CLONING AND EXPRESSION OF *VIBRIO CHOLERAE* VIRULENCE GENE, ACCESSORY CHOLERA ENTEROTOXIN (*ACE*)

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Abstract. The cholera enterotoxin (CT) has been considered a major virulence factor of *Vibrio cholerae*. The accessory cholera enterotoxin (*ace*) gene is the third gene of *V. cholerae* virulence cassette. The gene coding for the *Ace* toxin was amplified from *V. cholerae* isolates producing a single band of 314 bp. The presence of *ace* gene was confirmed by hybridization as well as by sequencing. The gene was successfully expressed in *Escherichia coli* (LMG194) using expression, pBAD/Thio-TOPO vector. Optimal conditions for expression included choice of host strain, temperature used for culturing, and concentration of antibiotic and arabinose inducer. The *Ace* protein was obtained from the cell supernatant as a fusion protein with a molecular mass 34 kDa which was detected using an anti V5-HRP epitope tagged antibody.

INTRODUCTION

Cholera has been epidemic in southern Asia for at least 1,000 years and to date has caused seven pandemics since 1817. When untreated, cholera is a disease of extraordinarily rapid onset and potentially high lethality. Although clinical management of cholera has advanced over the last 40 years, cholera remains a serious threat in developing countries where sanitation is poor, healthcare is limited, and drinking water is unsafe. Neither apparent nor unapparent infection is known to occur naturally in any species of animal other than man, and there is no epidemiology evidence to suggest that animal reservoir play any role in the maintenance or spread of cholera. Cholera is characterized by a severe watery diarrhea caused by toxigenic *Vibrio cholerae* which colonize the small intestine and produce an enterotoxin, cholera toxin, CT (Norris, 1974; Rabbani and Greenough, 1990). CT has molecular weight of 84 kDa, and is composed of one A subunit of 27 kDa and five B subunits, each of 11 kDa (Ghosh *et al*, 1996) encoded by one or more copies of CT genes (*ctxAB*). *V. cholerae* produces other putative toxins such as zonula occludens toxin (*zot*) that increases the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction (Fasano *et al*, 1991; Baundry *et al*, 1992) and accessory cholera enterotoxin (*ace*) that increases the short-circuit current in Ussing chamber, alters ion transport and causes fluid accumulation in ligated rabbit ileal loops (Trucksis *et al*, 1993). The gene that encodes the *Ace* toxin is located immediately next to the *ctx* gene on a 4.5 kilobase (kb) region of *V. cholerae* chromosome termed the core region and when amplified produces a 314 basepair (bp) fragment (Aidara *et al*, 1998). According to Trucksis, Conn *et al* (1997), the *Ace* toxin was expressed efficiently in the methylotrophic yeast *Pichia pastoris* but not in *Escherichia coli* (*E. coli*). However, in this study the *ace* gene was successfully expressed in *E. coli* LMG194 strain using the pBAD/Thio-TOPO vector. We also report the optimum conditions suitable for the expression of *Ace* toxin in *E. coli*. The study was carried out with the future aim of including the toxin in the development of a vaccine for cholera.

MATERIALS AND METHODS

Sources of bacteria and DNA extraction

Twenty *Vibrio cholerae* clinical isolates were obtained from patients during a cholera epidemic

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(1996-1998) in Malaysia. Eighteen of the isolates belong to the Ogawa serotype, and one isolate to Inaba and the other to 0139 Bengal serotype. Genomic DNA was extracted from the *V. cholerae* isolates using QIAGEN QIAamp Tissue kit (QIAGEN, Germany) according to the manufacturer's instruction. DNA concentration and purity were determined by measuring the absorbance at 260 nm and 280 nm using UV-visible spectrophotometer (UV- 1601, Shimadzu).

Detection and amplification of *ace* gene

Polymerase chain reaction (PCR) was used to amplify the *ace* gene in the *V. cholerae* isolates. Two oligonucleotide primers, 5′-TAA GGA TGT GCT TAT GAT GGA CAC CC-3′ and 5′-CGT GAT GAA TAA AGA TAC TCA TAG-3′, were synthesized (Operon Technologies Inc) in accordance to published data (Aidara *et al*, 1998).

