UNIVERSITI PUTRA MALAYSIA

MOLECULAR AND IMMUNOLOGICAL CHARACTERISTICS OF *VIBRIO CHOLERAE* AND *VIBRIO ALGINOLYTICUS*

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By

VASANTHAKUMARI NEELA

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

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Dedicated to my husband and daughter
For their strength and courage.

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

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Chairman: **Dr. Mariana Nor Shamsudin**

Faculty: **Medicine and Health Sciences**

Twenty isolates of *Vibrio cholerae* and four isolates of *Vibrio alginolyticus* used in this study were identified using the conventional biochemical tests. All of the isolates were screened for the zonula occludens toxin gene (*zot*) by the Polymerase Chain Reaction (PCR) technique. The 1083 bp fragment of the *zot* gene was successfully amplified in all the tested isolates. Asymmetry PCR was used as a preliminary detection method and later confirmed through the gene probe method to test for the presence of the *zot* gene. Consequently, the *zot* gene was successfully cloned in pCR 2.1 TOPO vector and sequenced, in which the sequences were 99% homologous to the gene bank sequences.

The genetic distance and the percentage of the similarity between *V. cholerae* and *V. alginolyticus* isolates were investigated using RAPD-PCR. Thirteen 10-mer

oligonucleotide primers of arbitrary sequence were used to amplify the genomic DNA of eleven isolates of *V. cholerae* and four isolates of *V. alginolyticus*. These primers revealed a total of 185 DNA markers with size ranging from 100-10,000 bp. Three primers OPAE-1, 10 and 12 produced a distinct banding pattern for *V. cholerae* isolates. Therefore, these primers were identified as suitable primers to produce genetic markers that could be used as a species-specific diagnostic trait. The overall percentage of the similarity range from 41 to 89% for *V. cholerae* and 21 to 43% for *V.alginolyticus*. In addition, the genetic distance ranged from 0.320513 to 0.710843 for *V. cholerae* and 0.275229 to 0.846774 for *V. alginolyticus*.

The differences of the LPS profile between three strains of *V. cholerae* (Ogawa, Inaba and Bengal) and three strains of *V. alginolyticus* (203, 265 and 1003) were demonstrated in the SDS-PAGE. All the strains except for strain 265 of *V. alginolyticus* were shown to have high molecular weight bands and indicated that the LPS were immunogenic. The effectiveness of the isolated LPS from the three strains of *V. alginolyticus* and one strain of *V. cholerae* (Ogawa) to induce antibody production in rabbits was determined by using passive haemagglutination (PHA) technique.

The study on the molecular and immunological characteristics of *V. cholerae* will definitely contribute towards the development of a DNA vaccine against cholera and the LPS study suggests that the *V. cholerae* isolates are immunogenic indicating the suitability of these isolates as potential vaccine component.

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

CIRI-CIRI IMMUNOLOGI DAN MOLEKUL *VIBRIO CHOLERAE* DAN *VIBRIO ALGINOLYTICUS.*

Oleh

VASANTHAKUMARI NEELA

May 2000

Pengerusi : **Dr. Mariana Nor Shamsudin**

Fakulti : **Perubatan and Sains Kesihatan**

Dua puluh isolat *Vibrio cholerae* dan empat isolat *Vibrio alginolyticus* yang digunakan dalam kajian ini telah dikenalpastikan melalui ujian-ujian biokimia. Gen toxin zonula occludens (*zot*) telah dikesan daripada kesemua isolat menggunakan teknik tindak balas berantai polimerase (PCR). Bahagian *zot* gen bersaiz 1083 bp telah diamplifikasi dengan jayanya dalam kesemua isolat yang diuji. PCR asimetri digunakan sebagai kaedah pengesanan awal dan kemudian disahkan melalui kaedah prob gen untuk mengkaji kehadiran gen *zot*. Seterusnya, gen *zot* diklon dengan jayanya dalam vektor pCR 2.1 TOPO dan dijujukan di mana jujukan tersebut adalah 99% homologus dengan jujukan gen bank.

