UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERISTICS OF VIBRIO CHOLERAE 01 FROM MIRI, SARAWAK

MICKY AK VINCENT

MOLECULAR CHARACTERISTICS OF *VIBRIO CHOLERAE* 01 FROM MIRI, SARAWAK

By

MICKY AK VINCENT

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Food Science and Biotechnology
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March 2002

…. MY SOURCES OF STRENGTH & ENCOURAGEMENT….

MY FAITHFUL GOD

MAK

APAK

YEK

NETT & WATT

BABY GIRL & BABY BOY

HOUSEMATES

FRIENDS

…..SHAPERS OF MY LIFE….

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

MOLECULAR CHARACTERISTICS OF *VIBRIO CHOLERAE* 01 FROM MIRI, SARAWAK

By

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March 2002

Chairman : Associate Professor Dr. Son Radu

Faculty : Food Science and Biotechnology

The purpose of this case study was to evaluate the molecular characteristics of *Vibrio cholerae* 01 isolated during the biggest cholerae outbreak in Miri between November 1997 to April 1998. A total of 33 strains were examined. The randomly selected strains from over 1,000 fecal samples were studied for their antibiotic resistance, the occurrence of plasmids, RAPD-PCR fingerprinting and the presence of the *Ace, Ctxa, Ctxb* and *Zot* genes. This study has shown that all strains were found to be resistant to four or more of the nineteen antibiotics and antimicrobial agents tested with MAR indices ranging between 0.21 to 0.74. These high MAR indexes suggest that all the strains originated from high-risk sources. The isolates exhibited high resistance to bacitracin (96.97%), cefuroxim (96.97%), cephalotin (90.91%), streptomycin (87.88%), rifampin (75.76%) and tetracycline (72.73%). The isolates in this study also demonstrated various degrees of resistance toward other antimicrobial agents used such as, carbenicillin (69.70%), amikacin (57.58%), ampicillin (54.54%), erythromycin (51.52%), nalidixic acid (51.52%), kanamycin (48.48%), oxacillin (33.33%), penicillin G (27.27%), ceftriaxone

(21.21%), gentamycin (21.21%), vancomycin (21.21%) and cefoperazone (3.03%). All strains were, however, susceptible to chloramphenicol. According to the plasmid profile analysis, only one plasmid pattern was observed among the plasmids harboring isolates with the plasmid DNA bands ranging in sizes from 1.3 to 1.6 megadalton. Randomly amplified polymorphic DNA (RAPD) analysis was used to analyze the genetic differentiation and relatedness of the 33 *Vibrio cholerae* 01 strains, using two arbitrary primers (GEN15003 and GEN15005), after screening a set of 10 primers. The two primers generated polymorphism in all 33 strains, producing typeable and reproducible results. The RAPD profiles revealed a wide variability and high level of DNA sequence diversity within the *Vibrio cholerae* 01 strains tested. This revealed no correlation with the source of isolation. The results from the RAPD-PCR fingerprinting were used to construct a dendrogram. From the dendrogram generated, three main clusters were observed and further subdivided into several subclusters defining the genetic heterogeneity among the isolates. The detection of the specific genes by PCR yielded the following results; 32 of 33 (96.97%) isolates were positive for the *Ace, Ctxa, Ctxb* and *Zot* genes. Only 1 (3.03%) of the isolates exhibited the absences of the respective genes. The observations from all the investigations done on the isolates may indicate that multiple pathogenic strains of *Vibrio cholerae* 01, rather than a single type of infective strain cause these infections during the Miri cholera outbreak of 1997 and 1998.

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

CIRI-CIRI MOLEKULAR *VIBRIO CHOLERAE* 01 DARI MIRI, SARAWAK.

