane. The core and O-antigen portions extend outward from the bacterium cell surface (Abigail and Dixie, 1994).	28
4	Schematic diagram showing gene amplification of target DNA using the polymerase chain reaction (PCR)	38
5	Schematic diagram of the dot blot technique and the detection of target sequence using Chemiluminescent Detection Kit (New England Biolabs, USA).	45
6	Set-up of Southern blotting and detection procedure.	47
7	Accumulative mortality (%) of seabass after exposure to 5 <i>V. alginolyticus</i> isolates with different cell concentrations.	91
8	Scanning electron micrograph of non-infected lamellae of the gills.	98
9	Scanning electron micrograph of infected fish with swollen lamellae of the gills.	98
10	Scanning electron micrograph showing the clubbing gill lamellae.	99
11	Scanning electron micrograph showing the production of mucus on the surfaces of the gill lamellae.	99
12	Transmission electron micrograph of gill showing neutrophils beneath epithelial cells.	100
13	Transmission electron micrograph of the liver showing the dilatation of cristae and condensation of mitochondria.	100
14	Transmission electron micrograph of the spleen showing large numbers of bacteria within splenic ellipsoids.	101



15	Transmission electron micrograph of the kidney showing some alterations of rough endoplasmic reticulum, degenerated mitochondria with disorganized and loss of cristae, darkly stained and shrunken nucleous. Note also distension of perinucleous space.	101
16	Transmission electron micrograph of the muscle showing decomposition of cytoplasm and abnormal mitochondria.	102
17	Transmission electron micrograph of the muscles showing swelling of mitochondria and fibrous filaments.	102
18	Transmission electron micrograph of a negatively stained <i>V. alginolyticus</i> with sheathed single lateral flagellum.	103
19	Comparative LPS profiles of <i>V. alginolyticus</i> isolates 14, 17, 26, 78, ATCC 17749, <i>V. cholerae</i> serotype Inaba 569B and <i>Salmonella typhimurium</i> on SDS-polyacrylamide gel.	104
20	Graph showing mean antibody titer of juvenile seabass intraperitoneally injected with lipopolysaccharides (200 µg/fish) of <i>V. alginolyticus</i> (isolates 17, 26 and 78) and unvaccinated control fish.	106
21	Graph showing mean antibody titer of juvenile seabass intraperitoneally injected with 0.1 ml/fish of $11.9 \times 10^6$ CFU/ml formalin-killed cells (FKC) of <i>V. alginolyticus</i> (17, 26 and 78) and unvaccinated control fish.	107
22	Mean antibody titer of juvenile seabass immersed in $11.9 \times 10^6$ CFU/ml formalin-killed whole cells (FKC) of <i>V. alginolyticus</i> (17, 26 and 78) for 15 minutes and unvaccinated control fish.	107
23	The survival rates of vaccinated and unvaccinated control seabass following challenged by immersion with virulent <i>V. alginolyticus</i> ( $11.9 \times 10^6$ CFU/ml) for 15 minutes.	110
24	Electrophoretic profile of the total genomic DNA extracted from ten isolates of <i>V. alginolyticus</i> using QIAGEN QIAamp Tissue kit on 1% agarose gel.	111
25	RAPD banding profiles generated by primer OPAE-01 (5'-TGAGGGCCGT-3').	113
26	RAPD banding profiles generated by primer OPAE-03 (5'-CATAGAGCGG-3').	113



27	RAPD banding profiles generated by primer OPAE-04 (5'-CCAGCACTTC-3').	114
28	RAPD banding profiles generated by primer OPAE-05 (5'-TGAGGGCCGT-3').	114
29	RAPD banding profiles generated by primer OPAE-07 (5'-GTGTCAGTGG-3').	115
30	RAPD banding profiles generated by primer OPAE-08 (5'-CTGGCTCAGA-3').	115
31	RAPD banding profiles generated by primer OPAE-09 (5'-TGCCACGAGG-3').	116
32	RAPD banding profiles generated by primer OPAE-10 (5'-CTGAAGCGCA-3').	116
33	RAPD banding profiles generated by primer OPAE-11 (5'-AAGACCGGGA-3').	117
34	RAPD banding profiles generated by primer OPAE-12 (5'-CCGAGCAATC-3').	117
35	RAPD banding profiles generated by primer OPAE-13 (5'-TGTGGACTGG-3').	118
36	RAPD banding profiles generated by primer OPAE-15 (5'-TGCCTGGACC-3').	118
37	RAPD banding profiles generated by primer OPAE-16 (5'-TCCGTGCTGA-3').	119
38	RAPD banding profiles generated by primer OPAE-17 (5'-GGCAGGTTCA-3').	119
39	RAPD banding profiles generated by primer OPAE-18 (5'-CTGGTGCTGA-3').	120
40	Dendrogram based on Nei and Li's (1979) genetic distances demonstrating relationships among 10 isolates of <i>V. alginolyticus</i> .	121
41	Dot-blot hybridization analysis for the presence of the lipopolysaccharide biosynthesis gene ( <i>rfaZ</i> gene) of <i>Escherichia coli</i> K-12 in DNA isolated from <i>V. alginolyticus</i> .	123



42	Amplification and isolation of <i>rfaZ</i> gene at the position between 1.0 and 1.5kb on agarose gel.	124
43	Photograph of an X-ray film showing the positive hybridization signal confirming the isolated target gene in all <i>Vibrio alginolyticus</i> isolates and positive control consisting of the DNA of <i>E. coli</i> .	124



## LIST OF PLATES

		<b>Page</b>
1	Experimental aquaria	53
2	Mass culture of bacteria in a 3L flask of Luria Bertanii Broth	56
3	Harvesting of cells using a centrifuge.	56
4	Phenol-water bacterial mixture subjected to centrifugation showing the formation of three layers A) water layer B) phenol layer C) insoluble residue	59
5	The water-phase containing LPS was aliquoted to a sterile bottle	59
6	The combined water extracts containing LPS were dialyzed against distilled water at 40°C under gentle agitation on magnetic stirrer	60
7	Freeze dryer used for LPS lyophilization	60
8	SDS-PAGE Mini Protean II Vertical Slab Gel Apparatus	62
9	Silver Staining Kit (Bio-Rad)	64
10	Intraperitoneal injection vaccination	66
11	Immersion vaccination set up	67
12	Propagation of bacteria in a 100ml conical flask on an orbital shaker.	70
13	UV-Visible Spectrophotometer for DNA quantitation and purity measurement.	73
14	Agarose gel electrophoresis (Scie Plas) set up.	74
15	UV transilluminator and Polaroid camera for visualizing the DNA and photography.	74
16	The bright yellow colonies of <i>V. alginolyticus</i> on the thiosulphate citrate bile-salt sucrose (TCBS) agar containing 50% artificial seawater (ASW).	89



17	Antimicrobial sensitivity test on TSA agar showing that <i>V. alginolyticus</i> is sensitive to 150 µg and resistant to 10 µg of the vibriostat compound 0/129.	89
18	Gram-negative curve rods of <i>V. alginolyticus</i> .	90
19	Infected fish with ulcer on the abraded skin.	92
20	Clinical signs of fish experimentally infected with <i>V. alginolyticus</i> showing haemorrhagic lesions around the eyes, peripheral of operculum, skin and dorsal fin.	93
21	Clinical signs of fish experimentally infected with <i>V. alginolyticus</i> showing excessive mucus production of internal organs and tail rot.	93



## 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 <sub>4</sub>	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 <sub>2</sub> O	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

