

# ROLE OF *hlyA*-POSITIVE *VIBRIO CHOLERAE* NON-O1/NON-O139 ON APOPTOSIS AND CYTOTOXICITY IN A CHINESE HAMSTER OVARY CELL LINE

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**Abstract.** *Vibrio cholerae* non-O1/non-O139 is capable of producing sporadic outbreaks of cholera-like diarrhea; however, the pathogenic mechanisms of this bacterium remain unclear. The objectives of this study were to: 1) compare the apoptosis induction and cytotoxicity between *hlyA*-positive and *hlyA*-negative strains of *V. cholerae* non-O1/non-O139; 2) clarify the molecular mechanisms by which these strains induce apoptosis; and 3) compare clinical and environmental *V. cholerae* non-O1/non-O139 isolates with respect to cytotoxicity and ability to induce apoptosis. Using cytotoxicity and apoptosis assays, it was shown that *hlyA*-positive strains of *V. cholerae* non-O1/non-O139 had significantly higher cytotoxic activity (70.6%) and levels of apoptosis induction (59.6%) than *hlyA*-negative strains (37.0% and 37.5%, respectively). Western blot analyses revealed that *hlyA*-positive strains had significantly increased expression of Bax, active caspase-3 and -9; and significantly decreased expression of NF- $\kappa$ B and Bcl-2 relative to *hlyA*-negative strains. Expression of BID did not differ significantly between *hlyA*-positive and negative strains. The truncated BID was not found, indicating that *V. cholerae* non-O1/non-O139 induces apoptosis through a mitochondria-dependent apoptosis pathway and not an extrinsic pathway. *V. cholerae* non-O1/non-O139 isolated from clinical sources exhibited significantly higher cytotoxic activity (79%) and levels of apoptosis induction (65.2%) than bacteria isolated from environmental sources (63% and 54.6%, respectively), suggesting that the clinical isolates may have other virulence-associated genes besides *hlyA*. Our results indicate that *hlyA* products play a role in cytotoxicity and apoptosis induction and that a mitochondria-dependent apoptosis pathway is involved.

**Keywords:** apoptosis, cytotoxicity, enzyme, *hlyA*, toxin, *Vibrio cholerae* non-O1/non-O139

## INTRODUCTION

Toxigenic *Vibrio cholerae* O1 and O139 are a major cause of cholera disease, but *V. cholerae* non-O1/non-O139 strains can cause cholera-like disease despite their inability to produce cholera toxin, zonula

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occludens toxin and accessory cholera toxin (Ulloa *et al*, 2011; Kadkhoda *et al*, 2012). However, the mechanisms by which non-O1/non-O139 strains induce pathology have not been clearly elucidated.

Most *V. cholerae* O1 and non-O1/non-O139 strains carry the *hlyA* gene encoding the HlyA protein also known as *V. cholerae* cytotoxin (VCC) (Yamamoto *et al*, 1984; Figueroa-Arredondo *et al*, 2001). HlyA (VCC) has a number of effects on infected mammals—*viz*, hemolysis and cell lysis (Figueroa-Arredondo *et al*, 2001; Zhang and Austin, 2005); cytotoxicity (Bidinost *et al*, 2004). It can also cause fluid accumulation (in rabbit ileal loops) (Debellis *et al*, 2009). In addition, HlyA is also reported to be associated with apoptosis induction through caspase-3 activation in Caco-2 cells and peritoneal B-1a cells (Mukherjee *et al*, 2008; Saka *et al*, 2008) although the molecular mechanism by which *V. cholerae* non-O1/non-O139 achieves, this has not been clearly elucidated. In addition, the activities of *V. cholerae* non-O1/non-O139 isolated from different sources have not been characterized in Thailand.

Apoptosis can be induced through two major pathways. The intrinsic (mitochondrial) pathway is triggered by the perturbation of mitochondrial membrane permeability as a result of the intracellular signal which stimulates the Bcl-2 family proteins, including pro-apoptosis protein (*ie*, Bax) and anti-apoptosis protein (*ie*, Bcl-2), causes the release of cytochrome C from mitochondria into cytosol. Cytochrome C forms complex with apoptotic-protease-activating factor-1 (Apaf-1) and procaspase-9 called apoptosome leading to the activation of caspase-9, caspase-3 and apoptosis cell death (Bras *et al*, 2005). The extrinsic pathway is mediated through the activation of death receptors on cell surface leading to the activation

of caspase-8, caspase-3 and apoptosis cell death. Along this pathway, the pro-apoptotic protein Bid is cleaved by activated caspase-8 yielding truncated Bid (tBid). The tBid induces the oligomerization of Bax which then translocate to the mitochondrial outer membrane where it participates in pore formation and subsequent apoptosis induction through intrinsic pathway (Danial and Korsmeyer, 2004). Previously, the effect of cholera toxin on the “*in vitro*” apoptosis, PKA activation, Bax and Bcl-2 expression was evaluated (Pessina *et al*, 2006), however, the HlyA induced the apoptosis molecules and which pathway has not been clearly elucidated.

