MOLECULAR STUDIES OF VIBRIO CHOLERAE STRAINS ISOLATED FROM LOCAL OUTBREAK

WAN SOMARNY BT. WAN MD. ZAIN

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MOLECULAR STUDIES OF *VIBRIO CHOLERAE* STRAINS ISOLATED FROM LOCAL OUTBREAK

By

WAN SOMARNY BT. WAN MD. ZAIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirement for the Degree of Master of Science

July 2003
To Daddy, Mummy, sisters, brothers, in laws, and beloved husband,
Words cannot express how much I love you all and how blessed I am to have you in my life. Thank you for seeing me through with your loves, strengths and prayers.

To my friends:

We are not lovers
because of the love
we make
but the love
we have

We are not friends
because of the laughs
we spend
but the tears
we save

I don't want to be near you
for the thoughts we share
but the words we never have
to speak

I will never miss you
because of what we do
but what we are
together

“Thanks for Everything”
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

MOLECULAR STUDIES OF VIBRIO CHOLERAE STRAINS ISOLATED FROM LOCAL OUTBREAK

By

WAN SOMARNY BT WAN MD. ZAIN

July 2003

Chairman : Associate Professor Mariana Nor Bt. Shamsudin, Ph.D.

Faculty : Medicine and Health Sciences

Cholera caused by toxigenic Vibrio cholerae is a major public health problem in developing countries. Epidemiology surveillance of cholera and comparative analysis of strains collected during outbreaks has demonstrated clonal diversity among epidemic strains and a continual emergence of new clones of toxigenic V. cholerae. In the present study, the Random Amplified polymorphic DNA (RAPD) technique was performed to study the clonal diversity on twenty isolates of V. cholerae and five isolates of V. harveyi (as a comparison). Eighteen of V. cholerae isolates belong to the Ogawa serotypes, while two isolates were Inaba and 0139 Bengal serotypes, respectively. Fourteen out of twenty random primers yielded clear and reproducible bands. From the RAPD banding profiles, the polymorphism rates of bands were much higher between V. cholerae and V. harveyi than those found among V. cholerae isolates. According to the dendrogram generated from the RAPDistance software program,
*V. cholerae* and *V. harveyi* isolates were distinctly separated into their own groups. The Nei and Li’s genetic distance obtained in this study ranged from 0.024691 - 0.644860 among *V. cholerae* isolates and 0.25368 - 0.633028 among *V. harveyi* isolates. The percentage of similarity among all *V. cholerae* isolates ranged from 32.5% to 99.4% and among *V. harveyi* isolates ranged from 32.5% to 73.0%. Analysis of RAPD bands using GEL COMPAR software at 50% similarity level could distinguish these isolates as well as Inaba and Bengal serotypes.

In this study, the Accessory cholerae enterotoxin (*ace*) gene, the third toxin of the *V. cholerae* virulence cassette was successfully amplified and isolated from 15 out of 20 local outbreak isolates of *V. cholerae*. However, this gene could not be amplified from the DNA in any of the five *V. harveyi* isolates. The amplification of *ace* gene produced a single band of 314 bp. In addition, the lipopolysaccharide (LPS) biosynthesis gene, *rfaz* was also successfully amplified in all *V. harveyi* isolates and 7 of the *V. cholerae* isolates whereby a single fragment of 1.2 kb was amplified. The specificity of the amplified products, *ace* and *rfaz* genes were then confirmed by the Southern hybridization technique using Ace and ZB3 biotinylated probes. The Southern hybridization results showed that all the PCR products amplified by *AceI/AceII* and *ZPf/ZPr* primers are specific to Ace and ZB3 probes, respectively.
In order to allow the production of large quantities of the DNA fragment for physical and biology analysis, the ace and rfaZ genes were cloned into 2.1 TOPO TA vector and were transformed into TOP10, E. coli host strain. From the sequencing analysis using BLASTN package, several mutations were found in the sequence, resulting frameshift mutation. Due to the mutation in the ace gene sequence in 2.1 TOPO TA vector, a new expression vector pBAD/Thio TOPO was chosen to clone the ace gene. Since the gene was found to be toxic, the positive construct, pBAD10/ace2 (E. coli TOP10 as a host strain) was retransformed into another E. coli host strain, LMG194 which produced a new construct, pBADLMG/ace2. Both of the constructs, pBAD10/ace2 and pBADLMG/ace2 were successfully transcribed by the detection of the ace gene in mRNA samples by Reverse transcriptase-PCR (RT-PCR). However, the pBAD10/ace2 construct failed to express the Ace protein. On the other hand, the pBADLMG/ace2 construct successfully expressed the soluble Ace protein even though there was an addition of two bases, T and G after the first ATG in the ace gene sequence. Analysis using the Biology Workbench 3.2 showed that the start codon is GTG, encoding the amino acid valine instead of methionine. The Ace fusion protein with the expected size of approximately 34 kDa was expressed after 72 hours induction with 0.02% arabinose.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KAJIAN MOLEKULAR KE ATAS VIBRIO CHOLERAE STRAIN PENCILAN DARI WABAK TEMPATAN

