DETECTION AND MOLECULAR CHARACTERIZATION OF THE *ZOT* GENE IN *VIBRIO CHOLERAE* AND *V. ALGINOLYTICUS* ISOLATES

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Absract. A total of 11 *Vibrio cholerae* isolates from 1996-1998 outbreaks in Malaysia and 4 *V. alginolyticus* were analyzed. Isolates were characterized by polymerase chain reaction (PCR) and Southern hybridization for the presence of the gene encoding zonula occludens toxin (*zot*). Screening of *zot* gene by PCR revealed the presence of this gene in *V. cholerae* and *V. alginolyticus*. The *zot* gene from one *V. cholerae* Ogawa isolate that was cloned in a pCR 2.1 TOPO vector was sequenced. The sequences obtained were 99% homologous to the *zot* gene sequence from the Gene Bank.

INTRODUCTION

Vibrio species like *Vibrio alginolyticus*, *V. cholerae*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus* and others, are commonly found in aquatic environments (Waldor *et al*, 1996). *Vibrio alginolyticus*, a halophilic gram-negative bacterium is found to be pathogenic to marine organisms and occurs commonly, if not ubiquitously. Some strains are to shellfish and others (Baumann *et al*, 1984). It occasionally causes opportunistic self-limiting infections of wounds (Baumann *et al*, 1984). The disease is transmitted via contaminated water or by diseased fishes. In the United Kingdom, *V. alginolyticus* is the most common *Vibrio* found in seafood (Lewis *et al*, 1997).

Vibrio cholerae on the other hand is an etiological agent of severe diarrheal disease in humans known as cholera. The disease is endemic in Asia and has caused worldwide pandemics (Pollitzer, 1959). The transmission of the disease is either by consumption of contaminated water and food or often by person to person (Blake *et al*, 1980; Kelly *et al*, 1984; Depaola, 1981). The pathogenicity of *V. cholerae* is mainly associated with the ability to produce the virulence factor cholera enterotoxin (CT). This toxin is encoded by two contagious genes which forms the CTXAB operon, that catalyzes an ADP ribosylation reaction in epithelial cells (Pearson *et al*, 1993; Kaper *et al*, 1995; Spangler, 1992). The search for additional enterotoxins produced by *V. cholerae* led to the discovery of the second enterotoxin zonula occludens toxin (Fasano *et al*, 1991). The zonula occludens toxin increases the permeability of rabbit small-bowel mucosa by affecting the structure of the intercellular tight junctions (zonula occludens). The gene encoding for *zot* is located immediately upstream of the CT operon encoding 4.5 kb dynamic region of the *V. cholerae* chromosome termed the core region or virulence cassette (Baudry *et al*, 1992). The present study reports the screening for the presence of *zot* gene in local strains of *V. cholerae*. As a comparative study, the species of *V. alginolyticus* is also screened for this gene. The presence of *zot* gene in *V. cholerae* was confirmed by Southern

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hybridization, followed by cloning and sequencing.

MATERIALS AND METHODS

Bacterial isolates

All of the *V. cholerae* isolates (9 Ogawa, 1 Inaba and 1 Bengal) were isolated during the epidemic (1996-1998) in Malaysia from patients with cholerae and two of the four *V. alginolyticus* isolates were obtained from food sample and the other two isolates from water samples (265 and Thai).

DNA extraction and screening of *zot* gene by PCR

Genomic DNA was extracted using QIAGEN QIAamp Tissue kit (Qiagen Inc# 29306) according to the manufacturer's instructions. The oligonucleotide sequences of primers used for DNA amplification were *zot* 1 (5′-TGGCTTCGTCTGCTGCCGGCGATT-3′) and *zot* 2 (5′-CACTTCTACCCACAGCG CTTGCGC-3′). The primer sequences were as published by Aidara *et al* (1998). The amplification was performed in a 25 µl volume of a final mixture containing sterile ultra pure water, 2.5 µl of 10x BST buffer (1x), 0.5 µl of 1.8 mM $MgCl_2$ solution, 0.2 µl of 200 mM dNTP mix (Finzymes) 1 µl of 5 pmol *zot* 1 primer, 1 pmol of *zot* 2 primer, 1 µl of pure DNA (100 ng) and 1U of *Taq* polymerase (Biosyntech Inc). The DNA amplification steps performed were initial denaturation cycle at 94ºC for 5 minutes, followed by 35 cycles of denaturation cycle at 94ºC for 1 minute, primer annealing at 64ºC for 1 minute and elongation at 72ºC for 2 minutes, with a single final extension at 72ºC for 3 minutes. The amplicons were electrophoresed through agarose 1.4% gel (Nuseive GTG agarose, FMC Bioproducts, Rockland, Maine, USA), stained with ethidium bromide and photographed under UV exposure. 1 Kb ladder DNA was used as the molecular weight markers.

