GENOTYPE AND DRUG RESISTANCE OF CLINICAL AND ENVIRONMENTAL *VIBRIO CHOLERAE* NON-O1/ NON-O139 IN NORTHEASTERN THAILAND

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Abstract. A total of 124 *V. cholerae* non-O1/non-O139 isolates were collected in Khon Kaen, Thailand from diarrheal patients, asymptomatic carriers and environmental water. The presence of virulence-associated and regulatory genes including *ctxA, tcpA, zot, ace, ompU, stn, hlyA* and *toxR*) were examined using multiplex PCR. The genomic diversity of the various *V. cholerae* isolates were differentiated using the random amplified polymorphic DNA (RAPD) method. Antimicrobial susceptibility was tested using disk diffusion. All of *V. cholerae* non-O1/non-O139 isolates carried *hlyA* and *toxR* and none carried *ctxA* and *tcpA*. The *zot, ace* and both genes together were found in 1.6%, 4.7% and 4.7% of 64 clinical *V. cholerae* non-O1 isolates, respectively, while the environmental ones did not. The *stn* gene was found in 3.1% (2/64) of the clinical and 3.3% (2/60) of the environmental isolates. The RAPD patterns were differentiated into 45 types (A to 2S). RAPD type A (32.3%) was the most frequently found in both clinical and environmental *V. cholerae* non-O1 strains (34.4% and 30.0%, respectively); indicating that there was a clonal relationship between some clinical and environmental isolates whereas almost all of the environmental isolates belonged to different clones. All strains were sensitive to ciprofloxacin and norfloxacin. The environmental isolates (30%) were more resistant than the clinical ones (21.9%). Resistance to sulfamethoxazole/trimethoprim and tetracycline among the clinical isolates occurred in 9.4% (6/64) in 2007, during which period the prevalence of *V. cholerae* O1 increased. We conclude that *V. cholerae* non-O1/non-O139 from the aquatic environment are potentially pathogenic and this same aquatic environment may be a source of antimicrobial resistance in *V. cholerae*.

Keywords: *V. cholerae* non-O1/non-O139, genotype, environment, RAPD, drug resistant

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INTRODUCTION

Vibrio cholerae, a gram-negative enteric bacterium, is the causative agent of cholera (Shears, 2001). It persists as a public health problem in many developing

countries including Thailand, especially in the northeastern region (Tangkanakul and Hanpanjakit, 2007). The *V. cholerae* serogroup O1 and O139 are associated with severe diarrhea and can produce cholera toxin (CT) encoded by the *ctx* gene (Sharma *et al*, 1998). Although cholera toxin is the major virulence factor in causing cholera diarrhea, other factors such as toxin coregulated pilus (encoded by *tcpA*), zonula occluden toxin (*zot*), accessory cholera toxin (*ace*), outer membrane protein (*ompU*), heat stable enterotoxin (*sto*), hemolysin (*hlyA*) and ToxR regulatory protein (*toxR*) have been associated to pathogenicity (Rivera *et al*, 2001; Singh *et al*, 2002).

The non-toxigenic (NT) *V. cholerae* non-O1/non-O139 strains can also carry other toxin genes besides *ctx*, including *zot*, *ace*, *stn* and *hlyA*, whose expressions may cause cholera, gastroenteritis, septicemia and/or extra-intestinal infection (Sharma *et al*, 1998). A previous study showed that a non-O1/non-O139 *V. cholerae* strain 10259 belonging to the serogroup O53 harbors genes related to the vibrio pathogenicity island (VPI) and a cholera toxin (CT) genetic element. Strain 10259 also contains CTX element-associated toxin genes with sequences almost identical to those of O1 strains (Sarkar *et al*, 2002a).

The pathogenicity of *V. cholerae* O1 and O139 strains depend on several virulence factors. The major ones are present in clusters comprising at least three major pathogenicity islands (Kaper *et al*, 1995). The first is the CTX genetic element comprising the genome of a filamentous bacteriophage, CTXφ which includes the *ctx*, *zot* and *ace* and plays major roles in causing diarrhea (Baudry *et al*, 1992; Trucksis *et al*, 1993). The second is the *V. cholerae* pathogenicity island (VPI), which encodes a toxin co-regulated type IV pilus (TCP) that

plays a role in colonization and as a CTXφ receptor (Karaolis *et al*, 1998). The third, the RTX toxin gene cluster, encodes a presumptive cytotoxin, and acyl transferase associated with the ATP-binding cassette transporter system and causes cell rounding and inhibition of actin polymerization (Chatterjee *et al*, 2008). Other virulence factors genes have been reported in El Tor strains, such as *stn* (encoding the heat stable enterotoxin) (Sarkar *et al*, 2002b), *hlyA* (hemolysin) (Yamamoto *et al*, 1984), *ompU* (outer membrane protein) (Sperandio *et al*, 1996), and *toxR* (ToxR regulatory protein) (Miller *et al*, 1987).