Jarak genetik dan peratus kesamaan di antara *V. cholerae* dan *V. alginolyticus* isolat telah dikaji menggunakan RAPD-PCR. Tiga belas primer 10-mer oligonukleotid

jujukan rawak telah digunakan untuk mengamplikasikan DNA genomik dari sebelas isolat *V. cholerae* dan empat isolat *V. alginolyticus*. Primer-primer ini telah menghasilkan sejumlah 185 penanda DNA dengan saiz yang berada dalam julat 100 – 10,000 bp. Tiga primer OPAE-1, 10 dan 12 menghasilkan corak jalur yang berbeza bagi *V. cholerae* isolat. Oleh itu, primer-primer ini dikenalpasti sebagai primer yang sesuai untuk menghasilkan penanda genetik yang boleh digunakan sebagai trait diagnostik untuk species yang spesifik. Secara keseluruhannya, peratus kesamaan mempunyai julat dari 41% hingga 89% bagi *V. cholerae* dan 21% hingga 43% bagi *V. alginolyticus*. Sementara, jarak genetik pula adalah berjulat dari 0.320513 hingga 0.710843 bagi *V. cholerae* dan 0.275229 hingga 0.846774 bagi *V. alginolyticus*.

Perbezaan profil LPS di antara tiga strain *V. cholerae* (Ogawa, Inaba dan Bengal) dan tiga *V. alginolyticus*.(203, 265 dan 1003) didemontrasikan menggunakan SDS-PAGE. Kesemua strain kecuali strain 265 *V. alginolyticus* menunjukkan kehadiran jalur yang mempunyai berat molekul yang tinggi dan menunjukkan bahawa LPS adalah immunogenik. Keberkesanan LPS yang diisolat dari tiga strain *V. alginolyticus* dan satu strain *V. cholerae* (Ogawa) dalam merangsangkan pengeluaran antibodi dalam arnab telah ditentukan dengan menggunakan teknik hemaglutinan pasif.

Kajian ke atas molekul dan ciri immunologikal *V. cholerae* akan menyumbangkan ke arah perkembangan DNA vaksin terhadap cholera dan kajian ke atas LPS *V. cholerae* isolat yang immunogenik menunjukkan isolat ini mempunyai potensi yang sesuai sebagai komponen vaksin.

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LIST OF ABBREVIATIONS

Ace	Accessory cholerae toxin
ACF	Accessory colonization factor
AIDS	Acquired Immuno Deficiency Syndrome
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
ASW	Artificial sea water
CA	Cytophaga Agar
CMI	Cellular mediated immune response
HMI	Humoral mediated immune response
LB	Luria Bertanii
LPS	Lipopolysaccharide
OF	Oxidation Fermentation
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PHA	Passive Haemagglutination
RAPD	Random Amplified Polymorphic DNA
RBC	Red blood corpuscles
RFLP	Restriction Fragment Length Polymorphism
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
TCBS	Thiosulphate Citrate Bile salt Sucrose
TSA	Tryptic Soy Agar
TSB	Tryptic Soy broth

VBNC	Viable but non-culturable cells
VNTR	Variable number of tandem repeats
YAC	Yeast artificial chromosome
Zot	Zonula occludens toxin

CHAPTER I

INTRODUCTION

The genus *Vibrio* is the most extensively characterized and medically important bacterial group within the family *Vibrionaceae* and is classified as the "Facultatively Anaerobic Gram Negative Rods". *Vibrio* species which include *Vibrio alginolyticus*, *V. cholerae, V. harveyi, V. parahaemolyticus, V. vulnificus* and others, are commonly found in aquatic environment (Waldor and Mekalanos, 1996). Members of the genus *Vibrio* are short, non-sporing, gram-negative rods that are often curved and actively motile by a single polar flagellum.

