DEVELOPMENT OF DELTA AMINO LEVULINIC ACID AUXOTROPHIC OF *VIBRIO CHOLERAE* O1 EL TOR OGAWA

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2005

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BY

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Thesis submitted in fulfillment of the requirements

for the degree of Master of Science

April 2005

DEDICATIONS

I would like to dedicate this thesis to my parents, Mr. Abd Rashid Bin Yusoff and Mrs. Semek Binti Che Stapha. Special dedication to my caring husband, Wan Hashim Bin Wan Mamat and not forget my lovely son, Wan Amirul Hannan. Thank you for your support and encouragement during my research study.

ACKNOWLEDGEMENTS

For their invaluable contribution in the successful completion of the thesis, I would like to express my deepest gratitude to the following:

First and foremost to my supervisor Dr. Manickam Ravichandran whom I cannot thank enough. For his continuous assistance and advice for the duration of this study and for his great patience and effort in developing my knowledge, experience and understanding, especially in the field of research and laboratory management.

To my co-supervisor Prof Dr. Zainul Fadziruddin Zainuddin for his advice and encouragement, especially towards my laboratory experiments. Special thank also to Dr. P. Lalitha for her guidance and encouragement during the research progress.

To my very energetic colleague Mr. Atif Ali for his great assistance both technically and theoretically. Also a special thank you to Mrs. Chan Yean Yean, Mr. Halkimi and Ms. Katriena for their help.

Thank you to USM for giving Pasca Scholarship during my study. Special dedication to Malaysian Government for support this study under long term grant number 304/PPSP/6110257.

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LIST OF ABBREVIATIONS

2X	2 times
AA	Amino acid
AMP	Ampicillin
AMPK	Ampicillin and Kanamycin
AMPKA	Ampicillin, Kanamycin and ALA
ALA	Aminolevulinic acid
bp	Base pair
CTX	Cholera toxin
CTXA	Cholera toxin A subunit
CTXB	Cholera toxin B subunit
CTXΦ	*ctx* phage
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
GFP	Green fluorescent protein
GMO	Genetically modified organism
HCl	Hydrochloric acid
Hg	Mercury
IPTG	Isopropyl-beta-D-thiogalactopyranoside
KA	Kanamycin and ALA
Kan	Kanamycin
kb	kilo base
L	Liter
LB	Luria Bertani
MCS	Multiple cloning site
ml	milliliter
mm	millimeter
min	minute
mRNA	messenger ribonucleic acid
mw	Molecular weight
NaCl	Sodium chloride
nm	Nanometer

NS	Normal Saline
Nt	Nucleotide
^{o}C	Degree centigrade
ORS	Oral Rehydration Solution
ORT	Oral Rehydration Therapy
P	Polymyxin B
PA	Polymyxin B and ALA
PAMPKA	Polymyxin B, Ampicillin, Kanamycin and ALA
PCR	Polymerase Chain Reaction
PK	Polymyxin Band Kanamycin
PKA	Polymyxin, Kanamycin and ALA
PL	Plain
pH	Potential hydrogen ion
RE	Restriction endonuclease
SA	10% sucrose and amino levulenic acid
TAE	Tris/acetate/EDTA (buffer)
Taq	*Thermus aquaticus*
TBE	Tris/borate/EDTA (buffer)
TCBS	Thiosulfate Citrate Bile salt Sucrose
TCBSA	Thiosulfate Citrate Bile salt Sucrose and ALA
TE	Tris/EDTA (buffer)
Tm	Melting temperature
tRNA	transfer ribonucleic acid
u	unit
UV	Ultraviolet
USA	United State of America
V. cholerae	*Vibrio cholerae*
VCUSM3	Δ*hem*A-kan *Vibrio cholerae* El Tor
VCUSM4	Δ*hem*A*/M *Vibrio cholerae* El Tor
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
µm	Micrometer

