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# Genotypes Associated with Virulence in Environmental Isolates of *Vibrio cholerae*

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***Vibrio cholerae* is an autochthonous inhabitant of riverine and estuarine environments and also is a facultative pathogen for humans. Genotyping can be useful in assessing the risk of contracting cholera, intestinal, or extraintestinal infections via drinking water and/or seafood. In this study, environmental isolates of *V. cholerae* were examined for the presence of *ctxA, hlyA, ompU, stn*/*sto, tcpA, tcpI, toxR*, and *zot* genes, using multiplex PCR. Based on *tcpA* and *hlyA* gene comparisons, the strains could be grouped into Classical and El Tor biotypes. The *toxR, hlyA*, and *ompU* genes were present in 100, 98.6, and 87.0% of the *V. cholerae* isolates, respectively. The CTX genetic element and toxin-coregulated pilus El Tor (*tcpA* ET) gene were present in all toxigenic *V. cholerae* O1 and *V. cholerae* O139 strains examined in this study. Three of four nontoxigenic *V. cholerae* O1 strains contained *tcpA* ET. Interestingly, among the isolates of *V. cholerae* non-O1/non-O139, two had *tcpA* Classical, nine contained *tcpA* El Tor, three showed homology with both biotype genes, and four carried the *ctxA* gene. The *stn*/*sto* genes were present in 28.2% of the non-O1/non-O139 strains, in 10.5% of the toxigenic *V. cholerae* O1, and in 14.3% of the O139 serogroups. Except for *stn*/*sto* genes, all of the other genes studied occurred with high frequency in toxigenic *V. cholerae* O1 and O139 strains. Based on results of this study, surveillance of non-O1/non-O139 *V. cholerae* in the aquatic environment, combined with genotype monitoring using *ctxA, stn*/*sto*, and *tcpA* ET genes, could be valuable in human health risk assessment.**

Toxigenic *Vibrio cholerae* O1 and *V. cholerae* O139 are etiological agents of epidemic cholera. However, both *V. cholerae* O1 strains that do not produce cholera toxin, i.e., that are nontoxigenic (NT), and non-O1/non-O139 strains have also been associated with cholera, gastroenteritis, septicemia, and/or extraintestinal infections (49, 51, 52, 63, 66, 71, 81). Outbreaks of cholera were reported in Brazil during the third (1853 to 1854), fourth (1866 to 1868), and fifth (1893 to 1895) pandemics (3). In the early 1970s, when cholera spread to Africa and Southern Europe, it was forecast to arrive in countries across the Atlantic as well. This prompted the establishment of a surveillance program in S. Paulo State, Brazil, by the WHO (World Health Organization) and CETESB (Companhia de Tecnologia de Saneamento Ambiental, S.P.-Brazil—State Agency for Environmental Control). Sewage samples were monitored for *V. cholerae* in the community, and 12,867 samples were collected. From these samples, four NT *V. cholerae* O1 strains were isolated, the first *V. cholerae* O1 NT strains to be isolated in Brazil. The isolates were from sewage samples collected in 1978, 1980, and 1983 (42). Non-O1 *V. cholerae* strains were subsequently isolated from sewage (77.3%), seawater (40.4%), and freshwater (33.3%) samples collected in 1982 and 1983, at a time when no cases of cholera or gastroenteritis had been reported in S. Paulo State (41). At the same time, in Rio de Janeiro State, non-O1 *V. cholerae* was isolated from 12% of seawater and oyster samples (64).

The seventh pandemic reached the Americas on 29 January 1991 in Lima, Peru, and spread rapidly to the Peruvian Northern Andean and Amazon regions (74). Brazil reported its first case of cholera on 8 April 1991 in Tocantins. Cholera cases then occurred in the Amapa, Amazonas, Maranhão, Mato Grosso, Pará, and Rondonia States. However, in S. Paulo State, a more developed region of the country, the highest incidence of cholera in 1994 was 0.23 per 100,000 inhabitants compared to 33.4, the national average that same year (47).

