
UNIVERSITI PUTRA MALAYSIA

# DEVELOPMENT OF A DIAGNOSTIC OLIGONUCLEOTIDE DNA PROBE FOR THE RAPID DETECTION OF VIBRIO CHOLERAE O139

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# DEVELOPMENT OF A DIAGNOSTIC OLIGONUCLEOTIDE DNA PROBE FOR THE RAPID DETECTION OF *VIBRIO CHOLERAE* O139

**By**

**KHAW AIK KIA**

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

**December 2001**

**This piece of work is specially dedicated to**

**my father and mother**

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

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*Vibrio cholerae* O139 Bengal emerged as the second etiologic agent of cholera in the Indian subcontinent in late 1992, it then spread to several neighboring countries and also some developed countries. *V. cholerae* O139 Bengal is closely related to *V. cholerae* O1 El Tor strains associated with the seventh pandemic, and it causes a disease which is virtually indistinguishable from cholera caused by *V. cholerae* O1. *V. cholerae* O139 Bengal and *V. cholerae* O1 El Tor share several phenotypic and genotypic properties. However, all the genes of the *rfb* complex which encode the O antigen in *V. cholerae* O1 El Tor have been found deleted in *V. cholerae* O139. In their place, there is a new chromosomal region detected. Based on a published sequence, six set of *V. cholerae* O139 primers have been designed. Primer combination S1-AS2 (5'-AGATGCCGAAGACTATAA-3' and 5'-GAGGAATAAC AACTGAGA-3') was found to be specific for detection of *V. cholerae* O139 in a polymerase chain reaction (PCR) assay, as they produced an amplicon of 520 bp from all tested pure cultures of *V. cholerae* O139 strains but not from 39 pure cultures of other bacteria. The newly designed primer combination has been used to develop a diagnotic kit for the identification of *V. cholerae* O139 in our laboratory.

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

**PENGHASILAN PROBE DIAGNOSTIK OLIGONUKLEOTIDA DNA UNTUK PENGESANAN *VIBRIO CHOLERAE* O139 SECARA PANTAS**

Oleh

**Khaw Aik Kia**

**Disember 2001**

**Pengerusi:** **Profesor Madya Dr. Son Radu, Ph.D.**

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*Vibrio cholerae* O139 Bengal muncul sebagai agen kedua etiologi taun di wilayah India pada akhir tahun 1992. Selepas itu, ia terus merebak ke beberapa negara jirannya dan juga negara-negara membangun. *V. cholerae* O139 Bengal didapati berkait rapat dengan *V. cholerae* O1 El Tor yang menyebabkan wabak taun ketujuh, penyakit taun ini sukar dibezakan daraipada taun yang disebabkan oleh *V. cholerae* O1. *V. cholerae* O139 Bengal dan *V. cholerae* O1 El Tor mempunyai kesamaan dari segi fenotip dan genotip. Namun demikian, kesemua gen kompleks *rfb* yang menyebabkan translasi antigen O dalam *V. cholerae* O1 El Tor didapati lenyap dalam *V. cholerae* O139. Sebagai gantian, bahan kromosom baru dikesan. Berasaskan susunan gen yang diperolehi, enam set primer baru untuk *V. cholerae* O139 telah dihasilkan. Kombinasi primer S1-AS2 (5'-AGATGCCGAAGA CTATAA-3' dan 5'-GAGGAATAAC AACTGAGA-3') didapati sangat spesifik dalam pengesanan *V. cholerae* O139 dengan menggunakan reaksi rantaian polimerase (PCR). Ia telah mengamplifikasikan gen sepanjang 520 bp dalam kajian terhadap *V. cholerae* O139, malah tidak terhadap 39 bakteria yang lain. Kombinasi primer baru telah digunakan dalam makmal kami untuk pengesanan *V. cholerae* O139.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| apw | Alkaline peptone water |
| CAMP | Cyclic adenosine monophosphate |
| CCD | Chemiluminescent and colorimetric detection |
| COAT | Coagglutination test |
| ct | Cholera toxin |
| DDPCR | Differential display polymerase chain reaction |
| dna | Deoxyribonucleic acid |
| dNTPs | Deoxyribonucleoside triphosphates |
| EAggEC | Enteroaggregative *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetate |
| EEO | Electroendosmosis |
| EHEC | Enterohemorrhagic *Escherichia coli* |
| EIEC | Enteroinvasive *Escherichia coli* |
| EPEC | Enteropathogenic *Escherichia coli* |
| ETEC | Enterotoxigenic *Escherichia coli* |
| $\Delta G$ | Free energy |
| GC% | Guanine-Cytosine contents |
| GET | Glucose-EDTA-Tris |
| HCl | Hydrogen chloride |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigens |
| KCl | Potassium chloride |
| lps | Lipopolysaccharide |
| $MgCl_2$ | Magnesium chloride |
| MRS | de Man, Rogosa and Sharpe |
| NAD | Nicotinamide adenine dinucleotide |
| ORS | Oral rehydration solution |
| PCI | Phenol-Chloroform-Isoamyl alcohol |
| PCR | Polymerase chain reaction |
| RACE | Rapid amplification of cDNA ends |
| RAPD | Random amplification polymorphic DNA |
| *rfb* | Reading frame-B |