ABSTRACT

Cholera is an important diarrheal disease in developing countries. WHO estimates that cholera caused 111,575 cases with 1,894 deaths in the year 2003 worldwide. To overcome that problem, a number of cholera vaccine candidates have been developed by mutation or deletion of various genes such as Δ*thy*A and Δ*gln*. However, these auxotrophic strains were leaky and able to grow in the small intestine of experimental animal's *in-vivo*. Objective of the study was to develop an auxotrophic vaccine strains by mutating the housekeeping gene, *hem*A gene in *V. cholerae*. The *hem*A gene codes for glutamyl tRNA reductase. The *hem*A gene plays a major rate-limiting step in delta aminolevulinic acid (ALA). The *hem*A gene was PCR amplified from *V. cholerae* O1 El Tor and cloned into pARO180 vector at *Eco*RI site. To mutate the *hem*A gene, a kanamycin cassette was inserted at the *Bst*XI site. Δ*hem*A-kan was first subcloned into conjugative suicide vector pWM91 which was then, conjugatively transferred into *V. cholerae* O1 El Tor and the mutant obtained was designated as VCUSM3. In order to remove the kanamycin cassette, the *hem*A gene was inserted with GFP gene flanking with *Smi*I site and subcloned onto pWM91. GFP gene was then excised and left a +1 frame shift mutation in *hem*A gene (Δ*hem*A*/M). Δ*hem*A*/M was conjugatively transferred to VCUSM3 and the mutant obtained was designated as VCUSM4. ALA auxotrophy of VCUSM3 and VCUSM4 were confirmed by their growth on ALA supplemented medium. The *hem*A mutants were confirmed by PCR using *hem*A specific primers and by DNA sequencing. Thus in this study ALA auxotrophs of *V. cholerae* O1 El Tor were created by mutating the *hem*A gene.

PEMBANGUNAN AUXOTROPIK DELTA ASID AMINO LEVULINIK PADA *VIBRIO CHOLERAE* O1 EL TOR OGAWA

ABSTRAK

kolera merupakan penyakit cirit-birit yang penting di negara-negara membangun. Pada tahun 2003, WHO menganggarkan sebanyak 111,575 kes kolera dengan 1,894 kematian berlaku di seluruh dunia. Untuk mengatasi masalah ini, beberapa vaksin kolera telah dicipta melalui memutasikan atau mendelesikan berbagai-bagai jenis gen seperti Δ*thy*A dan Δ*gln*. Walaubagaimanapun, strain auxotropik ini didapati berupaya hidup di dalam usus kecil haiwan kajian di makmal. Oleh itu, kajian ini bertujuan untuk membangunkan vaksin auxotropik melalui mutasi gen "*housekeeping*" pada *V. cholerae* iaitu gen *hem*A. Gen *hem*A mengkodkan untuk enzim glutamyl tRNA reductase. Ia berperanan sebagai penentu kadar di dalam pembentukan ALA. Gen *hem*A di PCR amplifikasikan daripada *V. cholerae* O1 El Tor dan diklonkan kedalam vector pARO180 pada kedudukan *Eco*RI. Kaset kanamycin dimasukkan pada kedudukan *Bst*XI untuk memutasikan gen *hem*A. Δ*hem*A-kan disubklonkan kedalam vector pWM91. Seterusnya, ia dipindahkan secara konjugatif kepada *V. cholerae* O1 El Tor, dan menghasilkan VCUSM3. Kaset kanamycin disingkirkan melalui pengantian dengan GFP yang mempunyai *Smi*I pada kedua-dua hujungnya dan disubklonkan ke dalam vector pWM91. "*Frame shift*" +1 terhasil apabila gfp disingkirkan. Mutan VCUSM4 dihasilkan apabila Δ*hem*A*/M dipindahkan secara konjugatif kepada VCUSM3. ALA auxotropik VCUSM3 dan VCUSM4 disahkan melalui pertumbuhan pada medium agar yang dibekalkan dengan ALA. PCR menggunakan primer spesifik terhadap *hem*A dan

analisis penjujukan DNA dilakukan untuk mengesahkan mutan *hem*A. Kajian ini telah berjaya, menghasilkan *V. cholerae* O1 El Tor ALA auxotropik melalui kaedah mutasi pada gen *hem*A.