The pathogenicity of *V. cholerae* O1 and O139 strains depends on a combination of properties, including enterotoxin (cholera toxin [CT], *ctxA*) and the ability to adhere to, and colonize, the small intestine (colonization factor, *tcpA*) (27). The major virulence-associated factors are present in clusters (23), with at least three regions in the *V. cholerae* chromosome. The first is the CTX genetic element (45), which has now been reported to comprise the genome of a filamentous bacteriophage (CTXΦ) (80). The second region is a large pathogenicity island for *V. cholerae* (VPI) (35) that encodes a toxin-coregulated pilus (TCP) gene cluster, a type IV pilus that functions as an essential colonization factor (75) and acts as CTXΦ receptor (35). The third gene cluster, the RTX toxin gene cluster, was described by Lin et al. (40) in a *V. cholerae* O1 El Tor strain and encodes cytotoxic activity for Hep-2 cells in vitro. However, the implication of RTX in pathogenesis has yet to be confirmed (16). Other factors have been associated with enteropathogenicity and include an El Tor-like hemolysin (*hlyA*)

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TABLE 1. Genotypic traits of toxigenic and NT *V. cholerae* O1, *V. cholerae* O139, and *V. mimicus* isolates examined in this study

| Strain (no. of strains) | Country | Reference(s) | Yr | Source | Code (*n*) | Genotype (presence [+] or absence [−] of genes) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *ctxA* | *hlyA*ET | *ompU* | *stn*/*sto* | *tcpA*ET | *tcpI* | *toxR* | *zot* |
| Toxigenic *V. cholerae* O1 (19) | Brazil | 61 | 1991–1994 | Sewage | RC7, RC221, RC226, CT2578, CT8514, CT31644, CT25017, CT24082 (8) | + | + | + | − | + | + | + | + |
| | Peru | 61 | 1991, 1993 | Clinical | RC8, IAL 1941 (2) | + | + | + | − | + | + | + | + |
| | Brazil | 61 | 1993 | Sewage | RC243, CT15861 (2) | + | + | + | − | + | − | + | + |
| | Chile | | 1991 | Clinical | RC11 | + | + | + | − | + | + | + | + |
| | Mexico | | 1991 | Clinical | RC25 | + | + | + | − | + | + | + | + |
| | Brazil | 41 | 1994 | River | CT7021 | + | + | + | − | + | + | + | + |
| | Brazil | 61 | 1992 | Clinical | CTMACM14 | + | + | + | − | + | − | + | + |
| | Brazil | 61 | 1993 | Sewage | RC224 | + | + | + | + | + | + | + | + |
| | Peru | | 1991 | Clinical | RC24 | + | + | + | + | + | + | + | + |
| | Brazil | 61 | 1993 | Sewage | CT15989 | + | + | + | *[b] | + | + | + | + |
| Total positives | | | | | | 19 | 19 | 19 | 2 | 19 | 16 | 19 | 19 |
| NT *V. cholerae* O1 (4) | Brazil | 41 | 1978 | Sewage | TM45 | − | + | + | − | + | + | + | − |
| | Brazil | 41, 61 | 1978 | Sewage | RC229, TM207831 (2) | − | + | + | − | + | − | + | − |
| | Brazil | 41 | 1980 | Sewage | RC231 | − | + | + | − | − | + | + | − |
| Total positives | | | | | | 0 | 4 | 4 | 0 | 3 | 2 | 4 | 0 |
| *V. cholerae* O139 (7) | India | 61 | 1993 | Clinical | RC4, RC30, RC120, RC138, RC139 (5) | + | + | + | − | + | + | + | + |
| | Bangladesh | | 1993 | Clinical | RC46 | + | + | + | + | + | − | + | + |
| | India | 61 | 1993 | Clinical | IGV | + | + | + | − | + | − | + | + |
| Total positives | | | | | | 7 | 7 | 7 | 1 | 7 | 5 | 7 | 7 |
| *V. mimicus* (5) | Louisiana | | | Environ[a] | RC54 | − | − | + | + | − | − | − | − |
| | Louisiana | | | Environ[a] | RC55 | + | + | − | + | − | + | − | + |
| | Louisiana | | | Environ[a] | RC56 | + | + | − | − | + | + | − | + |
| | Louisiana | | | Environ[a] | RC57 | + | + | − | + | − | − | − | − |
| | Louisiana | | | Environ[a] | RC59 | + | − | + | − | − | − | − | + |
| Total positives | | | | | | 4 | 3 | 2 | 3 | 1 | 2 | 0 | 3 |

[a] Environ, environmental.
[b] *, Amplicon with a different size.

(82), heat-stable enterotoxin (*stn*/*sto*) (1, 22, 55), hemagglutinins (14), neuraminidase (*nanH*) (20), a new CT (33), outer membrane protein (*ompU*) (72), Shiga-like toxin (*stx*) (33), a ToxR regulatory protein (46), and a zonula occludens toxin (*zot*) (18).