| | |
|---|---|
| RNA | Ribonucleic phskakacid |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SDS | Sodium Dodecyl Sulphate |
| SMART | Sensitive Membrane Antigen Rapid Test |
| TAE | Tris-acetate EDTA |
| $T_a$ Opt | Optimum annealing temperature |
| *Taq* | *Thermus aquaticus* |
| TBE | Tris-borate EDTA |
| TCBS | Thiosulphate-citrate-bile salt-sucrose |
| $T_m$ | Melting temperature |
| TTGA | Taurocholate-tellurite-gelatin agar |
| UV | Ultra-violet |
| WHO | World Health Organization |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Background

In October 1992, *Vibrio cholerae* O139, a new serogroup, emerged as a second etiologic agent of cholera after *Vibrio cholerae* O1 in the Indian subcontinent. The disease infected several thousands of individuals and caused many deaths wherever it spread, including neighboring countries and some developed countries, and this firmly indicated that the population was virgin to this organism and there was no existing immunity against the new serogroup of *Vibrio cholerae*.

## 1.2 Statement of Problems

In the years before 1992, cholera was originally detected using O1 antiserum which served as a serological marker. However, in late 1992, there was a cholera-like disease causing an epidemic in the Bay of Bengal, India, designated *Vibrio cholerae* O139. This bacteria does not agglutinate with O1 antiserum and this proved that it belongs to *Vibrio cholerae* serogroup non-O1. However, it shows similarities on several characteristics such as morphology, culture, fimbrial antigens and cholera toxin with *Vibrio cholerae* O1 El Tor which is under *Vibrio cholerae* serogroup O1. Studies have shown that immunity to *Vibrio cholerae* O1 does not protect patient from *Vibrio cholerae* O139 infection. Thus, it is of epidemiological interest to be able to diagnose *Vibrio cholerae* O139 as early as possible so that monitoring and

proper prevention actions can be taken by public health authorities. In this study, we are interested in the development of a molecular diagnostic test, such as PCR, which can be use for both clinical and environmental monitoring of specimens. Our rationale was that if the probes were specific, such probes could be used as an adjunct to serological methods for laboratory diagnosis of *Vibrio cholerae* O139.

### 1.3 Objectives of Study

The objective of this study is mainly to develop a specific diagnostic oligonucleotide DNA probe for rapid detection of *Vibrio cholerae* O139, and the measurable objectives are as follow:

1. To design primers based on published sequence.
2. To test the newly designed primers against *Vibrio cholerae* O139.
3. To test the primers against other bacterial strain

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Morphology and Biochemical Characteristic of *Vibrio cholerae*

*Vibrio cholerae* is a gram-negative facultative anaerobic bacteria, which has the shape of curved bacillus, measuring 2 to 3 μm by 0.5 μm and is actively motile by a single polar flagellum (Albert, 1994).