The aims of this study were to: 1) compare apoptosis induction and cytotoxicity between *hlyA*-positive and *hlyA*-negative strains of *V. cholerae* non-O1/non-O139; 2) determine the levels of expression of pro-apoptotic molecules (Bax, caspase-9, caspase-3 and BID) and anti-apoptotic molecules (NF- $\kappa$ B and Bcl-2) of these *V. cholerae* strains in the Chinese hamster ovary (CHO) cell line; and 3) compare the cytotoxicity and apoptosis inducing activities of *V. cholerae* non-O1/non-O139 isolated from clinical and environmental sources.

## MATERIALS AND METHODS

### Bacterial strains

*Vibrio cholerae* non-O1/non-O139 strains used in this study were 32 clinical isolates, of which 27 were *hlyA*-positive and five were *hlyA*-negative; and 30 environmental isolates that were all *hlyA*-positive. All the strains used were *toxR*-positive but *ctxA*, *tcpA*, *zot*, *ace* and *stn*-negative.

For detection of the apoptotic pathway, the *V. cholerae* non-O1/non-O139 strains used included four isolates, of

which two were *hlyA*-positive with the highest apoptosis induction activity and two were *hlyA*-negative with the lowest apoptosis induction according to three methods shown in Table 2.

#### Detection of *hlyA* by PCR

DNA was extracted from *V. cholerae* non-O1/non-O139 using the boiling method. The primer pair for amplification of the *hlyA* gene was F- TTAGCTGAGCTGCGCGATTTG and R- GAGTTGATCATTGAGA (Chomvarin *et al*, 2008).

PCR reactions were conducted in a 25- $\mu$ l reaction mixture containing 200 ng of target DNA, 1X PCR buffer, 0.2 mM of each deoxynucleoside triphosphate (dNTP) (Amresco, Solon, OH), 0.2  $\mu$ M of each primer, and 1.25 U of *Taq* polymerase (RBC bioscience, Taipei, Taiwan). The amplification was performed using a thermalcycler (Veriti 96 well Thermo Cycler, Applied Biosystems; Amresco, Solon, OH) as follows: 95°C for 2 minutes, 25 cycles of 95°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute and a final hold at 72°C for 5 minutes (Chomvarin *et al*, 2008). The amplified product was subjected to 1.5% agarose gel electrophoresis and stained with ethidium bromide. The band was examined using a UV light transilluminator. DNA extracted from *V. cholerae* O1 *hlyA*-positive was used as a positive control and sterile distilled water as a negative control.

#### Culture supernatant preparation

*V. cholerae* non-O1/non-O139 was inoculated into the brain heart infusion (BHI) broth and incubated at 37°C for 16 hours under continuous shaking. Cultures were then centrifuged at 12,000g at 4°C for 20 minutes and the supernatants were filtrated through 0.22- $\mu$ m filters. The concentration of protein was determined using the Bradford method (Bidinost *et al*, 2004).

#### Cell culture

Chinese hamster ovary (CHO) cells were cultured in Ham's F12 medium (Gibco, BRL, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum and 100 U/ml of penicillin and 100  $\mu$ g/ml streptomycin (Gibco, BRL) at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

#### Cytotoxicity assay

The cytotoxic assay was performed according to Begum *et al* (2006). CHO cells (10<sup>4</sup>cells/well) were seeded into 96-well microtiter plates and incubated at 37°C for 16 hours. Then, 100  $\mu$ l of culture supernatants of each strain were added to the wells in triplicate and incubated at 37°C for 4 hours. Cell morphological changes were observed (for necrosis, elongation and rounding) using a bright-field inverted Nikon microscope. Cytotoxicity was assessed as the percentage of cells showing any of these morphological changes, averaged over the three wells for each strain.

#### Detection of apoptosis-inducing activity

**Ethidium bromide/acridine orange (EB/AO) staining.** The method of EB/AO staining was performed according to Ribble *et al* (2005). CHO cells (10<sup>4</sup>cells/well) were cultured for 16 hours in 96-well microtiter plates, and then treated with 100  $\mu$ l (36  $\mu$ g) of supernatant from each culture for 4 hours. After treatment, 14  $\mu$ l of 100  $\mu$ g/ml ethidium bromide/acridine orange (EB/AO) mixture (Sigma, St Louis, MO) was added to each well and the apoptotic cells examined for condensed chromatin or fragmented chromatin using a Nikon fluorescent microscope. Apoptotic cells were randomly counted from a total of 500 cells and expressed as a percentage.

