Detection of the gene encoding the subunit toxin B from Malaysian *Vibrio cholerae* isolates

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Abstract

The gene encoding cholera toxin subunit B *(ctxB)* is the gene of focus in this study as the subunit toxin B is an effective immunogen. The *ctxB* was detected using polymerase chain reaction (PCR) based on the expected size (460 bp) in 20 isolates from local outbreaks of cholera in Peninsular Malaysia. These isolates consisted of *Ogawa*, *Inaba* and 0139 *Bengal* strains. The PCR conditions were optimised in different methods of template DNA preparations as well as primer and DNA concentrations. The PCR conditions were also specific against *Escherichia coli*, *Klebsiella* and *Salmonella* species. The identification of the *ctxB* PCR products was further confirmed using Southern hybridization and sequencing. For Southern hybridization, a 20-mer biotinylated oligonucleotide probe was used in detecting the PCR amplicon by a chemiluminescent detection method. The sequencing data obtained further confirmed that the amplicon is the gene of interest, and that the strain of *V. cholera* used in this study belongs to the Classical biotype.

Key words: *Vibrio cholerae*; *ctxB*; DNA vaccine

Introduction

Cholera pandemics have occurred since 1819 and the infection remains a public health problem in developing countries where safe water and sanitary facilities are suboptimal (WHO, 1996). Vibrio *cholerae* is differentiated into the three serotypes *Inaba*, *Ogawa* and 0139 *Bengal*, which is further separated into the two biotypes Classical and El Tor (Van Urk *et al.*, 1992).

This acute diarrheal disease is an important cause of morbidity and mortality, particularly in children, the elderly and immunodeficient persons. The clinical presentation is from self-limiting infection to severe dehydrating diarrhoea (Lima, 1994). Effective treatment is by rehydration therapies but these can be expensive, wasteful and less efficient in times of administrative machinery breakdown (Siddique *et al.*, 1995). Hence, a safe and efficacious vaccine is the best solution.

Vaccine development was initiated a year after the discovery of the etiological agent, *V. cholerae* using various strategies including purified proteins, heat-treated toxin, life attenuated vaccines as well as oral and parental killed whole cell vaccines (Van Urk *et al.*, 1992; Ghosh *et al.*, 1996). Despite protective efficacy of 60%, the live attenuated oral vaccine is serotype-specific (Lima, 1994). No vaccine is satisfactory thus far.

DNA vaccine, the latest evolution in vaccine development, involves direct intradermal or intramuscular introduction of a plasmid encoding antigens of interest. The foreign protein expressed within the host cells may induce both life long humoral and cellular immune responses. DNA vaccines are believed to be safer than the traditional vaccine as only relevant antigenic epitopes are selected (Ulmer *et al.*, 1993).

The purpose of this study is to screen and isolate genes that may be potential candidates for a DNA vaccine. We chose for this study the subunit toxin B (CTB) gene of *V. cholerae* which is responsible for binding cholera toxin to the mucosal GM1-ganglioside, and facilitate the entry of the active toxin (CTA) into the host cell. We have successfully optimised the conditions to amplify the target DNA sequence using several template DNA preparation techniques and confirmed the product by both Southern Hybridization and sequencing.

Materials and Methods

Vibrio cholerae isolates

Twenty *V. cholera* clinicalisolates (one *Inaba*, one *Bengal* and *Ogawa* of 1995, 1996 and 1998 outbreak strains) were obtained from the Bacteriology Division, Insti-

tute for Medical Research and the Medical Microbiology Department, University of Malaya. The bacteria were cultured in prepared TCBS Selective Medium (Bacto®, Difco Laboratories).

Preparation of Template DNA

Genomic DNA Isolation

DNA isolation from cell colonies was performed using the Qiagen Tissue Extraction Kit®following the manufacturer's protocol.

Template DNA from Boiled Cell Colonies

Bacteria colonies from an overnight growth on TCBS agar were suspended in 0.5 ml of sterile water to a concentration of 10^5 to 10^6 cells per ml, boiled for 30 min, and stored at 4°C prior to use (Olsvik *et al.*, 1993).

DNA Template from Broth Cultures

One ml of broth medium prepared using Broth powder (Gibco BRL), was cultured to approximately 10^2 to 10^3 of cells and heated at 94°C for 5 min. The lysate was used immediately or stored at 4°C for further use (Shirai *et al.*, 1991).