*V. cholerae* can be found in the environment both as a free-living bacterium and in association with zooplankton (30). Therefore, not surprisingly, non-O1/non-O139 *V. cholerae* is frequently isolated from the aquatic environment and seafood (5, 8, 9, 29, 30, 31, 32, 41, 43, 44, 78). In fact, *V. cholerae* is a heterogeneous species, with 206 serotypes identified to date (G. B. Nair, personal communication).

The emergence of the new serogroup O139 as a second etiologic agent of cholera epidemics (48), along with the discovery of horizontal and vertical genetic transfer by phages (80) and the elucidation of pathogenicity islands and other mobile genetic elements (36), has revived interest in the non-O1/non-O139 *V. cholerae* strains that had been previously implicated in cholera-like epidemics (2, 13, 51, 71, 81). In addition, the possible conversion of non-O1 to O1 serotype has provided added interest (10).

Cholera surveillance is now under way in many countries, based primarily on detection of *V. cholerae* O1 and O139 and determining the presence of cholera toxin using biological and molecular methods. However, virulence-associated factors in *V. cholerae* isolates from environmental sources are of concern.

The primary objective of this study was to evaluate the presence of virulence-associated factors in *V. cholerae* populations as potential pathogenic markers suitable for environmental monitoring. Virulence-associated factors studied here included cholera toxin (*ctxA*), hemolysin (*hlyA*), non-O1 heat-stable enterotoxin (*stn*/*sto*), outer membrane protein (*ompU*), TCP (*tcpA* and *tcpI*), ToxR regulatory protein (*toxR*), and zonula occludens toxin (*zot*).

## MATERIALS AND METHODS

**Bacterial strains.** A total of 69 *V. cholerae* isolates were included in this study. *V. cholerae* O1 strains comprised 19 toxigenic clinical and environmental isolates from Brazil (14 isolates), Peru (3 isolates), Chile (1 isolate), and Mexico (1 isolate) and four nontoxigenic *V. cholerae* O1 isolates from Brazil (1978 to 1980). Thirty-nine environmental isolates of non-O1/non-O139 *V. cholerae* from Brazil, seven *V. cholerae* O139 clinical isolates from India, and five *V. mimicus* environmental isolates from Louisiana (United States) were included in the study (Tables 1 and 2). The Brazilian environmental strains were isolated in the CETESB Laboratory, S. Paulo, Brazil. All of the isolates are part of a culture collection (RRC) at the Center of Marine Biotechnology (Baltimore, Md.). Frozen stock cultures were subcultured on Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.), streaked onto LB agar, and then onto TCBS agar (Oxoid) to verify purity.

TABLE 2. Genotypic traits of non-O1/non-O139 *V. cholerae* Brazilian isolates examined in this study