**Caspase-3 activity.** Caspase-3 activity was measured using caspase-3 (CASP-3-C)

Table 1  
Cytotoxic activity in *V. cholerae* non-O1/non-O139.

<i>hlyA</i> status	Strain	Morphological characteristic in cytotoxic activity			Cytotoxic activity (%)
		Elongation (%)	Rounding (%)	Necrosis (%)	
<i>hlyA</i> <sup>+</sup>	Clinical isolates ( <i>n</i> =27)	4	41	34	79 <sup>a</sup>
	Environmental isolates ( <i>n</i> =30)	3	43	17	63 <sup>a</sup>
	Total average ( <i>n</i> =57)	3.5	42	25.1	70.6 <sup>b</sup>
<i>hlyA</i> <sup>-</sup>	Clinical isolates ( <i>n</i> =5)	6	21	10	37 <sup>b</sup>

<sup>a</sup>Significantly higher in clinical isolates than environmental isolates ( $p < 0.01$ ); <sup>b</sup>Significantly higher in *hlyA*-positive *V. cholerae* non-O1/non-O139 strains than in *hlyA*-negative strains ( $p < 0.01$ ).

apoptosis detection colorimetry (Sigma), according to the manufacturer's instructions. CHO cells ( $10^4$  cells/well) were grown in 96-well microtiter plates at 37°C for 16 hours, and treated with 100  $\mu$ l (36  $\mu$ g) of supernatant of each culture for 4 hours. Cells were washed twice with phosphate buffer saline (PBS) and centrifuged at 600g for 5 minutes. Cells were re-suspended in 100  $\mu$ l of cell lysis buffer, incubated on ice for 20 minutes and centrifuged at 16,000g for 10 minutes. The supernatant was transferred to new wells and mixed with assay buffer. Then, 10  $\mu$ l of caspase-3 substrate (Ac-DEVD-pNA (N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide) was added and the wells were incubated at 37°C for 2 hours. The absorbance of each well was measured for caspase-3 activity using ELISA plate reader (Sunrise-TECAN, Morrisville, NC) at 405 nm. Three independent experiments were performed.

**DNA fragmentation.** DNA fragmentation was determined using a Cell Death Detec-

tion ELISA Plus kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. CHO cells ( $10^4$  cells/well) were grown in 96-well microtiter plates and incubated at 37°C for 16 hours, then treated with 36  $\mu$ g of supernatant from each culture for 4 hours. After treatment, the cells were pelleted by centrifugation at 200g for 10 minutes. The supernatant was discarded. Cells were re-suspended with lysis buffer for 30 minutes and centrifuged at 200g for 10 minutes. The supernatants (20  $\mu$ l) were transferred to a streptavidin-coated well of a microtiter plate. Then, 80  $\mu$ l of immunoreagent was added into each well and incubated for 2 hours. After removing the solution, each well was rinsed with incubation buffer. Then, 100  $\mu$ l of ABTS solution was added to each well and incubated for 20 minutes. To stop the reaction, 100  $\mu$ l of ABTS stop solution was added; after which absorbance was measured using an ELISA plate reader (Sunrise-TECAN, Mannedorf, Switzerland) at 405 nm.

Table 2  
Apoptosis induction by strains of *V. cholerae* non-O1/non-O139.

<i>hlyA</i> status	Strain	Average of each activity			Average of three methods
		EB/AO (%)	Caspase-3 (%)	DNA fragmentation (%)	
<i>hlyA</i> <sup>+</sup>	Clinical isolates (n=27)	65.5	63.5	66.7	65.2 <sup>a</sup>
<i>hlyA</i> <sup>+</sup>	Environmental isolates (n=30)	54.6	53.1	56.3	54.6 <sup>a</sup>
	Total average (n=57)	59.8	58.0	61.2	59.6 <sup>b</sup>
<i>hlyA</i> <sup>-</sup>	Clinical isolates (n=5)	36.7	37	39	37.5 <sup>b</sup>

<sup>a</sup>*p* < 0.01 significantly higher in clinical isolates than environmental isolates; <sup>b</sup>*p* < 0.01 significantly higher in *hlyA*-positive *V. cholerae* non-O1/non-O139 strains than in *hlyA*-negative strains.

### Detection of apoptotic pathway

**Protein extraction and western blot analysis.** Protein extraction was performed according to Niles *et al* (2003). In brief, 10<sup>6</sup> cells were seeded into 100-mm petri dishes for 16 hours, and treated with 36 µg of supernatant from each culture for four hours. After treatment, cells were washed with cold PBS and lysed in ice-cold lysis buffer. The cell lysate was homogenized and cleared by centrifugation at 10,000g for 30 minutes. Protein concentration was determined using Coomassie Protein Assay Kit then the protein was separated by SDS-polyacrylamide gel electrophoresis and electro-transferred to a Hybond ECL nitrocellulose membrane. The membrane was blocked with non-fat dry milk at 37°C for one hour, followed by incubation with the primary antibodies against Bax, Bcl-2, NF-κB, β-actin, caspase-9, BID (Santa Cruz Biotechnology, CA), and active caspase-3 (Sigma) at 4°C overnight, then re-incubated with the corresponding horseradish peroxidase-conjugated

secondary antibody at room temperature for one hour. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (Thermo Scientific, Rockford, IL). The intensity of the protein bands was quantified using Scion Image software (<http://softadvice.informer.com/>).

