# ASSOCIATION OF *ompU* GENE IN *VIBRIO CHOLERAE* FROM PATIENTS AND ENVIRONMENT WITH BILE RESISTANCE

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Abstract. The objective of this study was to determine whether *Vibrio cholerae*, possessing *ompU* isolated from patients and the environment, conferred bile resistance and whether other virulence genes were also related to bile resistance. Fifty-two *V. cholerae* O1 and non-O1 isolates were examined by PCR for the presence of the virulence-associated and regulatory genes, *ctxA, tcpA, zot, ace, ompU, toxR, hlyA* and *stn/sto*. *V. cholerae* possessing *ompU* resistant to equal or greater than 10% sodium deoxycholate were found in 93% of isolates but only in 9% of *V. cholerae* isolates not possessing *ompU*. The effects of other virulence genes on bile resistance could not be ascertained in this study. Thus *V. cholerae* non-O1 with *ompU* and possibly other virulence genes isolated from the environment have the potential of affecting public health.

## INTRODUCTION

Bile is found at relatively high levels within the intestine. Sodium deoxycholate (SDC), a major bile component, has been recently reported to act as an external signal for multiple cellular responses of the intestinal pathogen (Pagel *et al*, 2007) and is generally used to determine bile resistance (Provenzano and Klose, 2000; Provenzano *et al*, 2000). Bacterium that can survive in the intestine and is resistant to bile has been reported (Provenzano *et al*, 2000; Wang *et al*, 2003).

*Vibrio cholerae* is a gram-negative bacterium that causes diarrhea. This bacterium colonizes the small intestine and, upon induction of virulence factors, stimulates diarrhea (Wibbenmeyer *et al*, 2002). The ability of *V. cholerae* to cause cholera or diarrhea depends on a combination of virulence genes and virulence factors. Virulence genes include *ctxA* and *ctxB*, encoding for cholera toxin subunit A and B, *zot* encoding zonula occludens toxin (Zot), *ace* encoding accessory cholera toxin (Ace), *tcpA* encoding for toxin coregulate pilus (Tcp), *hlyA* encoding Eltor hemolysin (HlyA), and *stn/sto* encoding heat stable enterotoxin. Virulence gene expression is controlled by regulatory genes, particularly *toxR* and *tcpA* (DiRita *et al*, 1991; Rivera *et al*, 2001; Radu *et al*, 2002).

ToxR regulates the outer membrane porins OmpU and OmpT, which influence intestinal colonization and bile resistance (Provenzano and Klose, 2000; Provenzano *et al*, 2001; Wibbenmeyer *et al*, 2002). OmpU-mediated resistance to bile and anionic detergents, such as sodium dodecyl sulfate and BPI (bactericidal/permeability-increasing), has been studied (Provenzano and Klose, 2000; Provenzano *et al*, 2000; Mathur and Waldor, 2004). ToxR-dependent *ompU* transcription is stimulated by the presence of bile, and *toxR*$^-$

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strains that express low amounts of OmpU are more bile resistant (Provenzano and Klose, 2000; Provenzano *et al*, 2000). OmpU is a porin that forms water-filled channels across bacterial outer membrane (OM), which function as channels for entry and exit of hydrophilic low molecular-mass molecules (<600 Da) such as iron, phosphate and sugar (Aeckersberg *et al*, 2001).

Bile has bacteriocidal activity via its membrane perturbation properties. Resistance to bile is essential for enteric pathogens including *V. cholerae*. The basic structure of the gram-negative bacteria provides some measure of resistance to bile by hiding the bile-sensitive cytoplasmic membrane beneath the relatively bile-resistant outer membrane (Provenzano and Klose, 2000; Provenzano *et al*, 2000). Some strains of *V. cholerae* have the ability to exist both in natural environment and in human intestine (Shears, 2001; Reidl and Klose, 2002). The ability of these strains to respond to specific signals in both environments is part of the complex regulon which is ultimately coordinately regulated by ToxR. *V. cholerae* with *ompU* isolated from water environment has been reported (Karunasagar *et al*, 2003).