Polymerase Chain Reaction (PCR)

The primers used for amplification of the *ctxB* (460 bp) (Olsvik *et al.*, 1993), were custom-made by Genemed Biotechnologies, Inc., USA. The sequences of the primers are as follows:

Primer 1: 5'-GGT TGC TTC TCA TCA TCG AAC CAC -3'

Primer 2: 5'-GAT ACA CAT AAT AGA ATT AAG GAT G -3'

Amplification was performed using the PCR Core System II (Promega) Kit and *Taq* DNA polymerase (BioSynTech) along with its buffer and $MgCl_2$. Amplification was carried out in a final volume of 50 µl containing 30.85 µl of distilled pure water, 5 µl of BST *Taq* DNA polymerase, 1X reaction buffer, 0.75 µl of 100mM $MgCl_2$, 1µl of PCR Nucleotide Mix (10 mM)[10mM each of dATP, dCTP, dGTP and dTTP], 1 µl of both Primer 1 and Primer 2 (15 pmol each), 10 µl template DNA from boiled colonies and 0.4 µl of BST *Taq* DNA polymerase (5 U/µl). The DNA template obtained from boiled cell colonies was centrifuged (8000 rpm, 3 min) and only supernatant was used.

The positive control supplied with the PCR Core System II Kit was applied in a final volume of 50 µl containing 35.25 µl of distilled water, 3.3 µl of Upstream and Downstream Control Primer (15 µM each), 1 µl of Positive Control Plasmid DNA (1 ng/µl in TE buffer) and the same volume of buffer, MgCl2, Nucleotide mix and *Taq* polymerase as above. The same conditions were used for the negative control except for the substitution of DNA template with distilled pure water.

Amplification was performed in a thermal cycler (Biometra-TRIO Thermoblock). The cycling conditions were: initial denaturation (95°C, 2 min), followed by 25 cycles of denaturation (95°C, 1 min), annealing (55°C, 1min), and extension (72°C, 1 min) with a final extension (74°C, 5 min) (Fields *et al.*, 1992). The PCR products prepared were used immediately or stored at 4°C.

Detection of amplification products

The samples and marker (100 bp DNA ladder or λ/*Hind* III [Fermentas]) were subjected to electrophoresis in a 1.5 % agarose I (Amresco®) in 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA) and visualised under an UV transilluminator (Biometra®).

Southern Hybridization

Ten (1 samples (PCR products) were subjected to electrophoresis in a 2 % agarose gel. The gel was treated in depurination solution (0.24 M HCl) for 15 min and two, 30 min treatments in both denaturation solution (1.5 M NaCl, 0.5 M NaOH) and neutralising solution (1.5 M NaCl, 1 M Tris-HCl, pH 8) and subjected to capillary blotting on a nylon membrane (Magna). The membrane was hybridised using ExpressHyb™ Hybridization Solution (Clontech) with a biotinylated probe (biotin-CC TCA TGC GAT TGC CGC AAT-3' [Chopra *et al.*, 1987]) for 1 hr at 49°C. Chemiluminescent detection was carried out using a Phototope®-Star Chemiluminescent Detection Kit (NEB). The membrane was exposed to a piece of film (Kodak Diagnostic Film) and the film was processed manually.

Cloning and Sequencing.

The gene of interest with the expected size was extracted out from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen) and cloned into pCR®2.1-TOPO plasmid (Invitrogen). Transformation was carried out using TOP10F' cells and the white colonies were screened for the presence of the insert by restriction digests using *Eco*R1. The vector containing the insert was then sent for automated sequencing (CGAT, UKM) for confirmation.

Results

Methods in Template DNA Preparation

Template DNA can be prepared using extracted chromosomal DNA, boiled cell colonies and broth cultures of *V. cholerae*. The method using boiled cell colonies was chosen to prepare the DNA templates in this study. All the *ctxB* in the templates were successfully amplified by PCR (Fig. 1).

Detection of ctxB

PCR using boiled cell colonies as template DNA was successfully carried out in all 20 isolates (Fig. 2). This showed that the PCR conditions and parameters have 100 % sensitivity for detection of the toxin producing strain of *V. cholerae*.

Species Specificity of PCR

In order to test the specificity of the PCR method, an estimated 10^5 to 10^6 diarrhoea-causing bacteria (*Escherichia coli*, *Klebsiella* sp. and *Salmonella* sp.) were boiled prior to use as template DNA in the PCR reaction. All the three species showed no PCR products (Fig. 3). Thus, the primers and the PCR conditions were specific for the *ctxB* gene of *V. cholerae*.