### Statistical analysis

The student's *t*-test was used for statistical analysis and a *p*-value < 0.05 was considered significant.

## RESULTS

### Cytotoxic activity

Fig 1 illustrates the cytotoxicity of the culture supernatants of *V. cholerae* non-O1/non-O139 *hlyA*-positive and negative strains on CHO cells inferred using the cytological parameters, elongation, rounding and necrosis. The cytotoxicity of *V. cholerae* non-O1/ non-O139 *hlyA*-positive strains was significantly higher (70.6%) than that of *hlyA*-negative strains (37%)



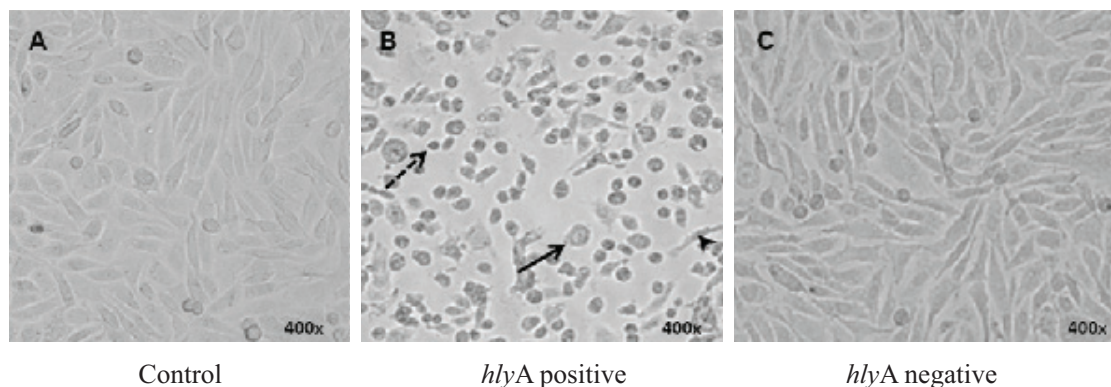


Fig 1—Determination of cytotoxic activity against CHO cells. A. Control CHO cells. B. CHO cells treated with the supernatant of cultures of *hlyA*-positive *V. cholerae* non-O1/non-O139. C. CHO cells treated with the supernatant of cultures of *hlyA*-negative *V. cholerae* non-O1/non-O139. Arrowhead indicates cell elongation, dash arrow indicates cell rounding and arrow indicates cell necrosis.

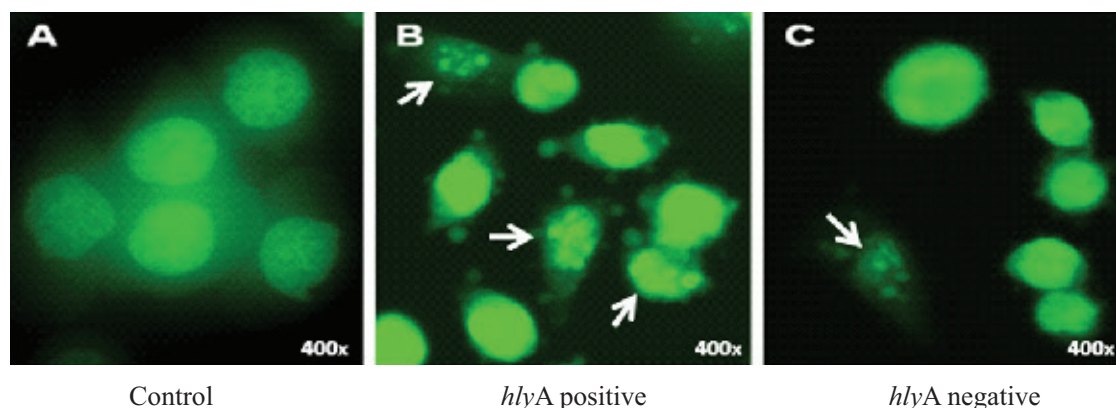


Fig 2—Apoptosis induction of *V. cholerae* non-O1/non-O139 in CHO cells using EB/AO staining. A. Control CHO cells. B. CHO cells treated with supernatant of cultures of *hlyA*-positive *V. cholerae* non-O1/non-O139. C. CHO cells treated with supernatant of cultures of *hlyA*-negative *V. cholerae* non-O1/non-O139. White arrows indicate the apoptotic body.