| Country (no.) | Yr | Reference(s) | Source | Code (*n*) | Genotype (presence [+] or absence [−] of genes) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *ctxA* | *hlyA*ET | *hlyA*Class | *ompU* | *stn*/*sto* | *tcpA*ET | *tcpA*Class | *tcpI* | *toxR* | *zot* |
| Brazil (9) | 1992 | 43 | Mussels | RC236, RC237, RC238, RC244 (4) | − | + | − | + | − | − | − | + | + | − |
| | 1992 | 43, 61 | Mussels | RC245, GM39 (2) | − | + | − | + | − | − | − | − | + | − |
| | 1992 | 43, 61 | Mussels | RC239, GM32 (2) | − | + | − | + | − | + | − | + | + | − |
| | 1992 | 44 | Oysters | RC247 | − | − | − | + | − | − | − | − | + | − |
| Brazil (9) | 1981, 1982 | — | Seawater | RC63, RC64, RC65 (3) | − | + | − | + | + | − | − | + | + | − |
| | 1982 | — | Seawater | RC62 | − | + | − | + | + | − | + | − | + | − |
| | 1983 | 61 | Seawater | TM16589 | − | + | − | + | − | − | − | + | + | − |
| | 1982 | — | Seawater | RC61 | + | + | − | + | + | − | + | + | + | − |
| | 1981 | 41 | Seawater | RC253 | + | + | − | + | − | + | − | − | + | − |
| | 1982 | — | Seawater | TMA52 | − | + | − | + | + | + | + | + | + | − |
| | 1982 | — | Seawater | RC60 | + | − | + | + | + | + | + | + | + | − |
| Brazil (7) | 1982 | — | Sediment | RC68 | − | + | − | + | *[a] | − | − | − | + | − |
| | 1981 | 61 | Sediment | TM50022 | − | + | − | + | − | + | − | − | + | − |
| | 1983 | — | Sediment | RC71 | − | + | − | + | − | + | − | + | + | − |
| | 1983 | — | Sediment | RC70 | − | + | − | + | + | − | − | − | + | − |
| | 1982 | — | Sediment | RC66 | − | + | − | + | + | + | − | − | + | − |
| | 1982 | — | Sediment | RC67 | − | + | − | + | + | − | − | + | + | − |
| | 1983 | — | Sediment | RC69 | − | + | − | + | + | + | + | + | + | − |
| Brazil (14) | 1977, 1978, 1979 | 41, 61 | Wastewater | RC235, RC248, TM41338, IG4 (4) | − | + | − | − | − | − | − | − | + | − |
| | 1977, 1978 | 41 | Wastewater | RC242, RC251 (2) | − | + | − | *[a] | − | − | − | − | + | − |
| | 1979, 1992 | 41 | Wastewater | RC234, RC252 (2) | − | + | − | + | − | − | − | + | + | − |
| | 1992 | 41 | River | RC241 | − | + | − | − | − | − | − | + | + | − |
| | 1983 | 41 | Wastewater | RC240 | − | + | − | − | − | + | − | + | + | − |
| | 1979 | 61 | Wastewater | TM31152 | − | + | − | *[a] | − | − | − | + | + | − |
| | 1992 | 61 | Wastewater | CT3481 | − | + | − | + | − | − | − | − | + | − |
| | 1977 | 41 | Wastewater | RC233 | + | + | − | + | − | + | − | − | + | − |
| | 1982 | 41 | Wastewater | RC246 | − | + | − | + | − | + | − | + | + | − |
| Total strains (39) | | | | | 4 | 37 | 1 | 30 | 11 | 12 | 5 | 22 | 39 | 0 |

[a] *, Amplicon with different size.

**Positive and negative controls.** *V. cholerae* O1 Classical ATCC 11623, *V. cholerae* O1 El Tor ATCC 14033 (*ctxAB* negative), *V. cholerae* O1 Classical ATCC 14035, *V. cholerae* non-O1 ATCC 14547, *V. cholerae* non-O1 ATCC 25872 (*ctxAB*$^+$), *V. cholerae* non-O1 ATCC 25874 (*ctxAB*$^+$), *V. mimicus* ATCC 33653, *V. cholerae* O22, and *V. cholerae* O31 were used as positive controls, and *Escherichia coli* was used as a negative control.

**Chromosomal DNA preparation.** DNA was extracted by the CTAB (cetyltrimethylammonium bromide) method previously described (62). DNA extracts were resuspended in Tris-EDTA (10 mM Tris-HCl, 0.10 mM EDTA [pH 8.0]) buffer and stored at 4°C for further analysis. Dilutions of template DNA were made with sterile distilled water to obtain a concentration of ca. 100 ng/μl.

**PCR primers and amplification conditions.** The oligonucleotide primers for each of the selected virulence-associated factors were designed based on available GenBank sequences for *V. cholerae* O1 Classical and *V. cholerae* O1 E1 Tor for all genes, except the *stn*/*sto* genes, for which *V. cholerae* non-O1 and *V. cholerae* O1 sequences were used. The sequence positions and accession numbers of the sequences or sources are listed in Table 3. Oligonucleotide primers were synthesized by Genosys Biotechnologies, Inc.

**Multiplex PCR.** The following reagents were added to each sample PCR mixture: 2.5 μl of 10× amplification buffer A (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 0.01% [wt/vol] gelatin) (Promega, Madison, Wis.); 0.5 μl each of 2.5 mM dATP, dCTP, dGTP, and dTTP (Promega); 1.0 μl each of forward and reverse primers (20 μM); 0.125 μl of *Taq* DNA polymerase at 5 U/μl (Promega); and Milli-Q water (to a final volume of 24 μl). PCR was carried out in 0.2-ml microcentrifuge tubes with 24 μl of the PCR mixture and 1 μl (ca. 0.10 μg) of template DNA. The solution was mixed, centrifuged briefly, and placed in an automated Peltier thermal cycler (PTC-200; M. J. Research).