CHAPTER 1

INTRODUCTION

1.1 Historical Background

There have been seven pandemics of cholera in recorded history. Even though the etiological agents of the first four pandemics are not known since they occurred in the time before such agents could be recognized, the last three pandemics are known to be due to *Vibrio cholera* serogroup O1. The modern history of cholera began in 1817, when cholera spread out of India described as the first of seven pandemics (Pollitzer, 1959). The disease poured out of Bengal, extending far beyond its normal habitat, and attacked large populations in many countries. The first pandemic recorded from 1817 to 1823, reaching China and Japan to the East; to the West, it spread to the shores of the Mediterranean and Zanzibar on the east coast of Africa (Pollitzer, 1959; Snow, 1855). The pandemic ended when the disease completely disappeared from any region outside India. In early 1830, the second pandemic of cholera reached the British Isles, Russia, Poland, Austria, Ireland and France. Cholera was first reintroduced into China in 1840 by British troops transferred from India.

The third pandemic was stated by Pollitzer to have begun in 1852, extending to Persia and Mesopotamia and to Europe. Whereas, the fourth pandemic occurred in 1873 and the outbreak was stopped in 1875. In 1881 the fifth pandemic extensively affected South

America; it caused large epidemics in many countries and was characterized by high mortality in Argentina, Chile and Peru (Laval, 1989). During the fifth pandemic, Robert Koch successfully isolated the causative organism of cholera, referred to as "comma bacilli" (Koch, 1884), from rice water stools of patients in Egypt in 1883 and in India in 1884. The sixth pandemic began in 1899; disease persisted in Eastern Europe until 1923. According to John *et al., (*2000), cholera has been epidemic in Southern Asia for at least 1000 years, but also spread worldwide to cause seven pandemics since 1817.

The seventh pandemic is the most extensive of the pandemics in geographic spread and in duration. The pandemic, which began in 1961 on the island of Sulawesi in Indonesia, spread to other islands, including Java, Sarawak, and Borneo and then to the Philippines, Sabah and Taiwan, thereby affecting the entire Southeast Asian archipelago by the end of 1962 (Kamal, 1974). In 1992, epidemic cholera was reported in Madras and other places in India and in Southern Bangladesh (Cholera Working Group, 1993; Ramamurthy *et al.,* 1993). Although the clinical syndrome was typical of cholera, the causative agent was a *V. cholerae* non-O1 strain, which was later serogrouped as O139 and given the synonym name "Bengal" (Shimada *et al.,* 1993). This new serogroup may represent the etiologic agent of a new eight pandemic of cholera.

The word "Cholera" has been used for over 2,500 years to describe any diarrhea and vomiting, not just that caused by bacteria. There is considerable debate over the origin of the term cholera and which ancient cultures the disease touched. According to the works of Hippocrates, it was believed that the word has been derived from the Greek word "chole" (bile) and "rein" (flow), which means, "flow of bile". Whereas another possible derivation is from the Hebrew, meaning "bad disease" (Kaper *et al*., 1995). Alexander Trallianus, however said that the word had come from "cholades" which

means intestine, as the evacuations were often serous and not bilious (Diman Barua and Greenough, 1992).

1.2 *Vibrio cholerae*

Members of the genus *Vibrio* are Gram negative, nonsporing, and straight or curved rods measuring about 0.5 to 0.8 μm in diameter and 1.4 to 2.1 μm in length (Kay *et al*., 1994). Stephen (2001) described *V. cholerae* as a "comma - shaped" bacterium. Only aerobic or facultatively anaerobic rods with fermentative metabolism have been retained in this genus. Most species are oxidase positive. A biochemical test characteristic between Vibrio cholerae and Enterobacteriaceae was showed in Table 1.1.