The role of associated-virulence genes and their regulator genes, including *ctxA, tcpA, zot, ace, hlyA, stn/sto*, *toxR* and *ompU*, in *V. cholerae* isolated from patients and environment, on bile resistance has not been studied in Thailand. Therefore the objective of this study was to determine whether *ompU* in *V. cholerae* O1 and non-O1 isolated from the environment and patients had an ability to confer bile resistance.

## MATERIALS AND METHODS

### Bacterial strains

A total of 52 *V. cholerae* isolates from the aquatic environment in Khon Kaen Municipality and from diarrheal patients presenting at Khon Kaen Hospital and Srinagarind Hospital, Thailand were included in this study. *V. cholerae* isolates consisted of 29 *ompU*-positive strains and 23 *ompU*-negative strains. Isolates were stored at -20ºC until used.

### Culture conditions

*V. cholerae* isolates were streaked onto thiosulfate-citrate-bile-salt-sucrose (TCBS) agar (Oxoid, Unipath Ltd, Basingstroke, Hamshire, UK) and incubated at 37ºC for 24 hours. The yellow sucrose-fermenting colonies were re-streaked on blood agar for further study (Koneman, 1997).

### Serotyping

*V. cholerae* colonies were determined for their serogroups by slide agglutination using polyvalent *V. cholerae* O1 antiserum (Oxoid). Isolates that agglutinated with the polyvalent *V. cholerae* O1 antiserum were then serotyped with specific monovalent antisera (Oxoid) against Inaba and Ogawa serotypes. *V. cholerae* isolates, which did not agglutinate with polyvalent *V. cholerae* O1 antiserum, were assumed to be *V. cholerae* non-O1.

### DNA extraction

DNA was extracted using a genomic DNA purification kit (Gentra SYSTEM, Minneapolis, Mn) according to the manufacturer's protocol. In brief, *V. cholerae* were cultured on blood agar overnight at 37ºC. Then, a full loop of *V. cholerae* was suspended in 300 μl of cell lysis solution (Gentra SYSTMES) and 1.5 μl of proteinase-K solution (20 mg/ml) and incubated at 55ºC for 3 hours. A 1.5 μl aliquot of RNase A solution (4 mg/ml) was added to the cell lysate which was then incubated at 37ºC for 60 minutes. A 300 μl aliquot of protein precipitation solution was added and the solution was centrifuged at 13,000*g* for 3 minutes. The supernatant was collected and 400 μl of 100% isopropanol was added, and the solution was centrifuged at 13,000*g* for 5 minutes. The supernatant was carefully discarded. A 300 μl aliquot of 70% ethanol was added to

the pellet and the suspension was centrifuged. The ethanol supernatant was poured off and the pellet was left to dry for 2 hours. An aliquot of 50 μl of DNA hydration solution was added and the solution was incubated for 1 hour at 65ºC. DNA was stored at -20ºC until used.

PCR assays

Specific primers designed for multiplex PCR analysis of *ctxA, tcpA, zot, ace, ompU, stn/sto, hlyA* and *toxR* were employed. The primer sets, amplicon sizes, and conditions of PCR amplifications are shown in Table 1. PCR was conducted in 50 μl of a reaction mixture containing 100-200 ng of target DNA, 200 μM of each deoxynucleoside triphosphates (Gibco-BRL, Life Technologies, Gaitherburg, Md), 0.75-1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 100-300 nM of each primer and 1.25 units of *Taq* polymerase (Gibco-BRL). Amplification was conducted in a thermal cycler (Perkin-Elmer, Gene Amp, PCR 2400). The amplicons were subjected to 2% agarose gel-electrophoresis and visualized under UV light (Imagemaster VDS, Pharmacia Biotech, USA) after ethidium bromide staining (1%).