PCR amplification conditions were as follows: denaturation at 94°C for 2 min, annealing for 1 min at the temperatures shown in Table 3, and extension at 72°C, as given in Table 3; with a final extension step at 72°C for 10 min at the end of 30 cycles, followed by maintenance at 4°C.

PCR products were separated by agarose gel electrophoresis (1.4%) in 1× TAE buffer (0.04 Tris-acetate, 0.001 M EDTA [pH 8.0]), stained with ethidium bromide, and visualized by using UV light.

## RESULTS

Multiplex PCR was carried out, using positive and negative controls and the primers designed for the genes studied. The size of each amplicon was verified. Optimal PCR conditions were determined for *ctxA*/*ompU*-PCR, *hlyA* (Classical and El Tor), *zot*/*toxR*-PCR, and *tcpA* (Classical and El Tor), using multiplex PCR. For *tcpI* and *stn*/*sto* genes, simple PCR was used. The multiplex and simple PCR products obtained for each gene investigated are shown in Fig. 1, and the corresponding amplicon sizes are given in Table 3.

The virulence-associated factors for specific *V. cholerae* serogroups are summarized in Tables 1 and 2.

All of the *V. cholerae* O1, O139, and non-O1/non-O139 strains, regardless of whether they were toxigenic or NT, were found to possess the *tox*R regulatory sequence, a gene absent in the five *V. mimicus* strains tested.

CT and zonula occludens toxin (ZOT) were present in all (100%) of the toxigenic *V. cholerae* O1 and O139 strains tested and were absent in NT *V. cholerae* O1. In *V. cholerae* non-O1/non-O139 strains, we found four strains (RC60, RC61, RC233, and RC253) to be *ctxA* positive and *zot* negative. These isolates

TABLE 3. Primers used in this study

| Gene(s), primers, and sequences (5′ to 3′) | Amplicon size (bp) | PCR conditions[a] | Source or reference; accession no. |
|---|---|---|---|
| *ctxA* and *ompU* (CT subunit A and outer membrane protein) | | 60–1 | |
| 94F, CGG GCA GAT TCT AGA CCT CCT G | 564 (*ctxA*) | | 19 |
| 614R, CGA TGA TCT TGG AGC ATT CCC AC | | | |
| 80F, ACG CTG ACG GAA TCA ACC AAA G | 869 (*ompU*) | | This study; AE004149, AF253529, U73751 |
| 906R, GCG GAA GTT TGG CTT GAA GTA G | | | |
| *zot* and *toxR* (zonula occludens toxin and operon ToxR) | | 60–1 | |
| 225F, TCG CTT AAC GAT GGC GCG TTT T | 947 (*zot*) | | This study; AE004224, AF123049, AF175708, M8363 |
| 1129R, AAC CCC GTT TCA CTT CTA CCC A | | | |
| 101F, CCT TCG ATC CCC TAA GCA ATA C | 779 (*toxR*) | | This study; M21249, AE004179 |
| 837R, AGG GTT AGC AAC GAT GCG TAA G | | | |
| *tcpA* (TCP A [Classical and El Tor]) | | 60–1 | |
| 72F, CAC GAT AAG AAA ACC GGT CAA GAG | 451 (El Tor) | | Modified from reference 39; AE004168, UO9807, X64098, X09807 |
| 477R, CGA AAG CAC CTT CTT TCA CGT TG | | | |
| 647R, TTA CCA AAT GCA ACG CCG AAT G | 620 (Classical) | | |
| *tcpI* (TCP I) | | 60–3 | |
| 132F, TAG CCT TAG TTC TCA GCA GGC A | 862 (*tcpI*) | | This study; AE004168, L25659, X64098 |
| 951R, GGC AAT AGT GTC GAG CTC GTT A | | | |
| *hlyA* (hemolysin [Classical and El Tor]) | | 60–1 | |
| 489F, GGC AAA CAG CGA AAC AAA TAC C | 481 (El Tor) | | Modified from reference 69; AE004362, AF117834, M36855, X51746, Y00557 |
| 744F, GAG CCG GCA TTC ATC TGA AT | 738/727 (ET/Clas) | | |
| 1184R, CTC AGC GGG CTA ATA CGG TTT A | | | |
| *stn*/*sto* (non-O1 heat-stable enterotoxin) | | 55–1 | |
| 67F, TCG CAT TTA GCC AAA CAG TAG AAA | 172 (*stn*/*sto*) | | This study; M85198, X74108 |
| 194R, GCT GGA TTG CAA CAT ATT TCG C | | | |

[a] Temperature of annealing (°C)–time of extension (min).

were positive for *ctxA*, using multiplex PCR assay, with a 564-bp amplicon identical to that of the O1 strains. The size of *ctxA* amplicons was confirmed by sequencing (data not shown).