*Vibrio cholerae* is detected positive for indole production and ferments a variety of sugars without the production of gas, it ferments D-(+)-mannose and sucrose but not L-(+)-arabinose and cellobiose. It is also identified positive for the Vogues-Proskauer reaction (Albert, 1994). In addition, it shows positive for oxidase test and decarboxylase test on lysine and ornithine but not arginine. It is also reported positive on nitrate reduction test (Gross, 1994).

## 2.2 Ecology and culture methods of *Vibrio cholerae*

*Vibrio cholerae* is a non-halophilic organism and it shows no growth at 10°C or below. It is detected worldwide in fresh water, brackish water and coastal water, because water is the most important vehicle for the spread of cholera. In endemic areas, *Vibrio cholerae* can frequently be detected in the environment due to contamination via irrigation water, sewage and the use of untreated night soil as

fertilizer (Fersenfeld, 1965). This phenomenon normally found in slum and rural area where the sanitation and sewerage system is poor.

In the natural habitat, food, especially seafood, is thought to be contaminated with pathogenic *Vibrio* spp. via water. The numbers of *Vibrio* spp. may increase in the seafood due to biological concentration in fish and shellfish, especially bivalve molluscus (Donovan and Netten, 1995). However, reports have shown that cholera is not always a waterborne disease (Robert, 1992). Contamination of food by vibrios may lead to foodborne cholera. In endemic areas, food such as vegetables can become contaminated with *Vibrio cholerae* via irrigation water, human faeces or sewage. Direct contamination by food handlers is another route of transmission to a wide variety of foods. Outside the natural habitat *Vibrio cholerae* can grow above 10°C on non-acid foods with a low number of competitive organisms (cooked foods) and a water activity greater than 0.93 (Fersenfeld, 1965; Roberts, 1992).

*Vibrio cholerae* is not nutritionally fastidious, growing well in simple peptone water. The optimum growth temperature is 37°C, and is one of the most rapidly multiplying bacteria, outgrowing for example the coliform bacilli in the early hours of incubation. It could unusually tolerance to alkaline, growing in high pH media as alkaline as pH 9.2; this property is sometimes utilized for purposes of primary isolation (Barua, 1970). Besides, it also grows in media containing 0 to 3%, but not 8%, salt. It grows on a variety of non-selective media such as nutrient agar and sheep blood agar and on selective media for *Vibrio cholerae* such as thiosulphate-citrate-bile salt-sucrose (TCBS) agar and taurocholate-tellurite-gelatin agar (TTGA) (Albert, 1994). However, as suggested by Ansaruzzaman *et al.* (1995), TTGA is a medium

superior to TCBS agar. However, unlike TCBS agar, TTGA is not commercially available. The combination of enrichment media using alkaline peptone water (APW) with thiosulphate-citrate-bile salt-sucrose (TCBS) as the plating medium is the most common method used in culturing *Vibrio cholerae*. APW has been the standard medium for the enrichment of *Vibrio cholerae* used since 1887. As in enrichment broth, a wide range of plating agar has been formulated based on the preference of *Vibrio cholerae* for alkaline conditions and its resistance to bile salts, sodium tellurite, bismuth sulphite and some dyes. TCBS, a highly selective differential medium that is widely used for pathogenic *Vibrio* spp. consists of ox bile (0.8%), sodium thiosulphate (1%), sodium citrate (1%), sodium chloride (1%) and alkaline pH of 8.6 which suppress the growth of most interfering organisms such as Enterobacteriaceae, pseudomonads, aeromonads and Gram-positive bacteria (Kobayashi *et al.*, 1963). The advantage of TCBS is its sucrose/bromothymol blue diagnostic system which readily distinguishes sucrose-positive vibrios such as *Vibrio cholerae* from other colonies (West, 1984). Ingredients of TCBS are shown in appendix A.2.