Oleh

WAN SOMARNY BT WAN MD. ZAIN

July 2003

Pengerusi: Profesor Madya Mariana Nor Shamsudin, Ph.D.

Fakulti: Perubatan dan Sains Kesihatan

Penyakit kolera yang disebabkan oleh Vibrio cholerae yang toksigenik merupakan masalah kesihatan yang utama di negara membangun. Kajian epidemiologi kolera dan analisa perbandingan strain yang dikumpul sepanjang wabak menunjukkan kepelbagaian klonal terhadap strain epidemic dan kewujudan klon baru V. cholerae yang patogenik secara berterusan. Dalam kajian ini, teknik DNA polimorifik menggunakan primer rawak (RAPD) diaplikasikan untuk mengkaji kepelbagaian klonal bagi 20 isolat V. cholerae dan 5 isolat V. harveyi (sebagai perbandingan). Lapan belas isolat V. cholerae merupakan serotip Ogawa, sementara dua isolat yang masing-masing merupakan serotip Inaba dan 0139 Bengal. Empat belas daripada duapuluhr primer rawak menghasilkan jalur yang terang dan kebolehulangan. Dari profil jalur RAPD kadar polimorfisma adalah lebih tinggi di antara V. cholerae dan V. harveyi berbanding yang ditemui di antara isolate V. cholerae. Merujuk kepada dendrogram yang dihasilkan dari

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program RAPDistance, isolat \textit{V. cholerae} dan \textit{V. harveyi} dibahagikan kepada kumpulan mereka tersendiri. Jarak genetik Nei dan Li yang diperolehi dari kajian ini, berjulat di antara 0.024691 – 0.644869 iaitu di antara isolat \textit{V. cholerae} dan 0.025368 – 0.633028 bagi isolate \textit{V. harveyi}. Peratus kesamaan di antara isolat \textit{V. cholerae} adalah berjulat 32.5% hingga 99.4% manakala antara isolat \textit{V. harveyi} adalah berjulat dari 32.5% hingga 73.0%. Analisis jalur RAPD dengan menggunakan software GEL COMPAR menunjukkan nilai kesamaan pada tahap 50% berupaya untuk membezakan isolat – isolat ini dan begitu juga serotip Inaba dan Bengal.