Oleh

MICKY AK VINCENT

Mac 2002

Pengerusi : Professor Madya Dr. Son Radu

Fakulti : Fakulti Sains Makanan dan Bioteknologi

Matlamat kajian ini dijalankan adalah untuk mengkaji ciri-ciri molekular *Vibrio cholerae* 01 yang telah dipencilkan semasa wabak kolera yang terbesar di Miri, Sarawak sepanjang tempoh November, 1997 sehingga April, 1998. Sebanyak 33 pencilan telah diuji. Kesemua pencilan-pencilan ini merupakan kutipan secara rambang daripada sebanyak 1,000 sampel najis dan telah dikaji untuk kerintangan terhadap antibiotik dan agen antimikrobial, kehadiran plasmid, pencirian RAPD-PCR serta kewujudan gen-gen *Ace, Ctxa, Ctxb* and *Zot.* Hasil kajian ini telah mendapati bahawa kesemuan pencilan mempunyai kerintangan terhadap empat atau lebih antibiotik serta agen antimikrobial yang telah diuji dengan nilai MAR dari julat 0.21 ke 0.74. Nilai-nilai MAR yang tinggi ini mencadangkan bahawa semua pencilan ini berasal dari sumber berisiko tinggi. Pencilan-pencilan ini menunjukkan kerintangan yang tinggi terhadap bacitracin (96.97%), cefuroxim (96.97%), cephalotin (90.91%), streptomycin (87.88%), rifampin (75.76%) dan tetracycline (72.73%). Pencilan-pencilan ini juga didapati menunjukkan pelbagai tahap kerintangan terhadap lain-lain agen antimikrobial dan antibiotik yang diuji seperti carbenicillin (69.70%), amikacin (57.58%), ampicillin (54.54%), erythromycin (51.52%),

nalidixic acid (51.52%), kanamycin (48.48%), oxacillin (33.33%), penicillin G (27.27%), ceftriaxone (21.21%), gentamycin (21.21%), vancomycin (21.21%) dan cefoperazone (3.03%). Walau bagaimanapun, kesemua pencilan ini adalah sensitif terhadap chloramphenicol. Menurut hasil daripada kajian terhadap kehadiran plamid, hanya satu pola yang diperolehi yang menunjukkan kehadiran dua plasmid bersaiz 1.3 dan 1.6 Megadalton. Analisis RAPD (Randomly amplified polymorphic DNA) telah dijalankan untuk mengkaji perbezaaan serta kesamaan genetik ke atas kesemua 33 pencilan dengan menggunakan dua primer rawak (GEN15003 dan GEN 15005) setelah menguji set sebanyak 10 primer. Kedua-dua primer yang telah digunakan ini telah menunjukkan polimorfisma terhadap kesemua 33 pencilan yang diuji dengan mendapat keputusan yang boleh-percaya dan dapat dihasilkan semula. Profil RAPD menunjukkan variasi yang pelbagai dan corak diversiti yang tinggi di antara pencilan *V. cholerae* 01 yang telah diuji. Oleh sebab itu, tiada korelasi dapat dikaitkan dengan sumber pencilan. Keputusan daripada ujian RAPD telah digunakan untuk menghasilkan sebuah dendrogram. Daripada dendrogram yang telah dibina, tiga kumpulan utama telah didapati sebelum pembahagian kepada sub-divisi yang lebih kecil yang menunjukkan kepelbagaian genetik di antara pencilan-pencilan. Kajian terhadap kehadiran gen-gen yang spesifik pula telah mendapati bahawa 32 daripada 33 (96.97%) pencilan adalah positif terhadap kehadiran gen *Ace, Ctx*a, *Ctx*b dan *Zot*. Hanya 1 (3.03%) pencilan yang menunjukkan ketiadaan keempat-empat gen yang diuji. Kajian yang telah dibuat ke atas kesemua pencilan telah menunjukkan bahawa strain *V. cholerae* 01 yang mempunyai kepelbagai sifat patogenik dipercayai telah menyebabkan wabak kolera di Miri, Sarawak pada tahun 1997 and 1998.

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God Is Good…. His Mercies Endureth Forever !!!

I start my acknowlegdement with one of the greatest phrase I've known to be true to reflect on The One who has always been faithful..

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God Is Good…. His Mercies Endureth Forever !!!

Approval Sheet No.1.

I certify that an Examination Committee has met on 19th February, 2002 to conduct the final examination of Micky Ak Vincent on his Master of Science thesis entitled "Molecular Characteristics of *Vibrio cholerae* 01 from Miri, Sarawak." in accordance with Universiti Putra Malaysia (Higher Degree) Act of 1980 and Universiti Putra Malaysia (Higher Degree) Regulations 1981. The Committee recommended that the candidate be awarded the relevant degree. The Committee Members for the candidate are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and was accepted as fulfillment of the requirements for the degree of Master of Science.

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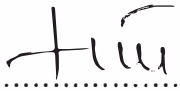
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously of concurrently submitted for any other degree at UPM or other institutions.