The oligonucleotide primers targeting *tcpA* exploited sequence differences between the *tcpA* of the El Tor (ET) and Classical *V. cholerae* biotypes. All toxigenic *V. cholerae* O1 and O139 and three of four nontoxigenic *V. cholerae* O1 isolates examined showed amplicons of the same size as that obtained for the *tcpA* El Tor gene. Of 39 non-O1/non-O139 *V. cholerae* strains, 14 yielded amplicons, using *tcpA* primers. The amplicon size (451 bp) of nine strains (RC66, RC71, RC233, RC239, RC240, RC246, RC253, GM32, and TM50022) was identical to that obtained for *V. cholerae* El Tor ATCC 14033, while the amplicon size (620 bp) of two strains (RC61 and RC62) was identical to that obtained for *V. cholerae* O1 Classical ATCC 14035. Interestingly, three strains carried both genes (RC60, RC69, and TMA52) (Table 2).

The *tcpI* gene was frequently found in toxigenic *V. cholerae* O1 (84.2%) and O139 (71.4%) serogroups. However, this gene was also present in 50% of the NT *V. cholerae* O1 and in 56.4% of the non-O1/non-O139 serogroups.

The *hlyA* El Tor gene was found in all toxigenic and NT *V. cholerae* O1 and in *V. cholerae* O139. Among non-O1/non-O139 serogroup strains, 94.9% showed homology to El Tor hemolysin, 2.6% were associated with Classical hemolysin, and 2.5% were negative for both genes. The amplified fragment sizes were 727 bp, specifically for the Classical biotype (ATCC 11623 and ATCC 14035) and both 481 bp and 738 bp for the El Tor biotype. Occasionally, a larger fragment (~1.4 kb) was observed in the El Tor biotype strains.

The *ompU* gene was found in all strains of toxigenic and NT *V. cholerae* O1, *V. cholerae* O139, and in 76.9% of the environmental non-O1/non-O139 *V. cholerae* isolates.

Genes homologous to *stn*/*sto* were observed in toxigenic *V. cholerae* O1 (2 of 19), *V. cholerae* O139 (1 of 7), and non-O1/non-O139 *V. cholerae* (11 of 39) strains, yielding an amplicon size of 172 bp. However, we observed an amplicon of 800 bp in one isolate from sediment (RC68).

The genotypes found in each *V. cholerae* serogroup are listed in Table 4. The genotype most frequently observed in clinical toxigenic *V. cholerae* O1 (68.4%) and O139 (71.4%) isolates was *ctxA hlyA*ET *ompU tcpA*ET *tcpI toxR zot*. Other genotypes found in toxigenic *V. cholerae* O1 strains were similar, but the *tcpI* and/or *stn*/*sto* genes were absent. In non-O1/non-O139 *V. cholerae* serogroups, the genotypes were diverse; however, the most frequent genotypes were *hlyA*ET *ompU tcpI toxR* (7 of 39) and *hlyA toxR* (6 of 39).

The presence of virulence-associated genes in non-O1/non-O139 *V. cholerae* isolates was analyzed by origin, i.e., water and sediment for marine ecosystems, mussels, and wastewater, and the results are summarized in Table 5.

## DISCUSSION

The majority of *V. cholerae* strains in the environment are considered to be harmless estuarine microorganisms. However, specific strains appear to have evolved that cause disease in humans by effectively colonizing the small intestine and releasing a potent enterotoxin. Eight chromosomal regions coding for virulence-associated factors in *V. cholerae* (*ctxA,*