The antimicrobial resistance of *V. cholerae* is a major concern, a phenomenon that has also been observed in other enteric pathogens. Previous reports have shown that multi-drug resistant *V. cholerae* O1 can cause serious outbreaks affecting the treatment of *V. cholerae* (Chomvarin *et al*, 2012)

Epidemiological surveillance of cholera in most public heath sectors are based on phenotypic characteristics, including biochemical tests, antigenic properties and antibiogram. However, these methods are of limited value for predicting the epidemiological potential of *V. cholerae* strains (Leal *et al*, 2004) because, even though *V. cholerae* isolates show similar phenotypic characteristics, they may differ in their genetic profiles indicating that they may have been derived from different strains or different clones. Molecular typing techniques, such as random amplified polymorphic DNA (RAPD), has been employed to study genetic relatedness (Bhowmick *et al*, 2007), and is a promising method for distinguishing individual bacterial strains and estimating nucleotide sequence diversity (Bhowmick *et al*, 2007; Leal *et al*, 2004). In addition, RAPD is simple, rapid and economical (Van Belum, 1994).

To the best of our knowledge, there

has been no simultaneous study of the virulence-associated genes, including *ctxA, tcpA, zot, ace, ompU, hlyA, stn* and regulatory gene (*toxR*), for the relationship between genomic diversity and antimicrobial resistance of clinical and environmental *V. cholerae* non-O1/non-O139 strains in Thailand. We therefore examined whether the strains isolated from clinical and environmental sources had the same virulence-associated genes, and whether they belonged to related clones. We also examined the antimicrobial resistances of *V. cholerae* strains isolated from different sources as to whether they had the same antimicrobial susceptibility patterns.

MATERIALS AND METHODS

Bacterial strains

A total 124 *V. cholerae* non-O1/non-O139 strains were used: these were isolated between 2003 and 2007 in Khon Kaen Province of Thailand. *V. cholerae* non-O1/non-O139 strains comprised 64 clinical (61 patients and 3 asymptomatic subjects) and 60 environmental (wastewater, river water, and water stored in the house of each patient) isolates, respectively. *V. cholerae* strains were presumptively identified using conventional bacteriological methods (Koneman, 1997). For complete identification, bacterial strains were sub-cultured in alkaline peptone water for selective enrichment followed by plating onto TCBS agar and subsequent biochemical tests.

The identification of the specific *V. cholerae* serogroup was determined by slide agglutination with polyvalent *V. cholerae* O1 and O139 antiserum (Oxoid, Unipath, Basingstroke, Hamshire, England).

Antibiotic susceptibility test

Antimicrobial activity of the *V. cholerae* strains was determined using the disk diffusion method (National Committee for Clinical Laboratory Standards, 2002) with commercially available disks (Oxoid, Unipath, Basingstroke, Hamshire, England). All of the *V. cholerae* isolates were examined for susceptibility to ampicillin (AMP, 10 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), sulfamethoxazole/trimethoprim (SXT, 23.75/1.25 µg), erythromycin (E, 15 µg), norfloxacin (NOR, 10 µg) and tetracycline (TER, 30 µg). *E. coli* ATCC 25922 was used as the control strain. For each antimicrobial agent, the zones were described as resistant (R), intermediate resistant (I) or susceptible (S).

DNA extraction

DNA was extracted using a genomic DNA purification kit (Puregene DNA purification system; Gentra System, Minneapolis, MN) according to the manufacturer's instructions. In brief, cells of *V. cholerae* from blood agar plates were lysed for DNA extraction and stored at -20 ºC until used (Chomvarin *et al*, 2012).