Bile salt sensitivity assay

The method of Provenzano *et al* (2000) was used to determine the minimum bactericidal concentration (MBC) of sodium deoxycholate (SDC, Sigma Chemical, St Louis, Mo). In brief, bacterial colonies, grown on the blood agar plates, were harvested after incubation at 37ºC for 24 hours. A bacterial colony was then inoculated in Luria-Bertani (LB) broth at 37ºC for 24 hours and then was diluted 1:100 with sterile normal saline. SDC was added into LB at a final concentration of 0, 2.5, 5, 7.5,10,

Table 1
Oligonucleotide primers, amplicon sizes, and PCR conditions used for detecting *V. cholerae ctxA, tcpA, zot, ace, ompU, toxR, stn/sto* and *hlyA.*

| Gene and size of amplicon (bp) | Primer sequence | PCR condition | Reference |
|---|---|---|---|
| *ctxA* , 302 | F-5′ CTCAGACGGGATTTGTTAGGGACG 3′<br>R-5′ TCTATGTCTGTAGCCATT 3′ | Multiplex PCR: 95ºC, 30 sec; 60ºC, 1 min; 72ºC, 1 min (25 cycles); | Kapley and Purohit, 2001; Rivera *et al*, 2001 |
| *tcpA*, 472 | F-5′ GAAGAAGTTTGTAAAAGAAGAACAC 3′<br>R-5′ GAAAGCACCTTCTTTCAGGTTG 3′ | | |
| *zot*, 947 | F-5′ TCGCTTAACGATGGCGCGTTTT 3′<br>R-5′ AACCCCGTTTCACTTCTACCCA 3′ | | |
| *ace*, 600 | F-5′ AGAGCGCTGCATTTATCCTTATTG 3′<br>R-5′ AACTCGGTCTCGGCCTCTCGTATC 3′ | Multiplex PCR: 95ºC, 30 sec; 60ºC, 1 min; 72ºC, 1 min (25 cycles); | Leal *et al*, 2004; Singh *et al*, 2002 |
| *toxR* , 779 | F-5′ CCTTCGATCCCCTAAGCAATAC 3′<br>R-5′ AGGGTTAGCAACGATGCGTAAG 3′ | | |
| *ompU*, 869 | F-5′ ACGCTGACGGAATCAACCAAAG 3′<br>R-5′ GCGGAAGTTTGGCTTGAAGTAG 3′ | | |
| *stn*/*sto*, 140 | F-5′ AAAACAGTGCAGCAACCACAAC 3′<br>R-5′ GCTGGATTGCAACATATTTCGC 3′ | Duplex PCR: 95ºC, 30 sec; 55ºC, 1 min; 72ºC, 1 min (25 cycles) | Rivera *et al*, 2001; Singh *et al*, 2002 |
| *hlyA*, 540 | F-5′ CTTAGCTGAGCTGCGCGATTTG 3′<br>R-5′ GAGTTGATCATTCAGA 3′ | | |

12.5, 15 and 17.5 % (w/v). Then 10 μl aliquot of bacterial suspension was inoculated into 5 ml of each SDC-containing LB solution. All tubes were incubated at 37ºC for 1 hour in a shaking incubator, then 100 μl aliquots of bacterial suspensions were added onto LB agar and the plates were incubated overnight at 37ºC. MBC was determined as being the lowest concentration of SDC in which visible cells grown on LB agar were absent.

## RESULTS

We tested 52 clinical and environmental *V. cholerae* O1 and non-O1 isolates, which possessed several virulence genes (*viz*, *ctxA*, *tcpA*, *zot*, *ace*, *toxR*, and *hlyA*) with or without *ompU* for bile resistance (Table 2). *V. cholerae* possessing *ompU* that were resistant to SDC at MBC of 7.5, 10, 12.5, and 17.5% comprised 2 (7%), 20 (69%), 6 (21%), and 1(3%) isolate(s), respectively (Table 3). *V. cholerae* not possessing *ompU* that were resistant to SDC at MBC of 5, 7.5, and 10% comprised 4 (17%), 17 (749%) and 2 (9%) isolates, respectively. Of the 29 *ompU*-containing *V. cholerae* isolates, 27 (93%) were resistant to SDC at MBC ≥ 10%, where as among 23 *ompU*-negative *V. cholerae* isolates, only 2 (9%) were resistant to SDC at MBC ≥ 10%.