( $p < 0.01$ ), and the cytotoxicity of clinical isolates was significantly greater (79%) than that of environmental isolates (63%) ( $p < 0.01$ ) (Table 1).

#### Apoptosis induction

Table 2 presents data on the ability of *V. cholerae* non-O1/non-O139 *hlyA*-positive and *hlyA*-negative strains to induce apoptosis on CHO cells examined using EB/AO staining, caspase-3 activity and DNA

fragmentation. The apoptosis-inducing activity of *hlyA*-positive strains was significantly higher than that of *hlyA*-negative strains by all three methods (Table 2). The apoptosis induction of *V. cholerae* non-O1/non-O139 in CHO cells using EB/AO staining is shown in Fig 2. The average apoptosis-inducing activity of the clinical isolates was significantly higher than that of the environmental isolates by all three

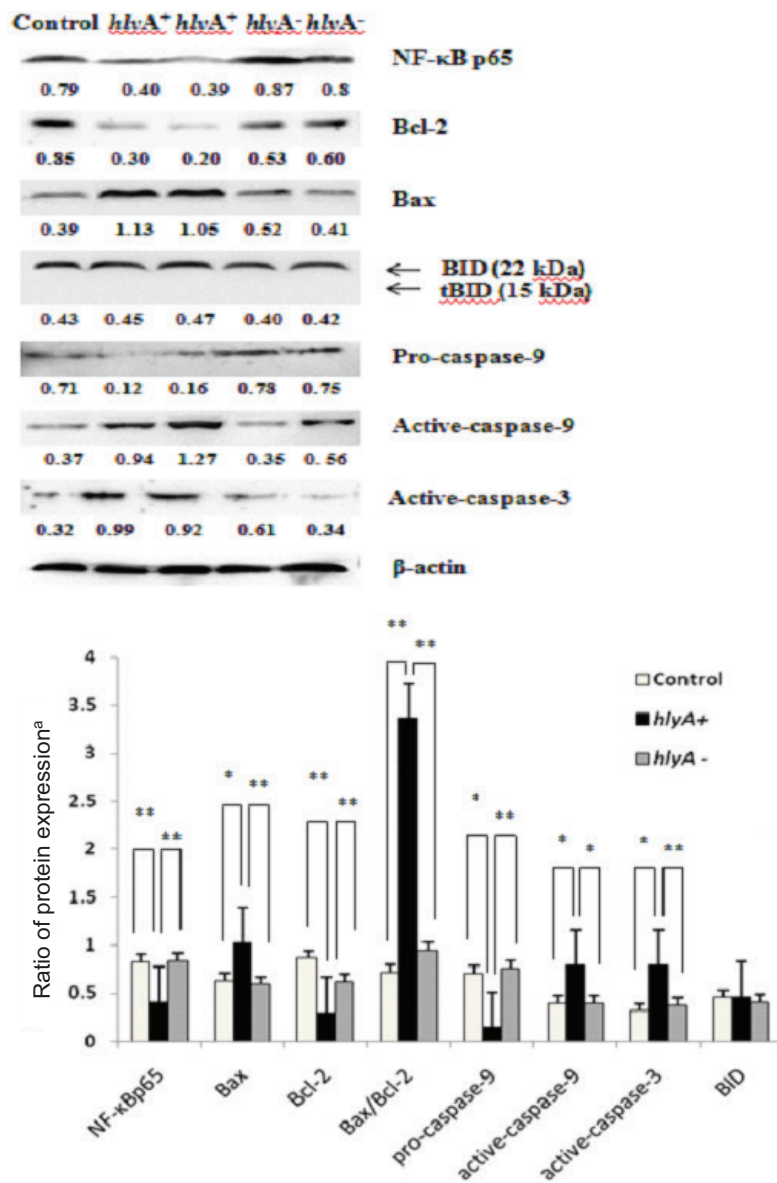


Fig 3—Assessment of apoptosis related-protein expression in CHO cells treated with the supernatants of *hlyA*-positive and negative strains of *V. cholerae* non-O1/non-O139. Upper panel: images of protein bands as revealed by western blots. Numbers below each band indicate the fold-change of treated cells compared to control cells (mean  $\pm$  SE) in the independent experiments ( $n = 3$ ). Lower panel: <sup>a</sup>the ratio between the density of each protein band and the density of  $\beta$ -actin for each experimental treatment. Pairwise  $p$ -values are indicated above each histogram.

<sup>a</sup> $p < 0.05$  and  $**p < 0.01$ .

methods (Table 2).