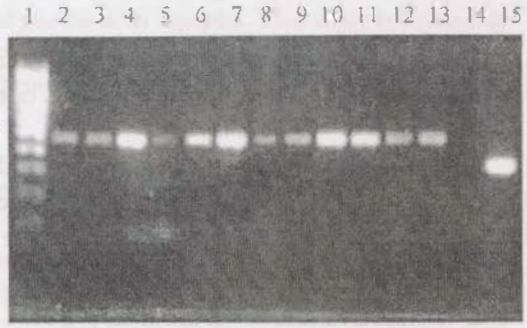

Fig. 1. Analysis of PCR products from the amplification of ctxB genes of *V. cholerae* using template DNA obtained from broth cultures, extracted chromosomal DNA.
Lane 1, a 100 bp molecular weight marker (Fermentas). DNA templates were obtained from broth cultures (lanes 2-5), chromosomal DNA extracted using DNAzol® (lanes 6-9) and boiled cell colonies (lanes 10-13) each with isolate 2,3,4 and 5. Lane 14, negative control; lane 15, positive control. The PCR products were separated by 1.5 % agarose gel electrophoresis and were visualised by staining with ethidium bromide and illumination under UV light. The size of the amplified fragment (460 bp) was confirmed by the positions of the bands relative to those of the molecular size markers.

Confirmation of the Correct PCR Amplification of ctxB

Randomly selected PCR amplicons were subjected to hybridization with the 20-mer non-radioactive labelled probe using the Southern Hybridization technique. Bands with the correct molecular weight for *ctxB* were seen on the nylon membrane (Fig. 4), confirming that the PCR products of the *ctxB* amplification had specifically hybridised with the probe.

The DNA sequence analysis confirmed that the amplicon was the *ctxB*. The amino acid sequence of the B subunit produced by the sequence analysis yielded a total of 124 amino acids with the termination codon of the A2 subunit overlapping the initiation codon of the B subunit by 4 bases (Fig. 5). The strain studied belongs to the Classical biotype of *V. cholera* since the amino acids at residues 39, 43, 68 and 91 are His, Asn, Thr and Asn respectively.

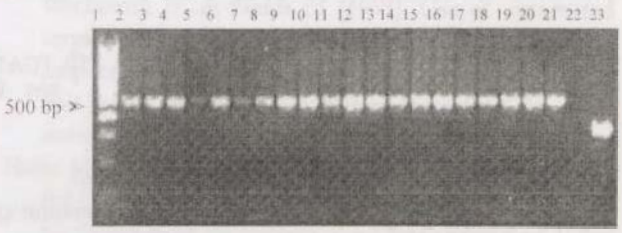

Fig. 2. Analysis of PCR products from boiled cell colonies of *Vibrio cholerae*.
Lane 1, 100 bp molecular weight marker (Fermentas); lanes 2-21, isolates 1-20; lane 22, negative control; lane 3, positive control.

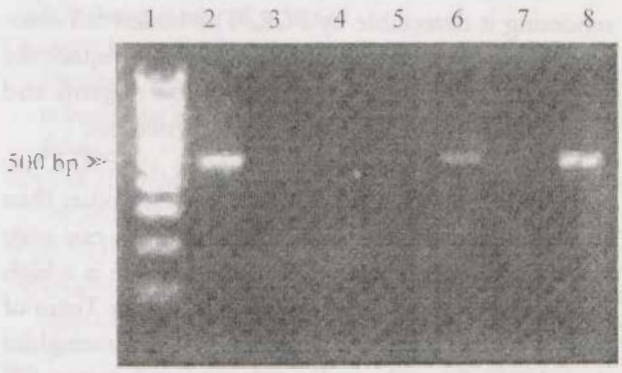

Fig. 3. Analysis of PCR products from boiled cell colonies of *Vibrio cholerae*, *Salmonella sp.*, *Klebsiella sp.* and *E. coli*.
Lane 1, 100 bp molecular weight marker (Fermentas); lanes 2, 6 and 8, isolates 2, 6 and 8 of *V. cholerae* respectively; lane 3, *Salmonella* sp.; lane 4, *Klebsiella* sp.; lane 5, *diarrheogenic E. coli* and lane 7, negative control.