Multiplex PCR assay

Specific primers designed for three sets of multiplex PCR analysis for *ctxA, tcpA, zot, ace, ompU, stn, hlyA* and *toxR* were employed as previously reported (Chomvarin *et al*, 2012). The primers and thermocycling conditions used in this study are listed in Table 1. PCR was conducted using a 50-µl reaction mixture containing 100-200 ng of target DNA, 200 µM of each dNTPs (Gibco-BRL; Life Technologies, Gaithersburg, MD), 0.75-1.5 mM MgCl₂, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 100-300 nM each primer and 1.25 U *Taq* polymerase (Gibco-BRL) in a thermocycler (Perkin-Elmer, GeneAmp, PCR 2400; Branchburg, NJ). The amplicons were subjected to 2% agarose gel-electrophoresis and visualized under UV light (Imagemaster VDS; Pharmacia

Table 1
Primers, amplicon sizes and PCR thermocycling conditions used for detection of virulence-associated genes and RAPD-PCR typing of *V. cholerae* non-O1/O139 strains.

Gene and size of amplicon (bp)	Primer sequence	Thermocycling conditions	Reference
ctxA, 302	F-5′ CTCAGACGGGATTTGTTAGGGACG 3′ R-5′ TCTATGTCTGTAGCCATT 3′	Multiplex PCR: 95°C, 30 sec; 60°C, 1 min; 72°C, 1 min (25 cycles)	(Kapley and Purohit, 2001); (Rivera *et al*, 2001).
tcpA, 472	F-5′ GAAGAAGTTTGTAAAAGAAGAACAC 3′ R-5′ GAAAGCACCTTCTTTCAGGTTG 3′		
zot, 947	F-5′ TCGCTTAACGATGGCGCGTTTT 3′ R-5′ AACCCCGTTTCACTTCTACCCA 3′		
ace, 600	F-5′ AGAGCGCTGCATTTATCCTTATTG 3′ R-5′ AACTCGGTCTCGGCCTCTCGTATC 3′	Multiplex PCR:	(Leal *et al*, 2004);
toxR, 779	F-5′ CCTTCGATCCCCTAAGCAATAC 3′ R-5′ AGGGTTAGCAACGATGCGTAAG 3′	95°C, 30 sec; 60°C, 1 min; 72°C, 1 min (25 cycles)	(Singh *et al*, 2002)
ompU, 869	F-5′ ACGCTGACGGAATCAACCAAAG 3′ R-5′ GCGGAAGTTTGGCTTGAAGTAG 3′		
stn, 140	F-5′ AAAACAGTGCAGCAACCACAAC 3′ R-5′ GCTGGATTGCAACATATTTCGC 3′	Duplex PCR: 95°C, 30 sec; 55°C, 1 min; 72°C, 1 min (25 cycles)	(Rivera *et al*, 2001); (Singh *et al*, 2002)
hlyA, 540	F-5′ CTTAGCTGAGCTGCGCGATTTG 3′ R-5′ GAGTTGATCATTCAGA 3′		
RAPD primer	5′ GTTTCGCTCC 3′	94°C, 4 min (1 cycle); 94°C, 1 min; 36°C, 1 min; 72°C, 2 min (45 cycles) 72°C, 7 min (1 cycle)	(Kondo *et al*, 2001)

Biotech, Piscataway, NJ) after ethidium bromide staining.

RAPD typing

The oligonucleotide primers and thermocycling conditions used for the RAPD protocol are shown in Table 1. PCR was conducted in a 50-µl reaction mixture containing 100 ng of target DNA, 250 mM dNTPs, 2 mM MgCl$_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 µM primer and 1.25 U *Taq* polymerase. The amplified product (10 µl) was electrophoresed in 1.5% NuSieve®

agarose gel with 0.5X TAE for 35 minutes at 100 V. Gels containing amplified DNA were stained with ethidium bromide and visualized under a UV transilluminator as described above.

Analysis of RAPD data

The RAPD types were examined based on the presence or absence of bands. The similarities between fingerprints were determined by construction of a similarity matrix using Restdist program, which generated a dendogram using UPGMA

Table 2
Distribution of virulence-associated genes in 124 *V. cholerae* non-O1/non-O139 strains.

