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Detection of RTX Toxin Gene in *Vibrio cholerae* by PCR

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A PCR that amplifies a recently discovered *Vibrio cholerae* RTX (repeat in toxin) toxin gene was developed. Among 166 clinical and environmental isolates of *V. cholerae* causing epidemics and sporadic cases of cholera in various parts of the world, all were found to be toxigenic by both PCR and HEp-2 cell cytotoxicity assay. Standard strains of the classical biotype containing a deletion within the gene cluster exhibited negative results by both assays. This is the first rapid genotyping method for differentiation of *V. cholerae* O1 classical biotype strains from El Tor biotype strains as well as strains of other non-O1 serogroups including serogroup O139. The PCR assay that was developed also specifically detects RTX toxin genes in *V. cholerae*, as clinical isolates of *Vibrio parahaemolyticus*, diarrheagenic *Escherichia coli, Aeromonas* species, and *Plesiomonas* species were all negative by the RTX toxin-specific PCR as well as the HEp-2 cytotoxicity assay. These findings highlight the characteristics of the RTX toxins in *V. cholerae*. Their role in the pathogenicity of the bacterium requires further investigation.

Vibrio cholerae is an important cause of diarrheal disease in many parts of Asia and Africa. It is the only enteric pathogen that has the potential to produce pandemics of disease and is of immense public health importance. Cholera is caused by *V. cholerae* serogroup O1, which has been highly prevalent in Southeast Asia in the past 20 years (2, 5, 13). *V. cholerae* O1 biotype El Tor was responsible for the cholera epidemic in Hong Kong between 1986 and 1997 (7, 22, 23). Although cholera toxin-producing *V. cholerae* O139 has been sweeping across the Indian subcontinent since 1992, epidemic spread of this novel strain of *V. cholerae* has not occurred in Hong Kong since the first imported case was detected in May 1993 (21, 27).

Traditionally, life-threatening diarrhea associated with the cholera syndrome is attributed to massive luminal secretion of electrolytes and water from enterocytes, with elevated cyclic AMP levels induced by the cholera toxin (CT). CT is encoded by the *ctxA* and *ctxB* genes of the core element (8). A novel toxin in *V. cholerae* that belongs to the RTX (repeat in toxin) family of toxins, which are generally produced by several pathogenic gram-negative bacteria (10), was recently discovered. The RTX toxins represent a family of important virulence factors that have disseminated widely among gram-negative bacteria (1). The RTX toxin gene cluster in *V. cholerae* encodes the presumptive cytotoxin (*rtxA*), an acyltransferase (*rtxC*), and an associated ATP-binding cassette transporter system (RtxB and RtxD, two proteins for toxin transportation) (Fig. 1). It is physically linked to the core element in the *V. cholerae* genome, although its activity is independent of the core element (10). Phenotypically, these genes are proven to be associated with cytotoxicity in HEp-2 cells. In the study described here, a highly specific PCR was developed to identify this toxin gene in more than 100 clinical and environmental isolates of *V. cholerae* causing sporadic and epidemic cases of cholera in various parts of the world.

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MATERIALS AND METHODS

Sources of strains. A total of 166 *V. cholerae* isolates from five different regions were included in our study (Table 1). The majority of the isolates were *V. cholerae* O1 El Tor, O139, and non-O1 serogroup strains collected from patients or the environment over a decade in Hong Kong (7, 21, 22, 23, 27). The remaining 51 clinical isolates of O1 El Tor and O139 were isolated from Hong Kong, China; Shenzhen, China; Singapore; Thailand; and Ukraine from 1991 to 1999. In addition, five strains each of diarrheagenic *Escherichia coli* (three isolates of verocytotoxigenic *E. coli* and two isolates of enteropathogenic *E. coli*), *Vibrio parahaemolyticus*, *Aeromonas* species, and *Plesiomonas* species isolated from patients suffering from diarrhea in Hong Kong were also included for comparative study (18, 19, 20).