Southern hybridization

The amplified PCR products were separated by agarose (1%) gel electrophoresis. The amplified DNA fragments were transferred to nylon membranes (Magna graph nylon) by the capillary transfer method according to the manufacturer's instruction with slight modification (MSI, Micron separation Inc). The modification included performing the prehybridization and hybridization steps at 65ºC. The membrane was immobilized by baking at 65-80ºC for 1 hour followed by hybridization with ZB3 (*zot*) probe (a biotin labeled non-radioactive HPLC purified) with a sequence of 5′GCCACTTTAACCGCGCCAC-3′ (Biosyntech Inc). The prehybridization, hybridization and detection steps were carried out according to the manufacturer's instructions.

Cloning and sequencing of *zot* gene

Multiple copies of *zot* gene amplified using the PCR method was cloned in pCR 2.1 Topo vector and transformed in *Eschericia coli* competent cells (Invitrogen company Inc). Plasmid DNA isolation was done using STT-lysozyme (Sucrose, Tris-HCl, Triton-100x and lysozyme) single tube plasmid DNA isolation method in accordance to Liu and Nichols, (1994). The isolated plasmid DNA was digested by restriction enzyme *EcoR1* to cleave the inserted DNA fragment (*zot* gene) from the vector by using standard molecular biological methods (Sambrook *et al*, 1989).

The *zot* gene from one Ogawa isolate cloned into the pCR 2.1 Topo vector was sequenced commercially by the automated sequencing (Biosyntech, Inc) method and the sequences were analyzed by computer assisted BLASTN package.

RESULTS

All the 11 *V. cholerae* isolates and 4 *V. alginolyticus* isolates were confirmed as *V. cholerae* and *V. alginolyticus* by the conventional methods. A 1083 bp fragment of *zot* gene was amplified from all 11 *V. cholerae* isolates and 4 *V. alginolyticus* isolates (Fig 1). The 1083 amplified product for the *zot* gene of the *V. cholerae* and *V. alginolyticus* isolates are reconfirmed by Southern hybridization using

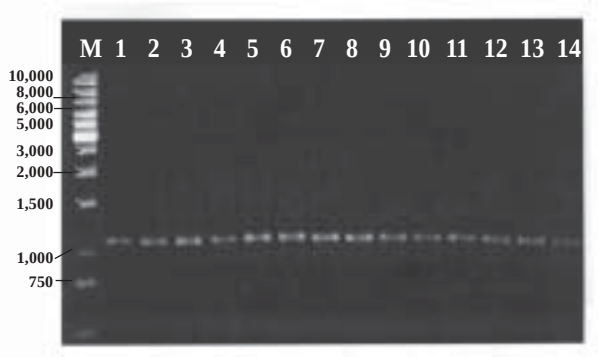

Fig 1-The detection of *zot* gene by the asymmetry PCR. Lane M represents 1kb DNA marker ladder, lanes 1-10 are the *zot* gene for *V. cholerae* isolates, lanes 11 - 14 shows the *zot* gene from *V. alginolyticus*.