Strain	Source	Gene								Total no. of isolates (%)
		ctxA	*tcpA*	*zot*	*ace*	*toxR*	*ompU*	*stn*	*hlyA*	
Clinical	Patients	-	-	-	-	+	-	-	+	47 (73)
V. cholerae		-	-	-	-	+	+	-	+	5 (8)
non-O1		-	-	-	-	+	-	+	+	2 (3)
(*n* = 64)		-	-	-	+	+	-	-	+	3 (5)
		-	-	+	+	+	-	-	+	1 (2)
		-	-	+	+	+	+	-	+	2 (3)
		-	-	+	-	+	-	-	+	1 (2)
	Carriers	-	-	-	-	+	-	-	+	3 (5)
Environmental	Water from	-	-	-	-	+	-	-	+	6 (10)
V. cholerae	*V. cholerae* O1									
non-O1	patient's house									
(*n* = 60)	Aquatic	-	-	-	-	+	+	-	+	23 (38)
	environment	-	-	-	-	+	-	-	+	29 (48)
		-	-	-	-	+	-	+	+	2 (3)

+, present; -, absent.

clustering algorithm (3.67 version) (Philip package, University of Washington, Seattle, WA).

Latex agglutination assay for cholera toxin production

Detection of cholera toxin was performed using the latex agglutination assay according to manufacturer's instructions (Oxoid, Columbia, MD). Each overnight culture of *V. cholerae* was centrifuged and a 25-µl aliquot of supernatant mixed with a 25-µl aliquot of latex suspension in a V-well microtiter plate. If toxin is present, agglutination results in the formation of a lattice structure (Chomvarin *et al*, 2012).

RESULTS

Prevalence of virulence and regularity genes in *V. cholerae* non-O1/non-O139 isolates

We examined 124 *V. cholerae* non-O1/non-O139 isolates, 64 from clinical source (61 patients and 3 asymptomatic subjects) and 60 from environmental source (54 wastewater and river water, and 6 water stored in the house of each patient) for the presence of *ctxA, tcpA, zot, ace, hlyA, ompU, stn* and *toxR*. The *toxR* and *hlyA* were detected in all of the *V. cholerae* non-O1/non-O139 isolates, whereas *ctxA* and *tcpA* were not detected (Table 2).

The occurrence of the heteromorphic genotypes present in either clinical or environmental *V. cholerae* non-O1 non-O139 isolates are shown in Table 2. Dimorphic genotype *toxR* and *hlyA* was the most frequently found in clinical (73%) and environmental (58%) isolates. Detection rate of trimorphic genotype *toxR, hlyA,* and *ace* was 5%, the tetramorphic genotype *toxR, hlyA, zot,* and *ace,* 2% and the pentamorphic genotype *toxR, hlyA, zot, ace,* and *ompU,* 3%. The trimorphic
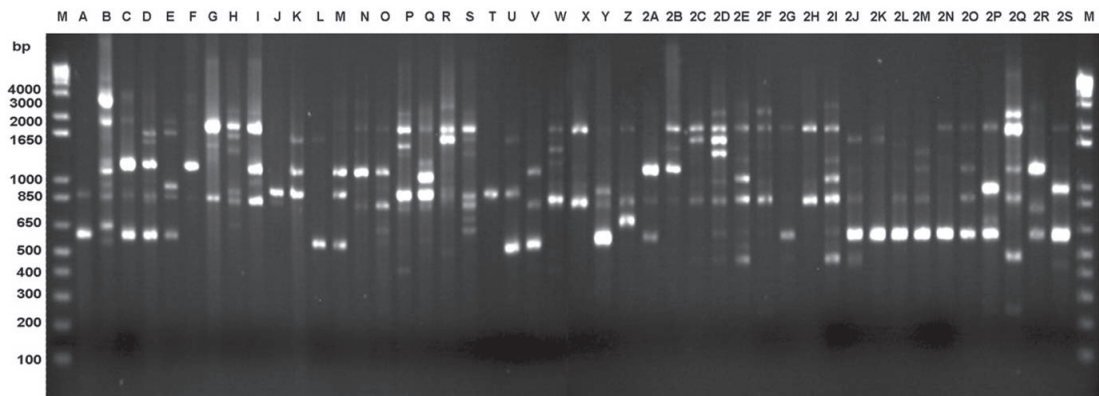
Fig 1–RAPD patterns of clinical and environmental *V. cholerae* non-O1/non-O139 strains. Primer used was 5' GTTTCGCTCC 3' and amplification was conducted for 1 cycle at 72°C for 7 minutes. Amplicons were analyzed by 1.5% NuSieve® agarose gel-electrophoresis. RAPD patterns (A to 2S) are indicated on top of each lane; M, molecular size markers.

genotype *toxR*, *hlyA*, and *zot* was present only in clinical isolates, whereas all these genotypes were not detected in all the environmental isolates tested in this study.