Vibrio cholerae is the etiological agent for the severe diarrheal disease cholerae in humans, transmitted by water or food. The actual reservoir of *V. cholerae* is unknown. 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 (Jiang *et al.*, 2000). The incidence of cholera is estimated to exceed five million cases each year (Tauxe *et al.*, 1994).

Historically, 200 serogroups of the *V. cholerae* have been described based on heat stable somatic O-antigen and genetic diversity (Shimada *et al.*, 1994; Yamai *et al.*, 1997)

and is known as O1 antigen group. Strains of other serogroups are collectively known as non-O1 antigen. The members of this O1 antigen group has been traditionally associated with cholerae. The O1 *V. cholerae* strains are further classified into two biotypes, Classical and El Tor and initially into 3 serotypes, Inaba, Ogawa and Hikojima. Apart from Ogawa, Inaba and Hikojima a new serogroup O139 Bengal has been recorded. An epidemic which began in India late in 1992 and spread to several neighboring countries was caused by the O139 Bengal strain (Albert *et al.,* 1993a ; Ramamurthy *et al.*, 1993; Nair *et al.*, 1994). The *V. cholerae* O139 strain proved to be genetically similar to *V. cholerae* 01 and is hypothesized as having evolved from the strain of the early seventh pandemic (Karaolis *et al.,* 1995; Dumontier and Berche, 1998).

Clinically, *V. cholerae* O1 and O139 Bengal strains caused cholerae of comparable severity in infected persons (Morris *et al.*, 1995). However, in striking contrast to O1 strains, O139 strains are encapsulated (Johnson *et al.*, 1994) possessing an O-antigen capsule and lipopolysaccharides (Waldor *et al.*, 1994). The LPS of O1 strains have a core region substituted with an average of 17 repeat limits of 4-NH_2-4, 6-dideoxymannose, each substituted with 3-deoxy-L-glycerol-tetronic acid (Manning *et al.*, 1994). The LPS of O139 strains appears to be an efficiently substituted core polysaccharide with only one or two additional sugar moieties (Weintraub *et al.*, 1994). These changes have made O139 organisms immunologically distinct from the O1 El Tor strains.

Vibrio cholerae produces a thermolabile enterotoxin, cholerae toxin (CTXϕ), whose action on the mucosal epithelium of the intestine is responsible for the disease

cholerae (Rabbani and Greenough, 1990). Although CTXϕ is responsible for severe dehydrating diarrhea associated with *V. cholerae*, the search for additional enterotoxins produced by *V. cholerae*, which has included the study of genetically engineered CTXϕ deleted *V. cholerae*, has led to the discovery of a new toxin (Kaper *et al.*, 1995). The zonula occludens toxin (*zot*) acts on the intestinal tight junctions (zonula occludens) to increase intestinal permeability. The genes encoding *zot* is located immediately next to the CTXϕ gene on a 4.5 kb dynamic region of the *V. cholerae* chromosome termed the core region or virulence cassette (Aidara *et al.*, 1998). The manifestation of this disease is a rapid onset of severe diarrhea or otherwise known as rice watery stool accompanied with vomitting. Untreated patient will succumb to death within 24 hrs after the onset of the symptoms, as a result of severe dehydration and cardiovascular collapse.

In recent years, cholerae has become a global health problem and endemic (a disease constantly present in a given location or geographical region) in South East Asia, Parts of Africa, Latin America and others, where the occurrence of seasonal outbreaks or epidemics (the sudden onset of a disease within a given geographical region, for a limited period of time) are particularly associated with poverty and poor sanitation (Coppo *et al.*, 1995; Echeverria *et al.*, 1995; Tamayo *et al.*, 1997). Seven distinct pandemics (an epidmic affecting large number of people in a major geographical area or worldwide) of cholerae has occurred where the onset of the first pandemic was in 1817 (Pollitzer 1959). The last pandemic began in Sulawesi, Indonesia in 1961 and since then had spread across Asia, affecting 19 countries over several years (Kamal, 1974).