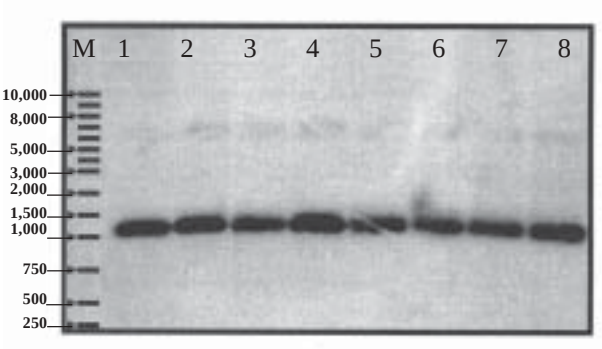

Fig 2-Photograph of the X-ray film confirming the presence of *zot* gene in *V. cholerae* isolates, indicated by a single band. Lane M represents 1kb DNA marker ladder.

ZB3 (*zot*) probe (Fig 2). With respect to the presence of the *zot* gene there were no difference between the *V. cholerae* and *V. alginolyticus* isolates. The *zot* gene of *V. cholerae* isolate serotype Ogawa was successfully cloned in pCR 2.1 cloning vector and sequenced (Fig 3). The nucleotide sequence of the *zot* gene is shown in Fig 4.

DISCUSSION

Cholerae has an ancient reputation as a killer disease (Jones *et al*, 1984). Presently, cholera is the most important diarrheal disease affecting human worldwide. It has ravaged humanity in a number of pandemics, the latest of which is the seventh (Barua, 1992). Therefore the methods for the effective prevention are necessary as the disease is causing serious problems. The existence of a second potential enterotoxin in *V. cholerae* as reported by Fasano *et al* (1991) and was named as *zot* for zonula occludens toxin, that affects the structure of epithelial tight junctions (zonula occludens) also existed in local *V. cholerae* and *V. alginolyticus* isolates, studied.

In the present study 11 strains of *V. cholerae* and 4 strains of *V. alginolyticus* produced an amplified product of 1083 bp. Based on the molecular weight of the product, it was tentatively identified as the genes encoding for the *zot* toxin. Interestingly, 4 strains of *V. alginolyticus* also produced an amplified band of 1083 bp, which suggests that the zonula occludens toxins are not only produced by the *V. cholerae*. A study by Johnson *et al* (1993) and Tamayo *et al* (1997) shows that the strains of *Vibrios* which harbor the cholerae toxin gene (CTX) will also harbor the *zot* gene. But in the case of *V. alginolyticus*, the presence of CTX gene cannot be confirmed since the study was not carried out. The identification of the presence or absence of the *zot* gene sequence in all the *V. cholerae* strains is important, as this toxin product could be selected as a potential candidate for the production of a recombinant DNA vaccine. To re-confirm the presence of *zot* gene in *V. cholerae* and *V. alginolyticus* amplified by PCR, the DNA (*zot* gene fragment) was hybridized with an oligonucleotide probe which was specifically designed to detect the presence of the *zot* gene by Southern hybridization technique. The presence of *zot* gene in all *V. cholerae* isolates that were confirmed by PCR were in homology with the hybridization results. The *zot* gene amplified by PCR from a *V. cholerae* Ogawa serotype was cloned and sequenced.

The complete nucleotide sequence of the *zot* gene from *V. cholerae* has been determined in this study. The deduced amino acid sequence revealed that *zot* gene codes for zonula occludens toxin, which is one of the member of the virulence cassette or core repron of *V. cholerae*. The DNA sequence data obtained spanned a 1083 base segment. The predicted amino acid

gb|AF123049.1|AF123049 *Vibrio cholerae* zonula occludens toxin gene (99%)

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Query: 6    gaatgagcgaggcttaaaccttgaacgcatggctaagtacttaaaaatggatgtctcgga 65
Sbjct: 111 gaatgtgcgaggcttaaaccttgaacgcatggctaagtacttaaaaatggatgtctcgga 170

Query: 66   catcagtatcgagtttattgatacagaccatcctgacggtcgcttaacgatggcgcgttt 125
Sbjct: 171 catcagtatcgagtttattgatacagaccatcctgacggtcgcttaacgatggcgcgttt 230

Query: 126 ttggcactgggcgagaaaggacgcgtttctctttatcgatgaatgtggtcgcatctggcc 185
Sbjct: 231 ttggcactgggcgagaaaggacgcgtttctctttatcgatgaatgtggtcgcatctggcc 290