The genus *Vibrio* contains more than 50 species, including three species that are frequently isolated from human clinical samples; *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, and four other species that are isolated more rarely; *V. alginolyticus*, *V. fluvialis*, *V. hollisae* and *V. mimicus*. About one third of these species in the genus *Vibrio* are pathogenic for humans. The most pathogenic *Vibrio. spp* are motile by monotrichous or loptrichous flagella which are surrounded by the sheath in liquid media (Kenneth, 2000). On solid media they may synthesize numerous lateral flagella, which are not sheathed. *Vibrio cholerae* grow in alkaline conditions up to a maximum of pH 10 but sensitive to acidic conditions, where most are dying in the stomach, where the pH is 6 or below (Pollitzer, 1959).

Table 1.1 Differential characteristics of selected members of the *Vibrionaceae* and *Enterobacteriaceae* (Kay *et al*., 1994).

Test	Reaction[a]						
	Vibrio cholerae	*Vibrio mimicus*	*Halophilic vibrios*	*Aeromonas hydrophila*	*Aeromonas veronii*	*Plesiomonas shigelloides*	*Enterobacteriaceae*
KIA	K/A	K/A	V	V	K/AG	K/A	V
TSI	A/A	K/A	V	V	A/AG	K/A	V
String	+	+	+[b]	–	–	–	–
Oxidase	+	+	+	+	+	+	–
Gas from glucose	–	–	–[c]	+	+	–	V
Sucrose	+	–	V	V	+	–	V
Lysine	+	+	V	V	+	+	V
Arginine	–	–	V	+	–	+	V
Ornithine	+	+	V	–	+	+	V
VP	V	–	V	V	+	–	V
Growth in 0% NaCl[d]	+	+	–	+	+	+	+
Growth in 1% NaCl[d]	+	+	+	+	+	+	+

a+, positive; a–, negative;V, variable reaction;K, alkaline; A, acid; G, gas produced.
[b] *V, parahaemolyticus*; *V. cincinnatiensis*, and *V. damsela* show variable reactions.
[c] *V. furnissii* and *V. damsela* are variable.
[d] Nutrient broth base.

*Vibrio*s are one of the most common organisms in surface waters of the world. They occur in both marine and freshwater habitats and in association with aquatic animals (Charles *et al*., 1994). Studies of the organism in the environment have found that *vibrio* is able to survive for extended periods in aquatic and estuarine areas. The survival of *V. cholerae* in the environment was thought to be short which less than one week is in seawater (Chavalier *et al*., 1980). However, since *vibrios* are typically marine organisms most species require 2–3% NaCl or a seawater base for optimal growth. Most *vibrios* have relatively simple growth factor requirements and will grow in synthetic media with glucose as a sole source of carbon and energy. *Vibrios* vary in their nutritional versatility, but some species will grow on more than 150 different organic compounds as carbon and energy sources, occupying the same level of metabolic versatility as *Pseudomonas*.

Most pathogenic *vibrio* species are found as part of the autochthonous microbial community in brackish and marine environments in temperate or tropical regions throughout the world. *Vibrio cholerae* and *V. mimicus* have been isolated from fresh water lakes and rivers and from birds and herbivores in areas geographically removed from marine and coastal water (Bockemuchl *et al.,* 1986; Ogg *et al*., 1989; Rhobes *et al.,* 1985 and 1986). The incidence and density of pathogenic *vibrio. spp* decrease significantly as water temperatures fall below 20°C. *Vibrio. spp* also can be found in the water column and surface sediment and are associated with mollusks and crustaceans. *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* are known to be pathogens of humans. Both *Vibrio cholerae* and *Vibrio parahaemolyticus* produce diarrhea, but in ways that are entirely different. *V. parahaemolyticus* is an invasive organism affecting primarily the colon, whereas *V. cholerae* is non invasive and