A typical PCR reaction was performed in a final volume of 25 µl containing 200 ng/µl of genomic DNA, 1.8 mM of $MgCl_2$, 1X of BST buffer (Biosynthec Inc), 200 µM of dNTPs, 5 pmoles of each primer and 1U of BST *Taq* polymerase (Biosynthec, Inc) in an automated DNA thermal cycler (Biometra-TRIO Thermoblock). Typically, the DNA amplification steps consisted of an initial denaturation at 94ºC for 5 minutes, followed by 35 cycles of denaturation at 94ºC for 1 minute, annealing at 65ºC for 1 minute, and elongation at 72ºC for 2 minutes. The amplification was ended with a single final extension at 72ºC for 7 minutes. Ten µl aliquot of the PCR product mixture was electrophoresed through 1.0% agarose gel.

Hybridization

The presence of *ace* gene in *V. cholerae* isolates was confirmed by hybridization employing the biotinylated non-radioactive Ace probe, 5′-CCG CTT ATC CAA CAG GCT AT-3′, and Phototope® Chemiluminescent kit (New England Biolab) following the manufacturer's instructions, following transfer onto MAGNA graph Nylon membrane (MSI, Micon Separation Inc).

Cloning of *ace* gene

The PCR product was cloned into an *E. coli* expression vector pBAD/Thio-TOPO (pBAD/TOPO ThioFusion™ Expression System, Version B, Invitrogen, USA, cat. No. K370-01), and trans-

fected into TOP10 *E. coli* host strain. Subsequently, the recombinant *ace* plasmids were retransfected into another *E. coli* host strain, LMG194, which was provided in the kit. The LMG194 competent cells were prepared according to Nishimura *et al* (1990). The transfected TOP10 and LMG194 *E. coli* strains were grown in LB-ampicillin broth at 37ºC overnight and subjected to plasmid extraction using QIAGEN following the protocol provided by the manufacturer. The extracted pBAD-*Ace*+ from both host cells was digested with *PmeI* and *NcoI* (Fermentas) to confirm for the presence of the insert. The pBAD-Ace+ recombinant plasmids were sent for commercial automated sequencing. Analysis of the sequencing data was performed using Biology Workbench 3.2 (http://workbench.sdsc.edu/CGI/bw.cgi). Alignment of the *ace* gene sequence in this study was compared with the published sequences in the database under three accession numbers, Z22569, AF175708.1 (Shin *et al*, 1999) and AF220606.

Messenger RNA (mRNA) analysis

The preparation of mRNA from transfected, TOP10-Ace+ and LMG194-Ace+ *E. coli* were carried out using Micro-FastTrack 2.0 kit (Invitrogen Corp) following the manufacturer's instructions with slight modifications. The generation of cDNA was carried out using cDNA Cycle kit (Invitrogen Corp) following the manufacturer's instructions. Two µl of each cDNA product were subjected to PCR using the protocol as described above.

Expression of fusion protein

The transfected TOP10-Ace+ and LMG194-Ace+ *E. coli* were subjected to protein expression in order to determine which host strain is suitable for expression of *Ace* toxin. Several modifications were made to the manufacturer's procedure (Invitrogen, USA) in order to obtain expression. A single colony from TOP10 and LMG194 host strain containing the *ace* gene clone was selected from the Luria Bertani (LB) and RM [(M9 salts, 2% casamino acids, 0.2% glucose, 1 mM $MgCl_2$) Invitrogen, USA] media, respectively, containing 100 µg/ml ampicillin and inoculated overnight in media containing 150 µg/ml ampicillin at 37ºC with shaking until the OD_{600} reached 1.5. This was followed by inoculating each of the five tubes of

100 ml LB and RM broth (100-200 µg/ml ampicillin) with 0.1 ml of the overnight culture. These flasks were incubated at 29°C and 37°C with vigorous shaking until OD_{600} was 0.5. The cultures were then induced with different concentrations of arabinose (20, 2, 0.2, 0.02, and 0.002%) and were further incubated at 37°C. At various incubation periods (after 4, 24, 36, 48, 60, 66, 72, and 90 hours), 1 ml or 10 ml aliquots of the cultures were centrifuged (13,000 rpm, Eppendorf 5403) for 30 seconds to sediment the cells, which were then stored at -20°C.