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Candidate
Micky Ak. Vincent
20 March, 2002

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Abbreviations

Amp	Ampicillin
B	Bacitracin
bp	base pair
C	Chloramphenicol
Cb	Carbenicillin
Cfp	Cefoperazone
Cro	Ceftriaxone
Cxm	Cefuroxim
ccc	covalently closed circular
Da	Dalton (unit of molecular mass)
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
E	Erythromycin
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
G	gram
Gm	Gentamycin
i.e.	that/example
K	Kanamycin
kbp	kilobase pair
Kf	Cephalotin
LB	Luria Bertani
M	Molar or molarity (moles of solute per liter of solution)
MDa	Megadalton
ml	milliliter
mm	millimeter
mM	millimolar
µg	microgram
µl	microliter
mol	mole
Na	Nalidixic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Ox	Oxacillin
P	Penicillin G
Psi	Pound(s) per square inch (lb/in^2)
R	Resistant
Ra	Rifampin
RAPD	Randomly Amplified Polymorphic DNA
RNA	Ribonucleic acid
Rpm	revolution per minute
s	sensitive

S	Streptomycin
sdH2O	sterile distilled water
SDS	Sodium dodecyl sulphate
Taq	Thermus aquaticus DNA (polymerase)
TBE	Tris-Borate EDTA electrophoresis buffer
Te	Tetracycline
Tris	Tris (hydroxymethyl) methylamine
UV	ultraviolet
V	volts
Va	Vancomycin

CHAPTER 1

INTRODUCTION

Vibrio cholerae is the bacterium that causes cholera, a potentially epidemic and life-threatening secretory diarrhea characterized by numerous, voluminous watery stools, often accompanied by vomiting, and resulting in hypovolemic shock and acidosis, and sometimes muscle cramps (Son *et al.*, 1999; Heilpern and Waldor, 2000). Cholera is an ancient disease, caused by certain members of the species *Vibrio cholerae* that can also cause mild or inapparent infections. The World Health Organization describes cholera as a tragedy because this theoretically "most preventable disease" is one of the top causes of human morbidity and mortality in the world in many areas of Asia, Africa, and Latin America (Osawa *et al.*, 1997). The incidence of cholera is estimated to exceed five million cases each year (Jiang *et al.*, 2000).

Vibrio cholerae is a natural inhabitant of aquatic environments. It is a Gram-negative bacterium that belongs to the subdivision of the family *Proteobacteriaceae*, from the genus of *Vibrio* and the family of *Vibrionacea*, (Trucksis *et al.*, 1998; Beltrán *et al.*, 1999; Walia *et al.*, 1999). Vibrios are one of the most common organisms in surface waters of the world, occurring in both marine and freshwater habitats and in associations with aquatic animals, often with a variety of algae and crustaceans (Byun *et al.*, 1999). This bacterium, in its extreme manifestation, can cause one of the most rapidly fatal diarrheal known. Cholera by itself has contributed to millions of death worldwide ever since it was first recorded. Outbreaks of cholera caused death are estimated at 120,00 worldwide annually, affecting mainly children (Faruque *et al.,* 1998). A healthy person may become hypotensive within an hour of the onset of symptoms and may die within 2-3 hours if no

treatment is provided. More commonly, the disease progresses from the first liquid stool to shock in 4-12 hours, with death following in 18 hours to several days later (Merson *et al.*, 1978). Untreated cholera frequently results in high mortality rates (50-60%).

Records of cholera pandemics started in 1817. However, incidences of cholera or at least cholera like outbreaks is not new nor is it just discovered during the modern-time era. Descriptions dating back to pre-Christian calendar (B.C.) have been found written in Sanskrit (~500 to 400 B.C.) from Sushruta Samshita in India. In fact, cholera existed in India for centuries without leaving the subcontinent until 1817. Gaspar Correa wrote in 1503, that when Vasco da Gama landed on the southwestern coast of India in 1498, about 20,000 men of Calicut died of "a disease which struck them sudden-like in the belly, so that some of them died in 8 hours" (Colwell, 1996).