Query: 186 gccgagactgacggtcaccaatttaaaggcgctcgacacgccgccggatttggtcgcaga 245
Sbjct: 291 gccgagactgacggtcaccaatttaaaggcgctcgacacgccgccggatttggtcgcaga 350

Query: 246 ggataggccggagagctttgaggtggcttttgacatgcatcgtcaccacggctgagatat 305
Sbjct: 351 ggataggccggagagctttgaggtggcttttgacatgcatcgtcaccacggctgggatat 410

Query: 306 ctgcctaaccacgcctaacattgccaaagtgcacaacatgataagagaggcggcggagat 365
Sbjct: 411 ctgcctaaccacgcctaacattgccaaagtgcacaacatgataagagaggcggcggagat 470

Query: 366 agggtatcgccactttaaccgcgccaccgtggggctaggggcaaagtttaccctgaccac 425
Sbjct: 471 agggtatcgccactttaaccgcgccaccgtggggctaggggcaaagtttaccctgaccac 530

Query: 426 ccatgatgcagccaactctggacagatggactcgcacgcgctgacacgccaagtc 480
Sbjct: 531 ccatgatgcagccaactctggacagatggactcgcacgcgctgacacgccaagtc 585

Query: 481 ttccaagtccgatttttaagatgtacgcaagcaccacgacaggcaaagcacgcgacacga 540
Sbjct: 593 ttccaagtccgatttttaagatgtacgcaagcaccacgacaggcaaagcacgcgacacga 652

Query: 541 tggccggaacggcgctgtggaaagacagaaagatccttttcttgttcggcatgg 594
Sbjct: 653 tggccggaacggcgctgtggaaagacagaaagatccttttcttgttcggcatgg 706

Query: 595 gatgttctcttattcgttttacggcttacacgacaatccaatttttacaggggaaatga 654
Sbjct: 714 gatgttctcttattcgttttacggcttacacgacaatccaatttttacaggggaaatga 773

Query: 655 tgcaactatcgagtcagagcaatccgagcctcagtcaaaggctactgttgggaatgctgt 714
Sbjct: 774 tgcaactatcgagtcagagcaatccgagcctcagtcaaaggctactgttgggaatgctgt 833

Query: 715 c 715
Sbjct: 834 c 834

Query: 716  ggaatgctgtcgggagcaaggcggttgctcctgcgtcttttggtttttgtattggtcggc 775
Sbjct: 824  ggaatgctgtcgggagcaaggcggttgctcctgcgtcttttggtttttgtattggtcggc 883

Query: 776  tttgtgtccaagatggttttgtcactgttggtgatgagcgttatcgcctcgtagacaatt 835
Sbjct: 884  tttgtgtccaagatggttttgtcactgttggtgatgagcgttatcgcctcgtagacaatt 943

Query: 836  tggacattccttatcgtggtctatgggcgacaggtcatcacatttacaaggatacgctta 895
Sbjct: 944  tggacattccttatcgtggtctatgggcgacaggtcatcacatttacaaggatacgctta 1003

Query: 896  cagtgttttttgaaaccgagagtggcagcgtcccaacagagctgtttgcatcgagctacc 955
Sbjct: 1004 cagtgttttttgaaaccgagagtggcagcgtcccaacagagctgtttgcatcgagctacc 1063

Query: 956  gctacaaggtgctaccgttaccggatttcaatcactt 1015
Sbjct: 1064 gctacaaggtgctaccgttaccggatttcaatcactt 1100
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Fig 3-Nucleotide sequence of *zot* gene of *V. cholerae* analysed with BLAST.

sequence of *zot* showed significant homology to sequences stored in the gene bank database (Fig 4), and analysis of the sequence with the program BLAST (2.0.11) also showed 99% homology to the Gene Bank database (gene bank ref GI: "4633654"). This *zot* gene consists of a 1.3 kb open reading frame, that encode a 44.8 kDa polypeptide (Baudry *et al*, 1992).

If the genetically engineered strains deleted for cholerae toxin is constructed and is still potential to cause diarrhea is confirmed, then *zot* represents a completely new mechanism of infectious diarrhea (Fasano *et al*, 1991). Thus identification of the *zot* gene is a potentially high significant step in the development of a safe and efficacious DNA vaccine against cholerae.

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