Protein analysis

The thawed pellets from 10 ml of arabinose-induced cultures were resuspended in 4 ml of lysis buffer (50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole) and sonicated for about 20 minutes or until the cells were lysed. The sonicated cell suspensions were centrifuged (13,000 rpm, Eppendort 5403) for 1 minute at 4°C and the supernatants transferred into new tubes. The pellet and supernatant was treated with 1X SDS and 2X SDS gel-loading buffer, respectively, vortexed and heated at 95°C for 5 minutes. The treated pellet and supernatant were then loaded on a 10% SDS polyacrylamide gel in a Laemmli buffer system (Laemmli, 1970). The electrophoresed gel was then 'sandwiched' to polyvinylidane difluoride (PVDF) membrane in a transfer buffer in order to facilitate the transfer of the protein from gel to the membrane. The transfer was conducted for 1 hour at 90V. The transferred protein was detected using anti V5-HRP as a primary antibody (1:1250, Fermentas) and a Chromogenic Detection kit (Amresco).

<div style="text-align:center">RESULTS</div>

Amplification of *ace* gene

Genomic DNA of high molecular weight was successfully extracted from twenty isolates of *Vibrio cholerae* using the QIAGEN QIAamp Tissue kit. Polymerase chain reaction (PCR) with the specific primers was sensitive enough to detect 100 ng/µl of *V. cholerae* genomic DNA (data not shown). The accessory cholera enterotoxin (*ace*) gene was successfully amplified from 15 out of 20 *V. cholerae* isolates producing a single

Fig 1–Amplification of *ace* gene by PCR. The *ace g*ene positive isolates have a single band present in the region between the size ladders of 250 bp to 500 bp. The actual band position is at a 314 bp.

Fig 2–Hybridization of *ace* gene. The presence of the *ace* gene in Fig 1 was confirmed using an Ace probe and detected on x-ray film by chemiluminescence. Lanes 1-15 show the presence of the *ace* gene with a size of 314 bp and lane C is the negative control.

band of 314 bp (Fig 1). The presence of the *ace* gene was confirmed by hybridization with an Ace gene probe (Fig 2).

Cloning and transformation of *ace* gene

The PCR product containing the *ace* gene was ligated to pBAD/Thio-TOPO expression vector (Invitrogen, USA). The recombinant *ace* gene was transfected into TOP10 *E. coli* and then retransfected into, LMG194 *E. coli* strain. Confirmatory PCR was carried out using the Ace specific primers on fifteen colonies. Restriction enzyme analysis was also carried out on the recombinant plasmids containing the *ace* gene, using *Pme*1 and *Nco*1 enzymes. Plasmid was also included from untransformed *E. coli* as negative control. As expected, plasmids from transformants produced two fragments of approximately 783 bp (314 bp from *ace* plus 469 bp from the pBAD vector) and the pBAD/Thio vector of 4.4 kb (Fig 3). The results indicated that the *ace* gene had
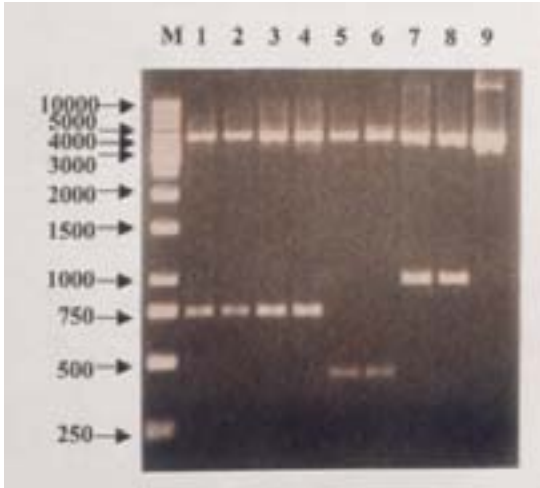
Fig 3–Restriction endonuclease digestion of pBAD/ TOP10 recombinant plasmids. Lanes 1-4 show recombinant clones producing two bands of 4.4 kb and 783 bp after digestion with *Pme*1 and *Nco*1. Lanes 5-6 (negative controls) and lanes 7-8 (positive controls) produced two bands of 4.4 kb and 469 bp and 4.4 kb and 969 bp, respectively. Lane 9 is undigested plasmid and lane M is DNA size markers (Fermentas, Germany).

been successfully cloned and transfected into both host cells. One clone from transformed TOP10 and LMG194 host, namely pBAD10/*ace*2 and pBADLMG/*ace*2, respectively, was selected and sequenced.