From an epidemiological standpoint, the species has been divided into serogroup O1 and serogroup non-O1 strains, which were long believed to differ in ability to cause epidemic cholera. Historically, O1 strains have been responsible for all major epidemics, including seven pandemics (Beltrán *et al.,* 1999). *V. cholerae* has the ability to cause global epidemics, or pandemics. It is believed that the first six cholera pandemics were caused by the classical *V. cholerae* biotype, while the seventh pandemic was caused by the El Tor biotype (Kimsey and Waldor, 1998; Provenzano *et al.,* 2000). The first pandemic is believed to have been fairly limited in its scope, being only about 7 years in duration (1817 – 1823). This first pandemic was related to two wars at the time – the Oman war and the war between Persia and Turkey (Colwell, 1996). The second pandemic is believed to have started in Russia in 1829 and before ending its reign in 1851, spreaded to the Americas, British Isles in 1830-1849 in London due to the mixing of drinking water and sewage waste in the streets of London (Faruque *et al.,* 1998). The person responsible

for the stopping of the London outbreak was Dr. John Snow who was also the first person to connect the disease's spread through unsanitized drinking water (Merson *et al.*, 1978). His action was an important understanding of the epidemiology of cholera. During the third pandemic which lasted from 1852- 1859, records showed that the illness was rampant in the United States where it was known to be hitting cities and towns along the Mississippi, Missouri and Ohio rivers until the outbreak ended in 1870's during the end of the forth pandemic (Faruque *et al.*, 1998). The fifth pandemic was marked by high incidences of death in Argentina, Chile and Peru when it spreaded to South America. It was during the fifth pandemic that Koch first isolated what he then referred to as "comma bacilli" from stools of patients between 1883 and 1884 in Egypt and India respectively. The sixth pandemic was presumably caused by *V. cholera* of the classical type marking its reappearance as a large outbreak in Egypt. It was also recorded that during the 1899–1923 pandemic, south and Southeast Asia, and also East and the Balkan peninsular was also affected by the disease (Colwell, 1996).

The seventh pandemic was initiated by the emergence of the "El Tor" biotype (distinguished from the classical type by production of hemolysins) in 1961 on the island of Sulewasi in Indonesia, also producing a major epidemic in the Philipines. By the end of 1962, it had affected the entire Southeast Asian Archipelago spreading like a storm to Borneo, Java and Taiwan. This particular pandemic is the most extensive of all the pandemics in geographical spread and duration (Faruque *et al*, 1998).

Until recently, cholera outbreaks have been attributed only to *Vibrio cholera* 01 of the *Vibrio* species, but in October 1992, epidemic cholera reported in Madras and other places in India and in Southern Bangladesh shocked the world by the appearance of a new cholera sinister (Karaolis *et al.*, 1995; Beltran *et al.*, 1999). This *V. cholera*e non-01

serogroup spreaded like wild fire in Bangladesh to the entire country by December 1992, with clinical syndrome typical that of cholera, affecting thousands of people, mainly adults and causing many deaths (Albert *et al.,* 1993; Boyd and Waldor, 1999). This new serogroup of *V. cholerae* was defined as 0139, with the synonym Bengal, to indicate its first isolation from coastal areas of the Bay of Bengal. Diarrhea cases due to this new serogroup then started to spread to other countries like Pakistan, Nepal, China, Thailand, Kazakhstan, Afghanistan and Malaysia with more reports from the United Kingdom and the United States. Epidemiologists believe that if outbreaks due to this new serogroup should affect more countries, this could initiate the eighth pandemic (Faruque *et al.*, 1998b).

Humans apparently are the only natural host for the cholera vibrios (Merson *et al.*, 1978). Cholera is acquired by the ingestion of water or food contaminated with the feces of an infected individual. After oral ingestion of contaminated food or water and even person-to-person transmission, this bacterium colonizes the human small intestine where it secretes a cholera enterotoxin (CT) (Blake *et al.*, 1980; De Paola, 1981; Jafrul *et al.*, 1994; Kimsey and Waldor, 1998). In fact, the pathogenicity of cholera is mainly associated with their ability to produce this toxin, which is encoded in its "virulence cassette" region of the chromosome that consists of the *Ace, Ctxa, Ctxb and Zot* genes (Trucksis *et al.*, 1993). Since not all *Vibrio cholerae* or *Vibrio cholerae* 01 are toxigenic, regular surveillance and examination of isolates for their potential to produce CT are needed to obtain a clearer picture of the public health hazard caused by toxigenic strains (Almeida *et al.,* 1990). Recently, CT was found to be encoded in the genome of an unusual lysogenic filamentous phage, name CTXϕ (Boyd and Waldor, 1999).