### Apoptosis induction pathway

To further elucidate the apoptosis mechanism of *V. cholerae* non-O1/non-O139 *hlyA*-positive and *hlyA*-negative strains on CHO cells, the level of apoptosis-related protein expression was examined using western blot analysis. Regarding the intrinsic apoptosis pathway, exposure of cells to the supernatant of *V. cholerae* non-O1/non-O139 *hlyA*-positive strains resulted in a significant decrease in the levels of NF- $\kappa$ B and Bcl-2, and a significant increase in the levels of Bax relative to changes induced by *hlyA*-negative strains (Fig 3). Changes induced by *hlyA*-positive strains would lead to an enhanced Bax/Bcl-2 ratio and activation of caspase-9 and caspase-3. The levels of active caspase-9 and active caspase-3 in cells treated with *hlyA*-positive strains were significantly higher than in cells treated with *hlyA*-negative strains (Fig 3). Regarding the extrinsic apoptosis pathway, the expression levels

of BID were not significantly different between cells treated with *hlyA*-positive and *hlyA*-negative strains of *V. cholerae* and the truncated BID was not found.

## DISCUSSION

The pathogenesis mechanism of the cholera-like syndrome produced by *V. cholerae* non-O1/non-O139 is not well understood. We therefore, examined the cytotoxic activities and apoptosis induction of *V. cholerae* non-O1/non-O139 with and without the *hlyA* gene in order to increase understanding of this pathogenesis.

The HlyA forms heptameric oligomers in the presence of cholesterol and ceramide-rich membranes, generating membrane pores (Zitser *et al*, 1995; Krasilnikov *et al*, 2007). These pores lead to changes in the permeability of host cells, affecting enterotoxicity, cytotoxicity, the lysis of erythrocytes, and can be lethal in mice (Zitser *et al*, 1995; Figueroa-Arredondo *et al*, 2001; Zhang and Austin, 2005). Notably, HlyA induced fluid accumulation and histopathological damage when a strain of *V. cholerae* non-O1 was injected into rabbit ileal loops (Saka *et al*, 2008). These cytotoxic effects are pathogenic and contribute to the virulence of *V. cholerae* non-O1/non-O139 strains.

Apoptosis induction is one of the most important mechanisms in the pathogenesis of *V. cholerae*. HlyA is a pore-forming toxin, similar to the alpha-toxin of *S. aureus* and listeriolysin O of *L. monocytogenes*, both of which can induce apoptosis in various cell types (Bantel *et al*, 2001; Carrero *et al*, 2004). Like other cytolytins, HlyA can induce apoptosis through caspase-3 activation (Saka *et al*, 2008) whereas Mukherjee *et al* (2008) reported that HlyA can induce apoptosis through activation of caspase-9 and cas-

pase-3 but not caspase-8.

We investigated the expression levels of other apoptosis regulated proteins—*ie*, the pro-apoptotic proteins (Bax, BID, caspase-9 and caspase-3) and anti-apoptotic proteins (NF- $\kappa$ B and Bcl-2)—and compared their expression between *hlyA*-positive and negative strains. The Bcl-2 family of proteins plays a major role in apoptosis regulation through the mitochondrial (intrinsic) pathway (Duprez *et al*, 2009). In the current study, the level of the pro-apoptotic protein, Bax, increased significantly whereas those of the anti-apoptotic proteins Bcl-2 and NF- $\kappa$ B decreased significantly, leading to an increased Bax/Bcl-2 ratio and the release of cytochrome c via pore-forming activity at the mitochondrial outer membrane. This is significant because cytochrome c forms a complex with Apaf-1 and pro-caspase-9, leading to activation of caspase-9 and caspase-3 (Duprez *et al*, 2009).

We also examined the expression of BID protein in order to confirm that HlyA is not involved in the extrinsic apoptosis pathway. The activation of caspase-8—which is involved in the extrinsic pathway—results in the cleavage of BID into truncated BID (tBID) (Duprez *et al*, 2009). The results showed that the expression of BID was not significantly different between cells treated with *hlyA*-positive and negative strains and there was no expression of tBID protein. These results strongly imply that *V. cholerae* non-O1/non-O139 *hlyA*-positive strains induced apoptosis at least in part through the mitochondrial pathway and not through an extrinsic pathway.

Our findings agree with previous studies on other pore-forming toxins; including the *Vibrio vulnificus* cytotoxin (Zhao *et al*, 2009), the EHEC hemolysin of enterohaemorrhagic *Escherichia coli*



(Bielaszewska *et al*, 2013) and the  $\alpha$ -toxin of *Staphylococcus aureus* (Bantel *et al*, 2001), all of which can activate apoptosis induction through a caspase-9/3-dependent pathway. Notwithstanding, the apoptosis induction pathway was different compared to other enteropathogenic bacteria, including *Shigella* and *Salmonella*, which activate macrophage apoptosis through a caspase-1-dependent pathway (Hilbi *et al*, 1998; Hersh *et al*, 1999) and a caspase-1-independent pathway (Suzuki *et al*, 2005; Grant *et al*, 2008). Evidently, diverse signaling pathways are involved in apoptosis and the pathway for apoptosis induction may depend upon each causative organism and the target cells.