Analysis of clones

The sequences obtained from pBAD10/*ace*2 and pBADLMG/*ace*2 (which showed the same sequence) were compared to the sequence of *ace* gene in the database using NCBI Blast software in The Biology Workbench 3.2 under CLUSTER W program. The *ace* gene sequence in this study was aligned to the *ace* gene sequence under three accession numbers, Z22569, AF175708.1 and AF220606 and is shown in Fig 4. The alignments showed that the *ace* gene sequences obtained were highly homologous (97- 99%). In this study, the start codon of *ace* gene is GTG, valine instead of methionine, due to insertion of T and G after the first ATG. However, the effect of this change is currently unknown.

Reverse transcription-PCR (RT-PCR)

Messenger RNA (mRNA) was extracted

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Z22569       ATGCTTATGATGGACACCCTTTATGACTGGCTAATTGATGGCTTTACGTG
AF175708.1   ATGCTTATGATGGACACCCTTTATGACTGGCTAATTGATGGCTTTACGTG
AF220606_    ATGCTTATGATGGACACCCTTTATGACTGGCTAATTGATGGCTTTACGTG
Ace          ATGTGCTTATGATGGACACCCTTTATGACTGGCTAATTGATGGCTTTACGTG
             * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Z22569       CTTGTGATCAAGCTCGGTATTATGTGGATTGAGAGCAAGATTTTTGTTAT
AF175708.1   CTTGTGATCAAGCTCGGTATTATGTGGATTGAGAGCAAGATTTTTGTTAT
AF220606_    CTTGTGATCAAGCTCGGTATTATGTGGATTGAGAGCAAGATTTTTGTCAT
Ace          CTTGTGATCAAGCTCGGTATTATGTGGATTGAGAGCAAGATTTTTGTTAT
             * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *  * *

Z22569       CAATTCTTCTGGGAGATGTCCCAGAAAGTGATTGATATGTTTACCATCT
AF175708.1   CAATTCTTCTGGGAGATGTCCCAGAAAGTGATTGATATGTTTACCATCT
AF220606_    CAATTCTTCTGGGAGATGTCCCAAAAAGTGATTGATATGTTTACCATCT
Ace          CAATTCTTCTGGGAGATGTCCCAGAAAGTGATTGATATGTTTACCATTT
             * * * * * * * * * * * * * * * * * * * * * *  * * * * * * * * * * * * * * * * * * * * * *  *

Z22569       ATCCGCTTATCCAACAGGCTATCGATATGCTGCCTCCTCAATACAGCGGC
AF175708.1   ATCCGCTTATCCAACAGGCTATCGATATGCTGCCTCCTCAATACAGCGGC
AF220606_    ATCCGCTTATCCAACAGGCTATCGATATGCTGCCTCCTCAATACAGCGGC
Ace          ATCCGCTTATCCAACAGGCTATCGATATGCTGCCTCCTCAATACAGCGGC
             * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Z22569       TTTCTGTTCTTTTTAGGGGTTAGACCAAGCGCTGGCTATCGTGCTTCAG
AF175708.1   TTTCTGTTCTTTTTAGGGGTTAGACCAAGCGCTGGCTATCGTGCTTCAG
AF220606_    TTTCTTTTCTTTTTGGGGGTTAGACCAAGCGCTGGCTATCGTGCTTCAG
Ace          TTTCTGTTCTTTTTAGGGGTTAGACCAAGCGCTGGCTATCGTGCTTCAG
             * * * * *  * * * * * * * * *  * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

Z22569       GCTTTGATGACCCGTTTTGCCCTGCGAGCGTTAAACCTATGA
AF175708.1   GCTTTGATGACCCGTTTTGCCCTGCGAGCGTTAAACCTATGA
AF220606     GCTTTGATGACCCGTTTTGCCCTGCGAGCGTTAAACCTATGA
Ace          GCTTTGATGACCCGTTTTGCCCTGCGAGCGTTAAACCTATGA
             * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
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*single, fully conserved residue.