The laboratory diagnosis of cholerae is normally carried out by morphology, physiology, nutrition requirements, antibiotic resistance, biochemical properties, phage sensitivity and more recently by DNA based techniques.

Rapid development in the field of molecular biology has led to an alternative DNA-based procedures for the detection of genetic variation in *Vibrio* species. A Randomly Amplified Polymorphic DNA (RAPD) was developed for the identification and differentiation of different species and strains based on the polymorphisms of the genomic DNA (Welsh and McClelland, 1990). RAPD, also known as Arbitarily Primed Polymerase Chain Reaction (AP-PCR), is a technique based on the PCR principle. This method provides a convenient and rapid assessment of the genetic composition of the particular organisms being studied.

This procedure is unique in which no prior sequence information of the genomic DNA sample is needed and also whole and cell cultures can be used as the source of the template DNA (Joshi *et al.,* 1991). In principle, the method employs a single, short 10-base oligonucleotide primer that binds to any region in the genome bearing the complementary sequence initiating the amplification of specific regions of the genome with the PCR (Welsh and McClelland, 1990; Williams *et al.,* 1990; Brauns *et al*., 1991; Caeteano-Anolles *et al*., 1991; Whitesides and Oliver, 1997).

A specific DNA fragment is produced by one individual but not by another can be represented as a genetic marker (Williams *et al*., 1990). The advantages of rapid

differentiation of strains are with respect to providing any form of treatment to problems such as an infection disease. If the strains were similar and closely related to each other, this would mean similar treatment can be used for all strains.

Even though cholera has been around for a long time and an enormous amount of information is known about this bacterium as well as the disease, still it remains as a public health concern. It is estimated that about 120,000 deaths per annum occur due to the outbreaks of cholerae world wide, of which majority occurs in children (WHO, 1995). The contributing factors could be due to the emergence of antibiotic resistant strains (Satcher, 1995), the clonal diversity of the epidemic strains and third factor is due to the lack of preventive measures Vaccination is an acceptable and widely need method of prevention

The first attempt to develop a cholerae vaccine was done more than 100 years ago by Jaime Ferranclue in 1884 The initial live attenuated vaccine and killed whole cell vaccines (Heyningen, 1983) developed were not successful, since it was less effective against children and also was not effective against more than one serotypes.

During 1980's, oral vaccines consisting of B subunit of cholerae had been developed. It provided short-term protection, where the maximum protection is only for 3 years with several doses. The rate of efficacy decreases from 85% to30% over the 3 years. This makes it unsuitable for use in large-scale vaccination program.

Later, a combination of the whole cells and subunit components like lipopolysaccharides (LPS), protein, toxoids and others were constructed as vaccines. Although this vaccine had shown encouraging results in preliminary animal studies, these vaccine strategies were not successful in preventing outbreaks of cholerae.

Then came the live attenuated vaccine, that induces rapid immunization, as short as 8 days after vaccination. It offers safe protection against classical strains, but was unsuccessful against El Tor types. Recent development of live attenuated vaccine against El Tor showed protective efficacy of about 83% in some of the cases (Tacket *et al.,* 1997).

All of the above factors indicate an immediate need of developing a safe and efficacious vaccine to prevent cholera. This leads to the need of a groundwork study to develop a new DNA based vaccine or Recombinant vaccine for cholerae. Basically, a DNA vaccine consists of a plasmid DNA encoded with an antigenic protein gene. When the gene is introduced into the host cells, the protein will be expressed, thus acts as an immunogen and will trigger an immune response against the antigen (Caldwell, 1997). The main advantage of a DNA vaccine is that both types of immune responses are induced. They are the cellular mediated immune response (CMI) and humoral mediated immune response (HMI). Developing a DNA vaccine against *V. cholerae* is a very feasible choice for the prevention of this disease because of the diversity in the serotypes of the species.