In the present study, the *hlyA*-positive strains of *V. cholerae* had significantly higher cytotoxicity and apoptosis induction levels than *hlyA*-negative strains, as described by Bidinost *et al* (2004), Saka *et al* (2008) and Ottaviani *et al* (2009). Additionally, the cytotoxic activity and apoptosis induction levels produced by the clinical isolates were significantly higher than those by the environmental isolates, as described by Kashimoto *et al* (2003) and Ottaviani *et al* (2009). Although the *hlyA*-negative strains in our study were only isolated from clinical sources, the results confirm that *hlyA* products can be involved in cytotoxic activity and apoptosis induction. Clinical isolates likely have virulence factors in addition to HlyA/VCC, thus enhancing cytotoxic and pro-apoptotic activities for example, *V. cholerae* WO7 can produce WO7-toxin that increases intracellular  $\text{Ca}^{2+}$  and can activate endonucleases, leading to apoptosis induction in HEP-2 cells (Bhattacharyya *et al*, 2004, 2008).

In conclusion, the *hlyA* gene product plays important roles in the cytotoxic activity and apoptosis induction, contribut-

ing to the virulence of *V. cholerae* non-O1/non-O139 strains. At the molecular level, our results confirm that *hlyA* products induce apoptosis through a mitochondria-dependent pathway and not through an extrinsic one. *V. cholerae* non-O1/non-O139 isolated from clinical cases had significantly greater cytotoxic activity and apoptosis-inducing activity than did environmental isolates, indicating that the clinical isolates may have more potential for expressing virulence factors or that they may have other virulence factors.

#### ACKNOWLEDGEMENTS

The authors thank Dr Potjanee Sri-manote (Graduate Program, Faculty of Allied Health Sciences, Thammasat University, Pathum Thani, Thailand) and Dr Munirul Alam (International Center for Diarrhoeal Disease Research, Bangladesh (icddr), Dhaka, Bangladesh) for providing the *hlyA* negative strains; Dr Sunee Sirivichayakul, (Division of Allergy and Clinical Immunology, Department of Medicine, Faculty of Medicine, Chulalongkorn University) for providing the CHO cell line and Mr Bryan Roderick Hamman for assistance with the English-language presentation of the manuscript. This project was partially supported by research grants from the Thailand Research Fund through the Royal Golden Jubilee PhD Program (Grant No.PHD/0178/2549) given to Chariya Chomvarin and Boon-apa Kanoktippornchai and Khon Kaen University, Thailand.

#### REFERENCES

- Bantel H, Sinha B, Domschke W, Peters G, Schulze-Osthoff K, Janicke RU.  $\alpha$ -Toxin is a mediator of *Staphylococcus aureus*-induced cell death and activates caspases via the intrinsic death pathway