Fig 4–Sequence analysis of *ace* gene by ClusterTree (phylogenetic analysis with CLUSTAL W program).
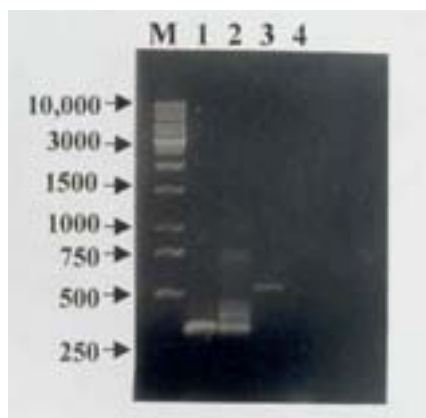
Fig 5–RT-PCR amplification of transformants expressing *ace* gene. Lanes 1 and 2 are RT-PCR products from pBADLMG/*ace*2 and pBAD10/*ace*2 transformant, respectively. Lane 3 and lane 4 are positive and negative controls, respectively. Lane M is DNA molecular weight markers (Fermentas).
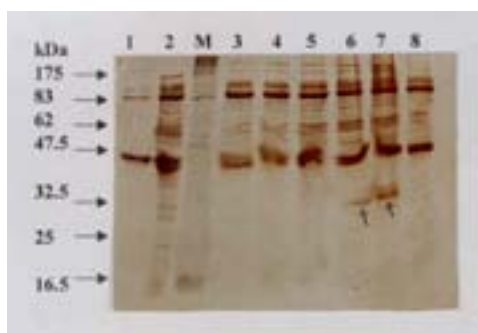

Fig 6–Analysis of proteins from pBADLMG/*ace*2 clone lysate by Western blot technique. Lane 1 shows sample at 0 hr induction time, 0.2% arabinose, lane 2 negative control, lane 3 at 4 hr, 0.2% arabinose, lane 4 at 4 hr, 2% arabinose, lane 5 at 24 hr, 0.2% arabinose, lane 6 at 60.5 hr, 0.2% arabinose, lane 7at 72 hr, 0.2% arabinose, lane 8 at 5 hr, 0.2% arabinose. Lane M is a broad range protein marker (Biolabs). The arrows show the 34 kDa band indicating the expression of *Ace* fusion protein.

from the pBAD10/*ace*2 and pBADLMG/*ace*2 transformants to determine if transcription of the *ace* gene had taken place. The RT-PCR was performed on the mRNA samples. The RT-PCR product was shown to be approximately the same size as the original amplified *ace* gene (314 bp in size)

indicating that transcription had occurred in the transformants (Fig 5). A single band of a size 500 bp was amplified from the mRNA positive control and no band was obtained from the mRNA negative control showing that amplified products were specific for the *ace* gene.

Expression of *Ace* fusion protein

Once the recombinant pBAD10/*ace*2 and pBADLMG/*ace*2 were confirmed to contain the *ace* gene in the correct orientation, expression of the *ace* gene product was performed. Several conditions for the expression were tested such as the temperature of induction, concentration of antibiotic, and concentration of arabinose. The *E. coli* host strain, TOP10, could not express the fusion protein under any of the conditions tested. However, the *E. coli* host strain, LMG194, was found to be a suitable host to express the *Ace* fusion protein in the presence of 200 µg/ml of ampicillin after induction with 0.2% arabinose. The induction time for this protein to be expressed was from 60 to 72 hours at 29ºC. These conditions produced the expected fusion protein of approximately 34 kDa (Fig 6) from 10 ml cell suspension. The fusion protein was not observed in the crude lysate pellet after sonication (20 minutes) in fresh lysis buffer, indicating the completion of lysis procedure.