Dalam kajian ini, gen \textit{ace} iaitu yang merupakan toksin ketiga bagi virulen kaset \textit{V. cholerae} telah berjaya diamplifikasi dan diisolat iaitu sebanyak 15 dari 20 keseluruhan isolat serangan tempatan bagi \textit{V. cholerae}. Namun begitu, gen ini tidak diamplifikasi dari DNA kelima – lima isolat \textit{V. harveyi}. Amplifikasi gen \textit{ace} menghasilkan satu jalur tunggal pada 314 bp. Di samping itu, gen biosintesis lipopolisakarida (LPS), \textit{rfaZ} juga berjaya diamplifikasi dalam kesemua isolat \textit{V. harveyi} dan 7 isolat \textit{V. cholerae} di mana satu jalur tunggal pada 1.2 kb telah diamplifikasi. Spesifikasi terhadap produk amplifikasi, gen \textit{ace} dan \textit{rfaZ} kemudiannnya dipastikan dengan teknik ‘Southern hybridization’ menggunakan probe Ace dan ZB3 yang dilabelkan dengan biotin. Keputusan ‘Southern hybridization’ menunjukkan kesemua produk PCR yang diamplifikasi oleh primer \textit{Acell/AceII} dan \textit{ZPf/ZPr} adalah spesifik terhadap probe Ace dan ZB3.
Untuk membenarkan penghasilan jalur DNA dalam kuantiti yang banyak untuk analisis fizikal dan biologi, gen ace dan rfaZ telah diklonkan dalam 2.1 TOPO TA vektor dan ditransformasikan ke dalam TOP10, strain perumah E. coli. Daripada analisi penjukan dengan menggunakan pakej BLASTN, terdapat beberapa mutasi telah ditemui dalam jujukan yang menghasilkan mutasi ‘frameshift’. Berdasarkan kepada mutasi dalam jujukan gen ace di dalam vector 2.1 TOPO TA, satu vektor ekspresi baru iaitu pBAD/Thio TOPO telah dipilih untuk pengklonan gen ace. Memandangkan gen tersebut adalah toksik, pembentukan positif pBAD10/ace2 telah ditransformasi sekali lagi ke dalam strain perumah E. coli yang lain iaitu LMG194 di mana pembentukan yang baru dihasilkan iaitu pBADLMG/ace2. Kedua-dua penghasilan ini pBAD10/ace2 dan pBADLMG/ace2 telah berjaya ditranskripsikan dengan pengenalpastian gen ace dalam sampel mRNA dengan menggunakan kaedah transkripsi berbalik – PCR (RT-PCR). Walaubagaimanapun, pembentukan pBAD10/ace2 telah gagal untuk mengekspres protein Ace. Sebaliknya, pembentukan pBADLMG/ace2 telah berjaya mengekspres protein Ace larut walaupun dengan penambahan dua bes, T dan G selepas ATG yang pertama dalam jujukan gen ace. Analisis dengan Biology Workbench 3.2 menunjukkan kodon permulaan ialah GTG yang mengkodkan asid amino valine selain dari methionine. Protein Ace yang bersaiz kira-kira 34 kDa diekspresikan setelah 72 jam selepas menginduksikannya dengan 0.02% arabinose.
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In the name of Allah S.W.T., the most Beneficent, the most Merciful. I would like to express the gratitude to Him, whom granted my ability and willing to start and complete this thesis.

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I certify that an Examination Committee met on 24th July 2003 to conduct the final examination of Wan Somarny bt. Wan Md. Zain on her Master of Science thesis entitled “Molecular Studies of Vibrio cholerae Strains Isolated from Local Outbreak” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that candidate be awarded relevant degree. Members of the Examination Committee are as follows:

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Date: 14 NOV 2003
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 or concurrently submitted for any other degree at UPM or other institutions.