PCR for *rtxA*, *rtxC*, and *ctxB*. DNA from all purified strains was prepared from overnight liquid cultures grown in 5 ml of brain heart infusion broth at 37°C. Cell pellets from 1 ml of culture were washed with normal saline, centrifuged, and resuspended in 100 μl of sterile Milli-Q H_2O. After the suspension was heated in a dry bath at 85°C for 15 min, the pellet was spun down at 15,900 × *g* for 15 min at 4°C, and the supernatant was saved for use as the DNA template in the PCR.

Two pairs of primers were derived from the *rtxA* and *rtxC* genes of *V. cholerae* N16961. The sequences of the primers extended within the deletion region of the RTX gene cluster in classical biotype strain O395 (Fig. 1). PCR amplified a 417-bp product of the *rtxA* gene (primers *rtxA-F* [5′-CTG AAT ATG AGT GGG TGA CTT ACG-3′] and *rtxA-R* [5′GTG TAT TGT TCG ATA TCC GCT ACG-3′]) and a 263-bp product of the *rtxC* gene (primers *rtxC-F* [5′-CGA CGA AGA TCA TTG ACG AC-3′] and *rtxC-R* [5′-CAT CGT CGT TAT GTG GTT GC-3′]). The total reaction volume was 25 μl, which contained 3 μl of DNA template and 2.5 μl of 10× PCR buffer (final concentrations, 1.5 mM $MgCl_2$ and 0.4 mg of bovine serum albumin per ml [Applied Biosystems, Foster City, Calif.]), 1 μM primers, deoxynucleoside triphosphates at a concentration of 0.2 mM, 1 U of AmpliTaq Gold polymerase (Perkin-Elmer), and 1 drop of mineral oil. After pretreatment by heating of the mixture at 94°C for 12 min to activate the enzyme polymerase prior to the cycling reaction, DNA amplification was carried out for 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. An extension period of 72°C for 10 min was added at the end of the cycling reaction. For product detection, 5 μl of the PCR mixture was subjected to electrophoresis in a 2% agarose gel.

The amplification of a 460-bp *ctxB* gene (primers $ctxB_2$ [5′-GAT ACA CAT AAT AGA ATT AAG GAT G-3′] and $ctxB_3$ [5′-GGT TGC TTC TCA TCA

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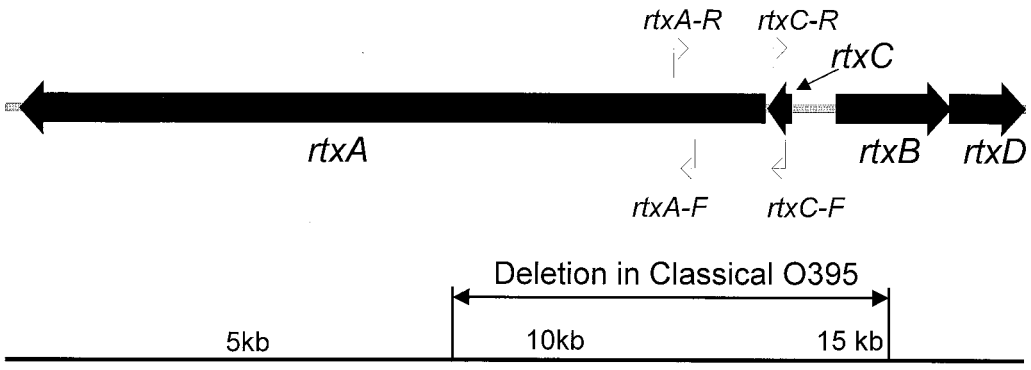

FIG. 1. Genomic structure of RTX toxin element in *V. cholerae.* Adapted from reference 10, with permission from the National Academy of Sciences, U.S.A.

TCG AAC CAC-3′]) from the sample strains was performed as described previously (3, 12).

Cytotoxicity and CT assays. All bacterial strains were tested to determine whether they had a cytopathic effect on HEp-2 cells, as described previously (10). Briefly, HEp-2 cells were cultured in RPMI 1640 medium (Gibco, Grand Island, N.Y.) with 10% fetal calf serum and supplement (without antibiotics) and were seeded onto dishes for assay. Washed bacterial cultures were added to the HEp-2 cells, and the dishes were incubated at 37°C in 5% CO_2 for 1 h. A positive result was indicated by rounding up and detachment of monolayer HEp-2 cells from the culture dishes.