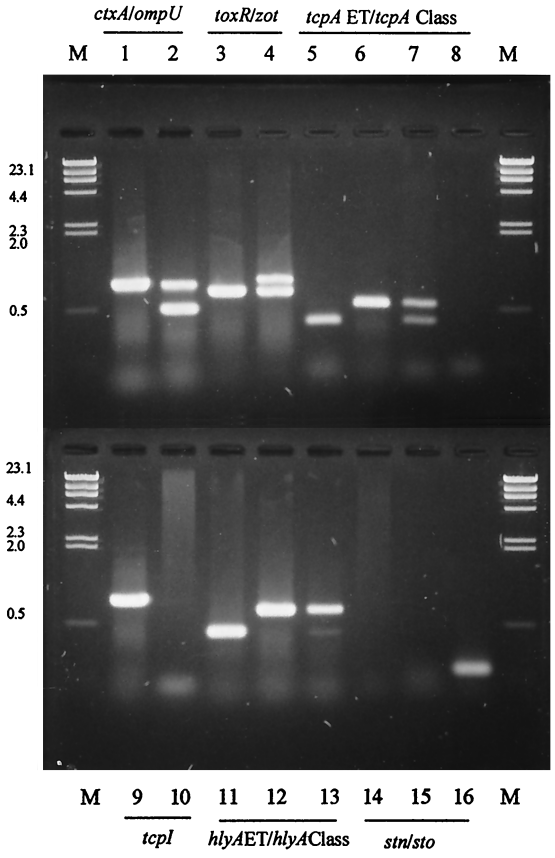


FIG. 1. Agarose gel electrophoresis of amplicons obtained using multiplex PCR. Lane M, molecular weight marker (lambda DNA *Hin*dIII); lanes 1, 3, 5, 11, and 14, *V. cholerae* O1 El Tor nontoxigenic ATCC 14033; lanes 2, 6, 9, 12, and 15, *V. cholerae* O1 Classical ATCC 14035; lane 4, RC224, *V. cholerae* O1 El Tor (Brazilian isolate from sewage, 1993); lane 7, TMA52, *V. cholerae* non-O1/non-O139 (Brazilian isolate from seawater, 1982); lane 8, negative control; lane 10, RC46, *V. cholerae* O139 (*tcpI* negative); lane 13, RC60, *V. cholerae* non-O1/non-O139 (Brazilian isolate from seawater, 1982); lane 16, RC66 (Brazilian isolate from sediment, 1982).

*hlyA, ompU, stn*/*sto, tcpA, tcpI, toxR*, and *zot*) were included in this study.

**Distribution of virulence-associated genes in *V. cholerae*.** The major CTX genetic element has the structure of a compound transposon, with a 4.5-kb central core region (*ctxAB, zot, ace, orfU*, and *cep*) that encodes for both CT and for functions required for virion morphogenesis and is flanked by one or more copies of a 2.7-kb repetitive sequence that encodes functions required for regulation, replication, and integration of CTXϕ (36, 80). In this region, we tested for the presence of two genes: CT subunit A (*ctxA*) and ZOT (*zot*). Interestingly, we found four of the non-O1/non-O139 *V. cholerae* isolates contained *ctxA* gene but not the *zot* gene. The ZOT was described by Fasano et al. (18) as a toxin that increases permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction, or zonula occludens. However, Waldor and Mekalanos (80) found that *zot* and *orfU* correspond to genes involved in CTXΦ morphogenesis and that the biological activity previously designated "zonula occludens toxin" is probably not directly associated with the *zot* gene product, unless its product possesses a dual function. Additionally, the presence of the *zot* gene and the absence of the *ctx* gene in *V. cholerae* and *V. mimicus* strains has been reported (7, 21, 38). To explore the CTXΦ genome integrated in the *V. cholerae* chromosome, sequencing of the CTX genetic element is under way.

The *V. cholerae* pathogenicity island (VPI) is 39.5 kb and contains genes associated with virulence (TCP-ACF cluster), the regulation of virulence (*toxT* and *tcpP*/*H*), the regulation of chemotaxis (*tcpI* or *acfB*), and mobility (*int* and *orfI*) (24, 36). Because the genes encoding TCP have been suggested to be part of a larger genetic element consisting of a cluster of genes, we looked for the presence of both *tcpA* and *tcpI* genes. The *tcpA*ET gene was present in all toxigenic *V. cholerae* O1 and O139 isolates, showing that despite different lipopolysaccharide structures, the two share common TCP-associated antigens (60). The *tcpA*ET gene was also found in 3 of 4 strains of NT *V. cholerae* O1 and in 9 of 39 non-O1/non-O139 Brazilian strains. Interestingly, in the non-O1/non-O139 serogroup, two strains had *tcpA* genes of the Classical biotype and three had *tcpA* gene of both biotypes, in agreement with previous reports (6, 54). These results suggest that these strains may have a selective advantage over nonpathogenic strains, with an ability to colonize the human intestine, and become toxigenic (35). However, while the El Tor TcpA pilin is 82% identical to TcpA of the Classical biotype (60), there are at least four major variants of *tcpA* genes that probably evolved in parallel, though independently, from a common ancestral gene (54). Sequencing of *tcpA* El Tor amplicons demonstrated the fragment size to be 451 bp, and the amplicon sizes for *tcpA* Classical was 620 bp in the *V. cholerae* non-O1/non-O139 strains (data not shown). These variations could indicate other functional significance as well, because TcpA also acts as a coat protein of the bacteriophage VPΦ, produced by vibrios containing VPI (37, 77).