## 2.3 Taxonomy and serology of *Vibrio cholerae*

The family Vibrionaceae includes the genera, *Vibrio, Aeromonas, Plesiomonas* and *Photobacterium* (Donovan and Netten, 1995). The specificity of the somatic (O) antigen of *Vibrio cholerae* resides in the polysaccharide moiety of the lipopolysaccharide present in the outer membrane, which forms the basis of the serological classification of this organism (Shimada *et al.*, 1994). In a study by

Yamai *et al.* in 1997, nearly 200 serogroups have been distinguished on the basis of epitopic variation in the cell surface lipopolysaccharide (LPS). From an epidemiological standpoint, the species has been divided into serogroup O1 and serogroup non-O1 strains. Cholera vibrio is placed in the O1 serogroup of *Vibrio cholerae*, while other isolates that do not agglutinate with the O1 antiserum are collectively referred to as *Vibrio cholerae* non-O1 (Sakazaki and Donovan, 1984). *Vibrio cholerae* O1 can be further categorized according to its biotype and serotype, which is classical, El Tor and Ogawa, Inaba, Hikojima respectively. *Vibrio cholerae* non-O1 serogroups were not known to cause epidemics of diarrhea; they were known to cause sporadic cases and small outbreaks of diarrheas and extraintestinal infections (Janda *et al.*, 1988). However in late 1992, an epidemic clone of cholera was detected. The epidemic strain was not related to the 138 known serogroups of *Vibrio cholerae* (serogroup O1 and 137 non-O1 serogroups); therefore, a new serogroup, O139 was assigned to the strain with the synonym Bengal to indicate its first isolation from the coastal areas of the Bay of Bengal (Shimada *et al.*, 1993).

### 2.3.1 *Vibrio cholerae* O139 serogroup

Since the first detection of cholera-like disease in the Bay of Bengal in late 1992, efforts have been put in the study of this bacteria. From a study of Johnson *et al.* in 1994, *Vibrio cholerae* O139 has been found not reacting with polyclonal Inaba- or Ogawa-specific sera or monoclonal antibodies specific for A, B and C antigens, and this indicated that the O1-antigen may be missing or altered. As the *Vibrio cholerae*

O139 strains were still typeable, virulent and did not produce rough colonies, the changes were proven not simply due to the loss of O antigen.

Like a majority of *Vibrio cholerae* non-O1, *Vibrio cholerae* O139 possess a capsule, preliminary analysis of capsular layer suggested that it was distinct from lipopolysaccharide antigen and has sugars such as 3,6-dideoxyhexose (abequose or colitose), quinovosamine and glucosamine, and trace of tetradecanoic and hexadecanoic fatty acids. In volunteer studies on other non-O1 strains, the presence of capsule appeared to mask certain critical surface antigen and eventually decreased the host immune response (Johnson *et al.*, 1994). The statement was supported by Waldor *et al.* (1994), whereby both O-antigen capsule and LPS-associated O side chains of *Vibrio cholerae* O139 have been proved to be virulence factors. Other than virulence characteristic, *Vibrio cholerae* O139 has also been found shifting between an encapsulated form with opaque colony morphology and an unencapsulated form that exhibits translucent morphology (Waldor *et al.*, 1994; Comstock *et al.*, 1995).

In a number of studies, *Vibrio cholerae* O139 has been related with *Vibrio cholerae* O1 due to the similarity in both synergistic hemolysis activity, epidemic potential and clinical profile of the disease (Bhattacharya *et al.*, 1993; Albert *et al.*, 1997). Additionally, studies on cholera toxin (CT) which is a virulence factor, has shown similarity between *Vibrio cholerae* O1 and *Vibrio cholerae* O139 strains but not the remaining 137 *Vibrio cholerae* non-O1 strains (O2 to O138) (Nair *et al.*, 1994). However, the major difference between *Vibrio cholerae* O1 and *Vibrio cholerae* O139 strains reported was the architecture of the cell envelope (Knirel *et al.*, 1995).

Serologically, the use of monoclonal antibodies against the various antigenic forms of *Vibrio cholerae* O1 strain has confirmed that *Vibrio cholerae* O139 did not bear any resemblance to the *Vibrio cholerae* O1 serogroup (Nair *et al.*, 1994). Genetically, *Vibrio cholerae* O139 was evolved from *Vibrio cholerae* O1 El Tor by the insertion of a large foreign genomic region encoding the O139-specific genes and simultaneous deletion of most of the O1-specific *rfb* gene cluster, including regions representing *rfbDEG*, *rfbNO*, *ompX*, *orf2* and *orf3* (Faruque *et al.*, 1997). The donor for the O139-specific DNA in this horizontal gene transfer event has been identified as *Vibrio cholerae* O22 (Dumontier and Berche, 1998).