- independently of death receptor signaling. *J Cell Biol* 2001; 155: 637-48.
- Begum KAC, Ansaruzzaman M, Dutta DK, Ahmad QS, Talukder KA. Toxin(s), other than cholera toxin, produced by environmental non-O1 non-O139 *Vibrio cholerae*. *Cell Mol Immunol* 2006; 3: 115-21.
- Bhattacharyya S, Ghosh S, Shant J, Ganguly NK, Majumdar S. Effect of W07-toxin on gut physiological response in mice. *Microb Pathog* 2004; 37: 1-9.
- Bhattacharyya S, Shant J, Ganguly NK, Majumdar S, Ghosh S. A potential epidemic factor from the bacteria, *Vibrio cholerae* W07. *Curr Microbiol* 2008; 56: 98-103.
- Bidinost C, Saka HA, Aliendro O, et al. Virulence factors of non-O1 non-O139 *Vibrio cholerae* isolated in Cordoba, Argentina. *Rev Argent Microbiol* 2004; 36: 158-63.
- Bielaszewska M, Ruter C, Kunsmann L, et al. Enterohemorrhagic *Escherichia coli* hemolysin employs outer membrane vesicles to target mitochondria and cause endothelial and epithelial apoptosis. *PLoS Pathog* 2013; 9: e1003797.
- Bras M, Queenan B, Susin SA. Programmed cell death via mitochondria: different modes of dying. *Biochemistry (Mosc)* 2005; 70: 231-9.
- Carrero JA, Calderon B, Unanue ER. Listeriolysin O from *Listeria monocytogenes* is a lymphocyte apoptogenic molecule. *J Immunol* 2004; 172: 4866-74.
- Chomvarin C, Jumroenjit W, Chaicumpar K, Namwat W. Association of *ompU* gene in *Vibrio cholerae* from patients and environment with bile resistance. *Southeast Asian J Trop Med Public Health* 2008; 39: 876-81.
- Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004; 116: 205-19.
- Debellis L, Diana A, Arcidiacono D, et al. The *Vibrio cholerae* cytolysin promotes chloride secretion from intact human intestinal mucosa. *PLoS One* 2009; 4: e5074.
- Duprez L, Wirawan E, Vanden Berghe T, Vandenabeele P. Major cell death pathways at a glance. *Microbes Infect* 2009; 11: 1050-62.
- Figueroa-Arredondo P, Heuser JE, Akopyants NS, et al. Cell vacuolation caused by *Vibrio cholerae* hemolysin. *Infect Immun* 2001; 69: 1613-24.
- Grant AJ, Sheppard M, Deardon R, et al. Caspase-3-dependent phagocyte death during systemic *Salmonella enterica* serovar Typhimurium infection of mice. *Immunology* 2008; 125: 28-37.
- Hersh D, Monack DM, Smith MR, Ghori N, Falkow S, Zychlinsky A. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc Natl Acad Sci U S A* 1999; 96: 2396-401.
- Hilbi H, Moss JE, Hersh D, et al. *Shigella*-induced apoptosis is dependent on caspase-1 which binds to IpaB. *J Biol Chem* 1998; 273: 32895-900.
- Kadkhoda K, Adam H, Gilmour MW, Hammond GW. Nontoxicogenic *Vibrio cholerae* septicemia in an immunocompromised patient. *Case Rep Infect Dis* 2012; 2012: 698746.
- Kashimoto T, Ueno S, Hanajima M, et al. *Vibrio vulnificus* induces macrophage apoptosis in vitro and in vivo. *Infect Immun* 2003; 71: 533-5.
- Krasilnikov OV, Merzlyak PG, Lima VL, Zitzer AO, Valeva A, Yuldasheva LN. Pore formation by *Vibrio cholerae* cytolysin requires cholesterol in both monolayers of the target membrane. *Biochimie* 2007; 89: 271-7.
- Mukherjee G, Biswas A, Banerjee KK, Biswas T. *Vibrio cholerae* hemolysin is apoptogenic to peritoneal B-1a cells but its oligomer shepherd the cells for IgA response. *Mol Immunol* 2008; 45: 266-70.
- Niles RM, McFarland M, Weimer MB, Redkar A, Fu YM, Meadows GG. Resveratrol is a potent inducer of apoptosis in human melanoma cells. *Cancer Lett* 2003; 190: 157-63.
- Ottaviani D, Leoni F, Rocchegiani E, et al. Prevalence and virulence properties of non-O1 non-O139 *Vibrio cholerae* strains from seafood and clinical samples collected in Italy. *Int J Food Microbiol* 2009; 132: 47-53.

- Pessina A, Croera C, Savalli N, *et al.* Bcl-2 down modulation in WEHI-3B/CTRES cells resistant to Cholera Toxin (CT)-induced apoptosis. *Cell Res* 2006; 16: 306-12.
- Ribble D, Goldstein NB, Norris DA, Shellman YG. A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnol* 2005; 5: 12.
- Saka HA, Bidinost C, Sola C, *et al.* *Vibrio cholerae* cytolysin is essential for high enterotoxicity and apoptosis induction produced by a cholera toxin gene-negative *V. cholerae* non-O1, non-O139 strain. *Microb Pathog* 2008; 44: 118-28.
- Suzuki T, Nakanishi K, Tsutsui H, *et al.* A novel caspase-1/toll-like receptor 4-independent pathway of cell death induced by cytosolic *Shigella* in infected macrophages. *J Biol Chem* 2005; 280: 14042-50.
- Ulloa FM, Porte L, Braun JS, *et al.* [Acute gastroenteritis caused by a *Vibrio cholerae* non-O1, non-O139 strain harboring a genetic region homologous to the Vpa1-7 pathogenicity island]. *Rev Chilena Infectol* 2011; 28: 470-3.
- Yamamoto K, Al-Omani M, Honda T, Takeda Y, Miwatani T. Non-O1 *Vibrio cholerae* hemolysin: purification, partial characterization, and immunological relatedness to El Tor hemolysin. *Infect Immun* 1984; 45: 192-6.
- Zhang XH, Austin B. Haemolysins in *Vibrio* species. *J Appl Microbiol* 2005; 98: 1011-9.
- Zhao JF, Sun AH, Ruan P, Zhao XH, Lu MQ, Yan J. *Vibrio vulnificus* cytolysin induces apoptosis in HUVEC, SGC-7901 and SMMC-7721 cells via caspase-9/3-dependent pathway. *Microb Pathog* 2009; 46: 194-200.
- Zitser A, Walev I, Palmer M, Bhakdi S. Characterization of *Vibrio cholerae* El Tor cytolysin as an oligomerizing pore-forming toxin. *Med Microbiol Immunol* 1995; 184: 37-44.