RAPD fingerprinting analysis

RAPD yielded 45 distinguishable banding patterns (identified by the letter 'A' to '2S', sequentially) (Fig 1). The discriminatory power (*D*-value) of the RAPD fingerprinting assay of *V. cholerae* non-O1/non-O139 strains in the study population was 0.995. RAPD pattern 'A' was the most frequently found (40/124, 32%) of the *V. cholerae* non-O1 strains, of which 34% and 30% were in clinical and environmental strains, respectively. The second most predominant RAPD pattern was 'W', which included 9 clinical and 4 environmental isolates. There was no association between RAPD patterns and time or source of collection. Other patterns were scattered among small groups and individual strains. *V. cholerae* non-O1/non-O139 strains were very diverse in both their banding patterns and source (patient and environment) (Fig 1).

Antimicrobial susceptibility

Twelve antimicrobial susceptibility (AS) types were found when the 124 *V. cholerae* non-O1/non-O139 strains were tested against 7 antimicrobial agents. These strains were resistant to TE (5%), SXT (9%), AMP (15%), and E (2%), but were sensitive to CIP and NOR (Table 3). The environmental isolates showed higher multi-drug resistance than the clinical isolates (Table 4). The AS type most frequently found was type 4 (AMP^S C^S CIP^S E^I SXT^S NOR^S TE ^S) (61%) (Table 5). Multi-drug resistance to SXT and TE were higher in strains isolated in 2007.

DISCUSSION

Between 2003-2007, we collected *V. cholerae* non-O1/non-O139 isolates in Khon Kaen, Thailand; from diarrheal patients and asymptomatic healthy individuals, natural aquatic environments and household water from the houses of patients with diarrhea. At least three localized outbreaks of diarrhea caused by non-O1/non-O139 serogroups have been

Table 3

Antimicrobial susceptibility of 124 *V. cholerae* non-O1/non-O139 strains from clinical and environmental sources.

Antimicrobial agent	Number of *V. cholerae* non-O1/non-O139 strains (%)		
	R	I	S
Ampicillin (AMP)	19 (15)	7 (6)	96 (77)
Chloramphenicol (C)	1 (1)	2 (2)	121 (98)
Co-trimoxazole (SXT)	11 (9)	0 (0)	113 (91)
Ciprofoxacin (CIP)	0 (0)	0 (0)	124 (100)
Erythromycin (E)	3 (2)	112 (90)	9 (7)
Norfloxacin (NOR)	0 (0)	0 (0)	124 (100)
Tetracycline (TER)	6 (5)	3 (2)	115 (94)

R, resistant; I, intermediate resistant; S, susceptible.

Table 4

Combination of antimicrobial resistance in *V. cholerae* non-O1/non-O139 isolates from clinical and environmental sources.

No. of combinations of antimicrobial resistance	Number of *V. cholerae* non-O1/non-O139 strains (%)		
	Patient (*n* = 64) No. (%)	Environment (*n* = 60) No. (%)	Total isolates (*n* = 124) No. (%)
0	50 (78)	42 (70)	92 (74)
1	7 (11)	17 (28)	24 (19)
2	7 (11)	1 (2)	8 (7)
3	0	0	0
Total	14 (22)	18 (30)	32 (26)

identified; including *V. cholerae* O10 and O12 in Lima, Peru (Dalsgaard *et al*, 1995), O10 in East Delhi, India (Rudra *et al*, 1996), and O10 in Khmer refugee camps in Thailand (Bagchi *et al*, 1993). Although most of our clinical and environmental *V. cholerae* non-O1/non-O139 strains did not carry *ctx* and *tcpA*, some carried *zot, ace, ompU* and *stn/sto* and all carried *hlyA* and *toxR*, in agreement with other reports (Brown and Manning, 1985; Sharma *et al*, 1998). *hlyA* expresses is a pore-forming exotoxin that contributes to cytotoxic activity and triggers apoptotic cell death (Saka *et al*, 2008). *V. cholerae* non-O1 may also produce several extracellular products; such as hemolysin, NAG-ST, Shiga-like toxin and hemagglutinin, which play roles in pathogenesis (O'Brien *et al*, 1984; Bagchi *et al*, 1993). In the current study, *stn* was present in 4 (3%) of the non-O1/non-O139 isolates; therefore, *V. cholerae* non-O1/O139 poses a public health threat as it can cause diarrheal disease (Singh *et al*, 2002).

ompU encodes the outer membrane protein OmpU, an aquaporin that allows

Table 5
Antimicrobial susceptibility (AS) types of 124 clinical and environmental *V. cholerae* non-O1/non-O139 strains.