## 2.4 Pathogenesis and symptoms of cholera

Cholera is a faecal-oral disease that infected through oral route. The bacteria is ingested when people consume contaminated food or drink, after passing the acid barrier of the stomach, *Vibrio cholerae* begins to multiply and penetrates the alkaline environment of the intestineal mucosa and attaches to microvilli of the brush border of the gut epithelial cells. *Vibrio cholerae*, usually of the serotype O1 (and O139), can produce enterotoxin. The toxin consists of five B subunits and a single A subunit. Subunit B binds to sugar residues of a specific ganglioside receptor on the cells lining the vili and crypts of the small intestine. This will then forms a hydrophilic transmembrane channel through which the toxic A subunit can pass into the cytoplasm. The cholera enterotoxin causes the transfer of adenosine diphosphoribose (ADP ribose) from nicotinamide adenine dinucleotide (NAD) to a regulatory protein which is part of the adenylate cyclase enzyme responsible for the

generation of intracellular cyclic adenosine monophosphate (cAMP). This will end up with irreversible activation of adenylate cyclase and overproduction of cAMP. This in turn causes (i) inhibition of uptake of sodium and chloride ions by cells lining the vili and (ii) hypersecretion of chloride and bicarbonate ions. Therefore the uptake of water, normally accompanied by sodium and chloride absorption, is blocked and there is a passive outflow of water and electrolytes (Gross, 1994).

As a symptom of this disease, patient infected with cholera will suffer from massive gastro-intestinal loss of an isotonic fluid with a low protein content. The loss of this fluid, sometimes at the rate of one liter per hour in the adult, rapidly leads to hypovolaemic shock and metabolic acidosis with the typical associated physical and laboratory abnormalities. The physical findings include apathy, cyanosis, thready or absent peripheral pulses, very poor skin turgor with scaphoid abdomen, sunken eyes and "washerwoman's hands", and weak or inaudible heart sounds. The laboratory abnormalities include severe metabolic acidosis, haemo-concentration, and marked elevation of the plasma protein concentration. Delayed or inadequate treatment may result in acute renal failure and problems associated with hypokalaemia (Carpenter, 1970).

**2.5 History of cholera epidemic**

There have been eight pandemics of cholera in recorded history, from 1817 to 1823, 1829 to 1837, 1852 to 1860, 1863 to 1875, 1881 to 1896, 1899 to 1823, 1961 to 1971 and 1992 to the present. Even though the etiological agents of the first four

pandemics were not known since they occurred in the time before such agents could be identified, the last three pandemics are known to be due to *Vibrio cholerae* serogroup O1. The seventh pandemic of cholera caused by El Tor vibrio originated in Celebes, Indonesia, and has spread far and wide over the last 30 years, reaching the Central and South American continent, Africa, Asia and Europe in 1992 (Barua, 1992; World Health Organization, 1993). In October 1992, when an epidemic of cholera due to a *Vibrio cholerae* O139 serogroup broke out in the southern Indian port city of Madras. Over the next few months, it spread to other southern Indian cities and reached the northeastern Indian city of Calcutta (Ramamurthy *et al.*, 1993). In December of that year, it spread to southern coastal Bangladesh, which over the subsequent several months spread to the entire country. The disease affected thousands of individuals, mainly adults, and caused many deaths in the Indian subcontinent, indicating that the population was virgin to the organism (Albert *et al.*, 1993). At last count, *Vibrio cholerae* O139 infection had been reported in India, Bangladesh, Nepal, Burma, Thailand, Malaysia, Saudi Arabia, China and Pakistan (Albert, 1993).

## 2.6 Diagnosis of *Vibrio cholerae* O139

With the emergence of new serogroup *Vibrio cholerae* O139, it has long been the epidemiological interest to develop a specific, sensitive and rapid diagnosis test in order to identify the bacteria as early as possible. In conventional bacteriologic techniques, which culturing stool on a selective medium, followed by biochemical testing of colonies and confirmation by slide agglutination test with specific