Malaysia is no exception for cholera outbreak cases. The period of 1991 to 1994 has seen serious outbreaks of the disease (Mahalingam *et al.*, 1994). In order to maintain the health security of our land, evolutionary and epidemiology research has to be carried out continuously either in the present or the future. Even if there were no emergence of new mutated strains, this effort is worthwhile since every year there are still sporadic outbreaks of cholera cases and there are trends of increasing cholera cases in recent years. This is proven from the fact that increased trend had been observed from various areas in Peninsular Malaysia that are well known for cholera outbreak cases. Penang, for instance, until the month of May in 1996, a total of 476 cases of cholera cases had been reported. Another report came from Shah Alam, Selangor, where in 1998 about 10 outbreak of cholera cases were reported. A cholera outbreak in an army camp in the month of December the same year had later been confirmed to be caused by the new mutated strain, *V. cholerae* serotype 0139. A statistics made by the Minisitry of Health from the year 1995 to 1999 reported a total of 5,915 cholera cases with 62 fatalities (Appendices). Hence, few alarming trends have shown the great importance of studies being conducted for *V. cholerae* in Malaysia. Various reports had also indicated sporadic cases of cholera in Kelantan, Putra Jaya and Kajang in the year 2000. This latest outbreak of cholera in Kota Baru, Kelantan started on the 26th of September, 2000, reaching Machang, Pasir Mas on the 8th of October, 2000 with about 180 reported infections.

All this while, identification and characterization of bacterial strains have been done with the more conventional method of biotyping, namely on the biochemical characteristics and serologic identification (Mahalingam *et al.*, 1994). But sceptics had proven that with the conventional methods, there are bound to be some loopholes that cause inaccuracies in studies. These are especially true, with the method focusing in detecting more on the phenotypic outlooks of bacteria strains. This would prove costly

since phenotypic differences would only reveal part of the full potential characterization of a certain bacteria strain. In addition, most biotyping methods have shown to be very tedious and time consuming. The recent global resurgence of cholera underscores the urgent need for effective and rapid epidemiological surveillance. Epidemiologic investigation of cholera requires the characterization of *V. cholerae* isolates by typing systems, which also allow determination of isolates relatedness.

Recently, various molecular biology-based techniques have been used to study the relationships among clinical and environmental isolate. Molecular tools have been considered as an alternative tools especially with the creation of Polymerase Chain Reaction (PCR) by Kary Mullis in 1987. Molecular tools are considered to be characterising or determining strains from the most basic structure of all living beings which is the genetic structure. Creation of PCR technique have improved in the specification, amplification and shortening the time factor of molecular techniques. Together with these molecular tools, traditional and molecular techniques has created a whole new armada of bacterial typing fleet which include randomly amplified polymorphic DNA (RAPD) (Yuherman *et al.,* 1998), multilocus enzyme electrophoresis (MLEE) (Beltran *et al.,* 1999), pulsed-field gel electrophoresis (PFGE) (Cameron *et al.*, 1994; Mahalingam *et al.* 1994), ribotyping (Popovic *et al.*, 1993) and amplified fragment length polymorphism fingerprinting (ALFP) (Jiang *et al.,* 2000). All these tools had differentiated isolates of the *V. cholerae* population into different electrophoretic (ETs or zymovars), PFGE types, ribotypes, ALFP types and RAPD fingerprint types, respectively. Applications of these molecular tools has shown the existence within the *V. cholerae* population of several pathogenic clones (Byun *et al.,* 1999).

It is now common to use rapid, practical and economical phenotypic and genotypic techniques for the characterization of organisms, and among them plasmid profiling, antibiotic resistance patterns, randomly amplified polymorphic DNA (RAPD) and specific PCR-based assays. A rapid diagnosis of cholera is important both for the immediate management of the patient with severe diarrhea and for epidemiological tracking. Hence, this research work done on *V. cholerae* 01 isolates from Miri, Sarawak has included molecular tools as part of the characterization criteria. In the work reported here, *V. cholerae* 01 isolated from the biggest and longest cholera outbreak from November 1997 to April 1998 in Miri, Sarawak were characterized by antibiotic resistance, plasmid profiling, randomly amplified polymorphic DNA (RAPD) and detection of the cholera toxin (CT) gene (consisting of the detecting *Ace, Ctxa, Ctxb and Zot* genes) by using specific primers in PCR.