WAN SOM ARNY BT. WAN MD. ZAIN

Date: 10/11/2003
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<td>ace</td>
<td>accessory cholera enterotoxin</td>
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<tr>
<td>AP-PCR</td>
<td>arbitrary primed polymerase chain reaction</td>
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<td>APS</td>
<td>ammonium persulphate</td>
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<td>ASW</td>
<td>artificial sea water</td>
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<td>ATCC</td>
<td>American type culture collection</td>
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<td>bp</td>
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<td>GTP</td>
<td>guanosine 5’-triphosphate</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>H₂O</td>
<td>water</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloride acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IF</td>
<td>initiation factors</td>
</tr>
<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pair</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium cloride</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo daltons</td>
</tr>
<tr>
<td>KDO</td>
<td>2-keto-3-deoxyoctonate</td>
</tr>
<tr>
<td>LB</td>
<td>luria bertani</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium cloride</td>
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xxi
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NJTREE</td>
<td>neighbour joining Tree</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OMPs</td>
<td>outer membrane proteins</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed field gel electrophoresis</td>
</tr>
<tr>
<td>pmoles</td>
<td>picomoles</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random Amplified Polymorphic DNA</td>
</tr>
<tr>
<td>RFLPs</td>
<td>Restriction Fragment Length Polymorphisms</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RS I</td>
<td>repetitive sequence</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-PCR</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBE</td>
<td>tris–borate-EDTA</td>
</tr>
<tr>
<td>TCBS</td>
<td>thiosulphate citrate bile salt sucrose</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>zot</td>
<td>zonula occludens toxin</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
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<tr>
<td>μl</td>
<td>microliter</td>
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CHAPTER ONE

INTRODUCTION

*Vibrio cholerae* is a highly motile gram-negative bacterium and is the causative agent of epidemic cholera. Cholera, a highly epidemic diarrheal disease, is caused by toxin-producing strains of *Vibrio cholerae* 01. *V. cholerae* of serogroup 01 produces a potent heat-labile enterotoxin, termed cholera toxin (CT). Cholera is caused by the action of cholera toxin, CT which through its stimulation of intestinal adenylate cyclase activity gives rise to the characteristic excessive electrolyte secretion and fluid loss from the small intestine (Osek et al., 1992). In its severe form, the disease can lead to fatal diarrhea dehydration and typically occurs in explosive epidemic whereby the small quantities of purified cholera toxin at 5 µg, can cause copious purging when fed to volunteers (Levine et al., 1983). In individuals infected with *V. cholerae*, cholera toxin is responsible for the drastic intestinal electrolyte secretion and fluid loss leading to the clinical state of cholera (Holmgren, 1981; Finkelstein, 1984). *V. cholerae* 01 are classified into two biotypes, classical and *El Tor*, and into two major serotypes, *Inaba* and *Ogawa* (Ghosh et al., 1996) while the Hikojima serotype has been rarely reported (Faruque et al., 1998). *V. cholerae* 01 is defined by agglutination in 0 group 1 in which 0 antigens (somatic antigens) is the target for the specific antiserum directed against the lipopolysaccharide component of the cell wall.
In 1992, cholera was caused by a new serotype 0139 synonym Bengal (Shimada et al., 1993). This serotype is the latest serogroup of *V. cholerae* that emerged in epidemic proportions in India and Bangladesh and is also known as a first non-01 group. *V. cholerae* non-01 serotypes are widely distributed in the aquatic environment and are free-living in nature. This serovar is identified by the absence of agglutination in 0 group 1 specific antiserum (Albert et al., 1993) but agglutinated in 0 group 139 specific antiserum and also by the presence of a capsule (Jonson et al., 1996). *V. cholerae* non-01, were not known to be associated with such a large outbreak of diarrhea before the present of epidemic (Faruque et al., 1994). Moreover, this serotype was known to produce cholera toxin at a very low frequency (Janda et al., 1988). It has often been identified as the causative agent of sporadic cases (Karaolis et al., 1998; Russell et al., 1992) and localized outbreaks (Craig et al., 1981). However, in 1992 a 0139 serogroup strain emerged and caused epidemic disease throughout India and Bangladesh which replaced 01 strains of *V. cholerae* as the predominant cause of cholera on the Indian subcontinent (Albert et al., 1993 and Bhattacharya et al., 1993).

Strains of *V. cholerae* 01 impaired in their ability to colonize the gut are able to produce their symptoms of the disease and elaborate a powerful exotoxin, CT. Cholera toxin (84 kDa) is a fairly complicated molecule which is composed of one A subunit of 27 kDa and five B subunits, each of 11 kDa (Ghosh et al., 1996). Although CT is responsible for severe dehydrating diarrhea associated with *V. cholerae*, the search for the additional enterotoxin produced by