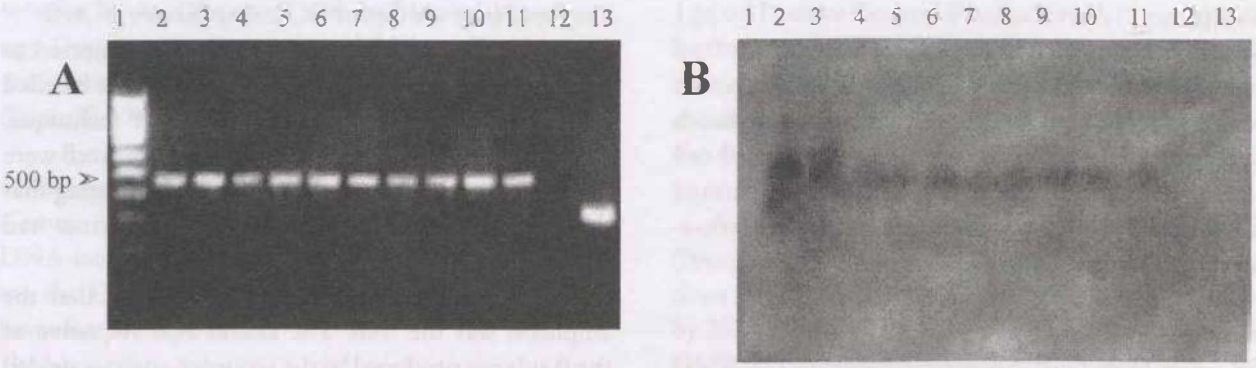

Fig. 4. Confirmation of the correct *ctxB* amplification by PCR
(A) Analysis of PCR products of boiled cell colonies of *Vibrio cholerae*.
Lane 1, 100 bp molecular weight marker (Fermentas); lanes 2-11, isolates 11-20; lane 12, negative control and lane 13, positive control. The PCR products were separated by 2 % agarose gel electrophoresis and were visualised by staining with ethidium bromide and illumination with UV light.
(B) The PCR products of the gel shown in A were transferred on to a nitrocellulose membrane, and hybridised with a biotinylated oligonucleotide probe specific to the *ctxB* using the Southern Blotting technique.

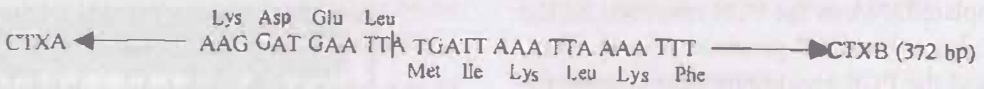

Fig. 5. Nucleotide sequence fragment of *ctxB*.
The sequencing data showed that the termination codon of the A2 subunit overlaps the start codon of the B subunit by 4 bases.

Discussion

The DNA template can be prepared using all three methods. Boiling of the cell colonies (Olsvik *et al.*, 1993) will cause the release of chromosomal DNA by lysis rendering it detectable by PCR. The boiled cell colonies method is cost effective as it does not require the usage of LB medium or any extraction reagents and simple boiling is much easier to be carried out.

The PCR conditions are species specific. The absence of bands from the PCR of bacteria other than *V. cholera* proves that the PCR condition can only amplify the *ctxB* from the *V. cholera*. There is a high degree of homology of the B subunit Labile Toxin of *E.coli* with cholera toxin (Splanger, 1992; Domenighini *et al.*, 1995). Some homology also exists between CT structural gene and *Salmonella* enterotoxin gene (Chopra *et al.*, 1987). The PCR conditions also showed 100% sensitivity in the amplification of the genes in all samples. The 460 bp (*ctxB*) band fragment obtained from the PCR corresponded to the published data (Lockman & James, 1983) and proven correct by Southern hybridization and by sequencing. The result revealed that the fragment was the *ctxB* and that no other fragments homologous to the CTX operon were amplified.

Based on the sequencing results, the local strain of *V. cholera* used in this study belongs to the classical biotype. Although there is evidence of some antigenic differences between the enterotoxins from both the biotypes (Classical and El Tor), the four differing residues might not be sufficient to determine unique antigenic sites within the molecule. The study by Olsvik *et al.* (1993) suggests that random mutations in the *ctxB* gene itself are uncommon. Although the toxin genotype seems to be more conserved, other portions of the *V. cholera* genome are more complex and variable. The sequence that was obtained is also identical to that published by Lokman & James (1983) in which the termination codon of the A subunit overlaps the initiation codon of the B subunit by four bases.

The *ctxB* gene is thus a potential candidate for a DNA vaccine against cholera as the gene was constantly found in the local *V. cholera* samples. As the sequence of the *ctxB* has been confirmed, we are in

the process of subcloning the gene into an appropriate eukaryotic expression vector and studying the expression of the gene *in vitro*. On-going work also include sequencing geographically and temporally diverse local *V. cholera* clinical isolates to identify heterogeneity in the B subunit since this could have implications to our work in DNA vaccine development. Other potential cholera toxin genes under study for the development of multivalent DNA vaccines include the ZOT (zonula occludens toxin) and the ACE (accessory cholera enterotoxin). ZOT increases the permeability of the intestinal mucosa (Fasano *et al.*, 1991) while the ACE alters the ion transport (Aidara *et al.*, 1998). However, as the vaccine formulation is complex, other antigenic determinants may contribute to differences in protection.

In conclusion, this study demonstrates the successful amplification and isolation of the *ctxB* gene from the local outbreak strains of *V. cholera* to a detectable amount. The PCR method employed was sensitive to all the *V. cholera* isolates tested and the amplification products were confirmed correct by Southern hybridization as well as by sequencing.

Acknowledgements

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