DISCUSSION

The accessory cholera enterotoxin (*Ace*) has recently been identified as a third toxin of *Vibrio cholerae* (Trucksis *et al*, 1997; Aidara *et al*, 1998) which causes a milder cholera manifestation, increases the potential differences across intestinal epithelium and alters ion transport. In this study, the *ace* gene was successfully amplified in 15 out of 20 *V. cholerae* isolates as a single DNA fragment of 314 bp which corresponded to published data (Trucksis *et al*, 1993; Aidara *et al*, 1998; Damian *et al*, 1998). The presence of the *ace* gene in *V. cholerae* isolates was then confirmed by hybridization with a biotinylated oligonucleotide *Ace* probe. This is in accordance to Aidara *et al* (1998) who confirmed the presence of *ace* gene in *V. cholerae* 01 by using an *Ace* probe labeled with digoxigenin-11-dUTP.

Initially, the *ace* gene was cloned in 2.1-

TOPO vector and the *ace* construct was sent out for automated sequencing after the clone was confirmed by confirmative PCR and restriction endonuclease analysis. An obvious difference between *ace* sequence in this study and other published *ace* sequences (Z22569, AF175708.1 and AF220606) in the Genbank was an extra two bases, T and G after the first ATG. Using the program Biology Workbench 3.2, the sequence of *ace* gene in this study was aligned where the start codon of *ace* gene is GTG (valine) instead of ATG (methionine), with no change in the sequence of the amino acids. In *E. coli*, the ATG (AUG) triplet is normally used as a translation start codon, but, GTG is used in 8% of the genes (Baneyx, 1999).

Finally, the *ace* PCR product was directly cloned in the expression vector pBAD/Thio-TOPO and transfected into an *E. coli* host strain, TOP10. The construct pBAD10/*ace*2 was retransfected in LMG194 *E. coli* host strain since *ace* is a toxic gene. A previous study has shown that the *Ace* toxin was expressed efficiently in the methylotrophic yeast, *Pichia pastoris* (Trucksis *et al*, 1997) but in the TOP10 *E. coli* expression system the *ace* construct was unstable and resulted in cell lysis. According to Guzman *et al* (1995) the *E. coli* strain LMG194 allows for the additional repression of low basal level of toxic genes. The authors also noted that the expression of protein in LMG194 as a host strain and pBAD as a vector produced 11% of total protein and 10 mg of purified protein per liter of culture. In the present study, the expression of *Ace* toxin was successfully obtained from the *ace* construct transformed in LMG194 *E. coli* host strain but not in the TOP10 host strain.

In this study, the results showed that a low concentration of arabinose, 0.2%, was able to induce the expression of *Ace* in LB medium (in RM medium the final concentration was 0.02%) after 60 to 72 hours induction. The protein was not expressed at all with induction of twenty four hours or less. Sugrue *et al* (1997) reported that a longer or shorter induction time than the optimum duration would produce a low protein level which may be due to *in vivo* proteolytic degradation of the recombinant protein in the cytoplasm of *E. coli* by intracellular proteins. The fusion protein

had a size of approximately 34 kDa where the *Ace* protein's molecular weight is estimated to be 18 kDa (Trucksis *et al,* 1997) and approximately 16 kDa for the V5-epitope protein. The optimized condition that enabled the expression of the *Ace* fusion protein includes temperature for culturing at 29ºC with a high concentration of ampicillin, 200 µg/ml in the minimal medium. The incubation temperature was important for the toxin to be expressed because the lower temperature frequently improves the solubility of the yields (Weickert *et al*, 1996). According to Wang *et al* (2003), the *E. coli* LMG194 strain expressing protein at lower temperature can reduce inclusion bodies and increase protein insertion into membrane. The recovery of the recombinant protein is difficult when induced at higher temperatures such as 37ºC since the use of higher induction temperature would result in an insoluble form of recombinant proteins (Sugrue *et al*, 1997). According to Lohrke *et al* (2001), high level expression of *virBp*::*lacZ* from *A. tumefaciens* in *E. coli* was observed at a low temperature of 28ºC while no expression was seen at 37ºC. In another study, the expression of tetanus toxoid control by P_{BAD} was obtained at a very low temperature of 20ºC after 3 hours induction of 0.2% arabinose (Clark *et al*, 1997). In the present study, the fusion protein was detected from 10 ml of culture but not from culture volume of 1 ml as suggested by the manafacturer's protocol (Invitrogen, USA). This is in accordance with Trucksis *et al* (1997) who reported that the *Ace* protein that was expressed in methylotrophic yeast, *Pichia pastoris* at only 7 mg of *Ace* toxin per liter. The researchers also noted that the expression of *Ace* protein was highly dependent on the composition of the growth medium and the density of the culture at the time of induction of protein expression.