AS type	Antibiotic							Number of strains		Total number of strains (%)
	AMP	C	CIP	E	SXT	NOR	TER	Patient ($n = 64$)	Environment ($n = 60$)	
1	S	S	S	S	S	S	S	5	5	10 (8.1)
2	S	S	S	R	S	S	S	0	1	1 (0.8)
3	R	S	S	R	S	S	S	1	1	2 (1.6)
4	S	S	S	I	S	S	S	45	31	76 (61.3)
5	R	S	S	I	S	S	S	5	12	17 (13.7)
6	I	S	S	I	S	S	S	0	5	5 (4.0)
7	S	S	S	I	R	S	S	0	1	1 (0.8)
8	S	I	S	I	R	S	S	1	0	1 (0.8)
9	I	I	S	I	R	S	S	0	2	2 (1.6)
10	I	S	S	S	S	S	I	0	1	1 (0.8)
11	S	S	S	I	R	S	I	1	1	2 (1.6)
12	S	S	S	I	R	S	R	6*	0	6 (4.8)

R, resistant; I, intermediate resistant; S, susceptible. AMP, ampicillin, C, chloramphenicol, CIP, ciprofloxacin, E, erythromycin, SXT, co-trimoxazole, NOR, norfloxacin, TER, tetracycline, *In 2007.

entry and exit of hydrophilic low-molecular mass molecules (Aeckersberg *et al*, 2001). OmpU is regulated by ToxR and influences intestinal colonization and resistance to bile acids (Provenzano *et al*, 2001). In this investigation, *ompU* was detected in 24% of the isolates, 75% of which surprisingly were isolated from environmental source.

Our study used RAPD fingerprinting for molecular typing because it is simple, sensitive and of low cost (Williams *et al*, 1990). This method has been used in genetic diversity analysis of different bacterial species, including *V. cholerae* (Makino *et al*, 1995; Radu *et al*, 1999). We found that RAPD type 'A' was the most prevalent among these isolates. Nonetheless, there was no specific association between the isolation sources (clinical and environmental) and collection times with the RAPD patterns. Our findings suggest that *V. cholerae* non-O1/non-O139 strains are genetically heterogeneous, as observed by other researchers (Rivera *et al*, 2001; Vital Brazil *et al*, 2002).

Interestingly, the intermediate resistant and multi-drug resistant *V. cholerae* non-O1/non-O139 strains isolated from the environmental and clinical sources were also found in the same period and some environmental isolates had higher antibiotic resistance than in the clinical isolates, indicating that the natural environment may serve as a reservoir of multi-drug resistance genes (Kruse and Sorum, 1994). It is well known that the conjugation and transfer of resistance plasmids (R plasmids) is a phenomenon that occurs in the environment and can occur between bacterial strains common to humans, animals and fish origins, unre-

lated either evolutionarily or ecologically, even in the absence of any antimicrobial agent. The presence in the environment of strains that are resistant to antimicrobial agents is a threat not only as a source of disease but also as a source from which R plasmids can easily spread to other pathogens of diverse origins (Kruse and Sorum, 1994). Consequently, *V. cholerae* non-O1/non-O139 should be carefully monitored for new serogroups with epidemic potential, and for environmental presence of antimicrobial-agent-resistance.

In conclusion, we demonstrated that clinical and environmental *V. cholerae* non-O1/non-O139 strains carry other genes, in addition to *ctxA*, that can cause diarrhea. *V. cholerae* non-O1/non-O139 strains in the aquatic environment are therefore potentially pathogenic and of significant public health concern. Surveillance of *V. cholerae* non-O1/non-O139 strains (including genotype monitoring) is necessary in both clinical and environmental sources, using detection of virulence-associated genes, RAPD typing and antibiogram, in order to provide prior warning of any impending public health risk.

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