Active CT production was also performed by the VET-RPLA (Oxoid Ltd., Basingstoke, United Kingdom) assay, with modifications, as described previously (24). Briefly, polymyxin B was added to the overnight bacterial cultures, followed by incubation at 37°C for 4 h with shaking. After centrifugation, the supernatants were diluted in V-type microtiter plates in duplicate until the last well contained diluent only. Latex suspensions sensitized with antibodies to CT were added to each well and were left undisturbed at room temperature for 20 to 24 h. Agglutination was examined macroscopically and recorded.

RESULTS

All 166 clinical and environmental strains of *V. cholerae* were collected from 1986 to 1999 in Hong Kong, China, Singapore, Thailand, and Ukraine (Table 1). *V. cholerae* isolates of the O1 El Tor, O139, and non-O1 serogroups exhibited positive PCR results for both *rtxA* and *rtxC* genes (Fig. 2). Concurrently, all these isolates were also positive by the HEp-2 cytotoxicity assay. Complete concordance was found between the genotypic and the phenotypic expressions of the RTX toxins in all strains tested, indicating the integrity of the RTX toxin gene cluster among the strains. Although no clinical strain of the *V. cholerae* classical biotype was collected during the period studied, the *rtxA* and *rtxC* genes were not amplified from standard *V. cholerae* classical biotype strains, strains ATCC 9458 and

TABLE 1. Characteristics of clinical and environmental isolates of *V. cholerae*

Strain	Yr of isolation	Origin	Source	No. of isolates	PCR result for:			HEp-2 cell cytotoxicity assay result	Reference(s)[a]
					rtxA	*rtxC*	*ctxB*		
V. cholerae									
O1 EI Tor	NA[b]	NEQ AS4043	Standard strain	1	+	+	+	+	NEQ
O1 classical	NA	ATCC 9458	Standard strain	1	−	−	+	−	ATCC
O1 classical	NA	ATCC 11628	Standard strain	1	−	−	+	−	ATCC
O1 EI Tor	1986–1999	Hong Kong SAR	Patient	81	+	+	+	+	7, 22, 23
	1989–1998	Hong Kong SAR	Environment	3	+	+	+	+	7, 22, 23
	1999	Shenzhen, China	Patient	7	+	+	+	+	This study
	1996	Singapore	Patient	1	+	+	+	+	This study
	1995	Thailand	Patient	29	+	+	+	+	This study
	1991–1994	Ukraine	Patient	3	+	+	+	+	This study
	1994–1995	Ukraine	Environment	3	+	+	+	+	This study
O139	1994–1999	Hong Kong SAR	Patient	10	+	+	+	+	21, 27, this study
	1999	Shenzhen, China	Patient	1	+	+	+	+	This study
Non-O1	1993–1999	Hong Kong SAR	Patient	5	+	+	−	+	19, this study
	1993–1999	Hong Kong SAR	Environment	23	+	+	−	+	19, this study
V. parahaemolyticus	1993	Hong Kong SAR	Patient	5	−	−	−	−	19
Diarrheagenic *E. coli*	1993–1998	Hong Kong SAR	Patient	5	−	−	−	−	18, 19, 20
Aeromonas species	1998–1999	Hong Kong SAR	Patient	5	−	−	−	−	This study
Plesiomonas species	1998–1999	Hong Kong SAR	Patient	5	−	−	−	−	This study

[a] NEQ, National External Quality Assessment Scheme for Microbiology, Colindale, United Kingdom; ATCC, American Type Culture Collection, Manassas, Va.
[b] NA, not available.

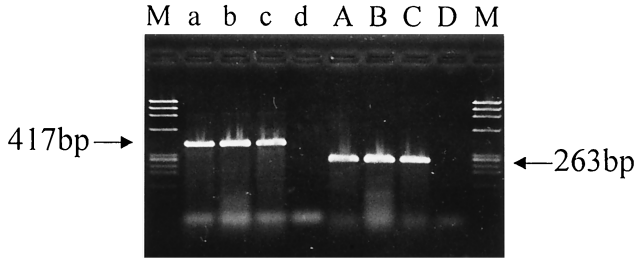

FIG. 2. Agarose gel electrophoresis of PCR products of *rtxA* (lanes a to d) and *rtxC* (lanes A to D). Lanes a and A, *V. cholerae* O1 El Tor; lanes b and B, *V. cholerae* O139; lanes c and C, *V. cholerae* non-O1; lanes d and D, *V. cholerae* classical strain ATCC 9458; lanes M, molecular mass markers (*Bsu*RI-digested ϕX174 DNA).