Table 2
MBC of sodium deoxycholate of 52 *V. cholerae* O1and non-O1 isolates isolated from patients and the environment.

| Strain | Source | Genotype | | | | | | | | No. of isolates (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | MBC of sodium deoxycholate (%) | | | | | | |
| | | *ctxA* | *tcpA* | *zot* | *ace* | *ompU* | *toxR* | *hlyA* | *Stn/sto* | 2.5 | 5 | 7.5 | 10 | 12.5 | 15 | 17.5 |
| *V. cholerae* | Patient | + | + | + | + | + | + | + | - | | | 1 | 13 | 3 | | |
| O1 | Environment | + | + | + | + | + | + | + | - | | | | 1 | | | |
| | | + | + | + | - | - | + | + | - | | | 1 | | | | |
| | | | | | | | | | | | | (10) | (74) | (16) | | |
| *V. cholerae* | Patient | - | - | - | - | + | + | + | - | | 1 | 2 | | | | |
| non-O1 | Environment | - | - | - | - | + | + | + | - | | | | 4 | 3 | | 1 |
| | | | | | | | | | | | | (9) | (54) | (27) | | (9) |
| | Patient | - | - | - | - | - | + | + | - | | 2 | 6 | 1 | | | |
| | Environment | - | - | - | - | - | + | + | - | | 2 | 10 | 1 | | | |
| | | | | | | | | | | | (18) | (73) | (9) | | | |

Table 3
MBC of sodium deoxycholate of *V. cholerae* isolates possessing and lacking *ompU*.

| Genotype | No. of isolates | | | | | | |
|---|---|---|---|---|---|---|---|
| | MBC of sodium deoxycholate | | | | | | |
| | 2.5 | 5 | 7.5 | 10 | 12.5 | 15 | 17.5 |
| *ompU* (+) | 0 | 0 | 2 | 20 | 6 | 0 | 1 |
| *ompU* (-) | 0 | 4 | 17 | 2 | 0 | 0 | 0 |

## DISCUSSION

Survival of *V. cholerae* in the gastrointestinal tract plays a critical role in pathogenicity (Shears, 2001; Reidl and Klose, 2002). The outer membrane proteins (OMPs) of *V. cholerae* have been reported to participate in bile resistance (Provenzano and Klose, 2000). OmpU, an OMP involved in bile resistance, has been reported *in vivo* (Wibbenmeyer *et al*, 2002). Karunasagar *et al* (2003) suggested that *ompU* is required for *V. cholerae* survival in the human gastrointestinal tract.

We tested clinical and environmental *V. cholerae* O1 and non-O1 isolates for presence of virulence genes, *ctxA*, *tcpA*, *zot*, *ace*, *toxR*, and *hlyA*, including *ompU,* and for bile resistance. The majority (93%) of *V. cholerae* that possessed *ompU* was resistant to equal or more than 10% SDC, whereas only 9% of *V. cholerae* without *ompU* were found to have this level of SDC resistance.

Although several genes were found in different isolates, the presence or absence of other virulence genes besides *ompU* had little effect on bile resistance. All 52 *V. cholerae* isolates, randomly selected to test for bile sensitivity, possessed both *toxR* and *hlyA*. Therefore, we could not test the role of these genes on *V. cholerae* bile resistance.

Although there was only 1 *V. cholerae* O1 *ompU*-positive strain isolated from the environment, it was resistant to 10% SDC. Our results agree with other reports showing that the *ompU*-negative strains have a lower resistance to SDC than the *ompU*-positive strains (Provenzano and Klose, 2000). Therefore we have confirmed that *ompU* gene plays a major role in enhancing bile resistance both in clinical and environmental isolates. We hypothesize that environmental *V. cholerae* isolates that possess *ompU* gene should be able to survive in the human intestine and cause diarrhea similar to those isolated from the patients because they also possess some of the virulence genes, such as *hlyA* encoding El Tor hemolysin that acts as a pore-forming toxin and lyses erythrocytes of some mammals (Zhang *et al*, 1999; Zhang and Austin, 2005).

In summary, we have demonstrated that *ompU* gene in *V. cholerae* isolated from patients and the environment is involved in bile resistance and may be required for survival in the gastrointestinal tract. Our results also indicate that *V. cholerae* O1 and non-O1 in aquatic environment may have the potential of affecting public health.

## ACKNOWLEDGEMENTS

This study was supported by a research grant from the Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. We thank Mr Bryan Roderick Hamman for assistance with the English language.

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