REFERENCES

Aidara KAS, Boye CS, Raphenon A, Gassama G, Grimont PA. Phenotypic and genotypic characterization of *Vibrio cholerae* isolates from a recent cholera outbreak in Senegal: comparison with isolates from Guinea-Bissau. *Am J Trop Med Hyg* 1998; 58: 163-7.

Baneyx F. Recombinant protein expression in *Escherichia coli*. *Cur Opin Biotechol* 1999; 10: 411-21.

Baudry B, Fasano A, Ketley J, Kaper JB. Cloning of a gene (*zot*) encoding a new toxin produced by *Vibrio cholerae*. *Infect Immun* 1992; 60: 428-34.

Clark MA, Hammond FR, Papaioannou A, Hawkins NJ, Ward RL. Regulation and expression of human Fabs under the control of the *Escherichia coli* arabinose promoter, P_{BAD}. *Immunotechnol* 1997; 3: 217-26.

Damian M, Koblavi S, Carle I, *et al*. Molecular characterization of *Vibrio cholerae* 01 strains isolated in Romania. *Res Microbiol* 1998; 149: 745-55.

Fasano A, Baundry B, Pumplin DW, *et al*. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. *Proc Nat Acad Sci USA* 1991; 88: 5242-6.

Ghosh A, Thungapathra M, Ghosh RK. Strategies for production of a potential candidate vaccine for cholera. *Indian J Med Res* 1996; 104: 60-75.

Guzman LM, Berlin D, Carson MJ, Beckwith J. Tight regulation, modulation and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* 1995; 177: 4121-30.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-86.

Lohrke SM, Yang HJ, Jin SG. Reconstitution of acetosyringone-mediated *Agrobacterium tumefaciens* virulence gene expression in the heterolo-gous host *Escherichia coli. J Bacteriol* 2001; 183: 3704-11.

Nishimura A, Morita M, Nishimura Y, Sugino Y. A rapid and highly efficient method for preparation of competent *Escherichia coli* cells. *Nucleic Acids Res* 1990; 18: 6169.

Norris HT. Cholera. In: Barua D, Burrows W, eds. The pathology of cholera. Philadelphia: The WB Saunders, 1974: 160-88.

Rabbani GH, Greenough WB. Cholera, In: Lebenthal E, Duffy M, eds. Textbook of secretory diarrhea. New York: Raven Press, 1990: 233-53.

Shin HJ, Park YC, Kim YC. Cloning and nucleotide sequence analysis of the virulence gene cassette from *Vibrio cholerae* KNIH002 isolated in Korea. *Misainmurhag Hoiji* 1999; 35: 205-10.

Sugrue RJ, Cui T, Xu Q, Fu J, Chan YC. The production of recombinant dengue virus E protein using *Escherichia coli* and *Pichia pastoris*. *J Virol Methods* 1997; 69: 159-69.

Trucksis M, Conn TL, Fasano A, Kaper JB. Production of *Vibrio cholerae* accessory cholera enterotoxin (ACE) in the yeast *Pichia pastoris*. *Infect Immun* 1997; 65: 4984-8.

Trucksis M, Galen JE, Michalski J, Fassano A, Kaper JB. Accessory cholera enterotoxin (*Ace*), the third toxin of a *Vibrio cholerae* virulence cassette. *Proc Nat Acad Sci USA* 1993; 90: 5267-71.

Wang DN, Safferling M, Lemieux MJ, Griffith H, Chen Y, Li XD. Practical aspects of overexpression bacterial secondary membrane transporters for structural studies. *Biohim Biophysic Acta* 2003; 1610: 23-36.

Weickert MJ, Doherty DH, Best EA, Olins PO. Optimization of heterologous protein production in *Escherichia coli*. *Curr Opin Biotechnol* 1996; 7: 494-9.