ATCC 11628. These two classical strains also exhibited negative results by the HEp-2 cytotoxicity assay. Except for the non-O1 serogroups, all *V. cholerae* O1 and O139 isolates were positive for CT by VET-RPLA and PCR assay. Five strains each of *V. parahaemolyticus,* diarrheagenic *E. coli*, *Aeromonas* species, and *Plesiomonas* species included in the present study exhibited negative results by all PCR and toxin assays.

DISCUSSION

On the basis of the discovery of RTX toxins in *V. cholerae*, we developed two assays specific for *rtxA* and *rtxC* and screened for the presence of functional RTX toxin genes in our collection of strains obtained from various parts of the world from 1986 to 1999. Our findings for chronologically and geographically disparate strains indicate that the presence of an intact RTX toxin gene cluster is consistent with the phenotypic expression of cytotoxic activity in all these isolates. Standard strains of the classical biotype exhibited negative results by both PCR and cytotoxicity assays, which was explained by the deletion of the gene cluster. In addition, the PCR detection assay described here was highly specific for *V. cholerae*, as all clinical isolates of *V. parahaemolyticus,* diarrheagenic *E. coli*, *Aeromonas* species, and *Plesiomonas* species exhibited negative results.

Although genotypic methods like PCR of the *ctx* gene are available for the rapid identification of toxigenic *V. cholerae*, definitive identification of the classical and El Tor biotypes within the O1 serogroup relies on conventional biochemical methods, which are tedious and time-consuming. Although only two *V. cholerae* classical strains were included in the present study, the PCR assays developed for *rtxA* or *rtxC*, when used in combination with PCR for CT, not only identify CT-producing *V. cholerae* but also differentiate the biotypes of strains within the *V. cholerae* O1 serogroup.

While CT is a principal virulence factor for *V. cholerae*, the contribution of the RTX toxins to its pathogenesis requires further investigation. At present, the cytolytic RTX toxins represent a family of important virulence factors for organisms that produce the toxin. Good evidence of the role of the hemolysin of *E. coli* as a cause of extraintestinal infections is available (17). In our study, the *rtx* gene cluster was absent only from the *V. cholerae* classical O1 serogroup strain, which has greater epidemic potential than strains of the other serogroups, despite its displacement by the El Tor biotype since the seventh pandemic. Nonepidemic *V. cholerae* non-O1 serogroup strains, which cause only sporadic, milder cases of diarrhea, do secrete the RTX cytotoxins but do not secrete CT. Several groups proposed that virulence factors account for the clinical manifestations of diarrhea caused by non-O1 serogroup strains (6, 15, 25, 26). One distinct finding indicated that *V. cholerae* non-O1 serogroup strains caused necrosis of the luminal epithelium in the colon and mild inflammatory cell infiltration in the adjacent lamina propria (14). Evidence of the inflammatory response due to a *V. cholerae* O1 El Tor strain from which all known toxin genes excluding the *rtx* gene cluster have been deleted has also been reported (16). The vacuolating activity of the *V. cholerae* El Tor hemolysin in nucleated mammalian cells may be associated with gastrointestinal symptoms caused by nontoxigenic *V. cholerae* (11). A recent investigation (4) demonstrated that the RTX toxins of *V. cholerae* caused actin depolymerization and cross-linking in HEp-2 cells. Similar actin rearrangement or condensation was observed in HEp-2 cells and was caused by a protein encoded by the *eaeA* gene of enteropathogenic and hemorrhagic *E. coli*, leading to the effacement of microvilli, with subsequent hemorrhagic colitis and bloody diarrhea (9). Our findings highlight the occurrence of RTX toxins in strains of *V. cholerae* except those exhibiting the classical biotype. Further investigation is required to determine the role of RTX in the pathogenicity of *V. cholerae.*

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