In this study, the presence of strains containing *tcpA* genes, either identical to those of biotype El Tor or of Classical, is in agreement with results obtained in Australia, where *V. cholerae* serogroup O6 was shown to contain the *tcpA*ET gene and another strain of serogroup O23 presented as *tcpA* Classical, both strains having been isolated from water (67).

TcpI, an integral inner membrane protein, involved in environmental sensing and signal transduction (chemosensors), negatively regulates the synthesis of the major pilin subunit of TCP (TcpA) (24, 76). Harkey et al. (24) suggested that regulators such as TcpI, that act downstream of ToxR and ToxT may function to fine-tune the expression of the TCP virulence determinant throughout the pathogenic cycle of *V. cholerae*. However, our results showing the presence of *tcpI* gene in 65.5% of *V. cholerae* strains suggests that the importance of this gene may be physiological and not pathogenesis alone.

Outer membrane protein, OmpU, was reported to be a potential adherence factor for *V. cholerae* (72). However, later studies suggested that OmpU is not involved in the adhesion of *V. cholerae* to the intestinal epithelium (53). In this study, the gene was present in 87% of the *V. cholerae* strains tested, except in nine non-O1/non-O139 *V. cholerae* strains isolated from wastewater. These findings suggest that this gene may be mainly physiological in its activity.

The hemolysin traditionally has been employed to differen-

TABLE 4. Genotypes of *V. cholerae* isolates listed by serogroup

| Genotypes | No. of isolates | | | | |
|---|---|---|---|---|---|
| | O1 | NT O1 | O139 | Non-O1/ non-O139 | Total |
| *ctxA*$^+$ *hlyA*$^+$ET *ompU*$^+$ *tcpA*$^+$ET *tcpI*$^+$ *toxR*$^+$ *zot*$^+$ | 13 | 0 | 5 | 0 | 18 |
| *ctxA*$^+$ *hlyA*$^+$ET *ompU*$^+$ *tcpA*$^+$ET *toxR*$^+$ *zot*$^+$ | 3 | 0 | 1 | 0 | 4 |
| *ctxA*$^+$ *hlyA*$^+$ET *ompU*$^+$ *stn*/*sto*$^+$ *tcpA*$^+$ET *tcpI*$^+$ *toxR*$^+$ *zot*$^+$ | 2+1[a] | 0 | 0 | 0 | 3 |
| *ctxA*$^+$ *hlyA*$^+$ET *ompU*$^+$ *tcpA*$^+$ET *toxR*$^+$ | 0 | 0 | 0 | 2 | 2 |
| *ctxA*$^+$ *hlyA*$^+$ET *ompU*$^+$ *stn*/*sto*$^+$ *tcpA*$^+$ET *toxR*$^+$ *zot*$^+$ | 0 | 0 | 1 | 0 | 1 |
| *ctxA*$^+$ *hlyA*$^+$ET *ompU*$^+$ *stn*/*sto*$^+$ *tcpA*$^+$Class *tcpI*$^+$ *toxR*$^+$ | 0 | 0 | 0 | 1 | 1 |
| *ctxA*$^+$ *hlyA*$^+$Class *ompU*$^+$ *stn*/*sto*$^+$ *tcpA*$^+$ET/Class *tcpI*$^+$ *toxR*$^+$ | 0 | 0 | 0 | 1 | 1 |
| *hlyA*$^+$ET *ompU*$^+$ *tcpI*$^+$ *toxR*$^+$ | 0 | 1 | 0 | 7 | 8 |
| *hlyA*$^+$ET *toxR*$^+$ | 0 | 0 | 0 | 6 | 6 |
| *hlyA*$^+$ET *ompU*$^+$ *tcpA*$^+$ET *tcpI*$^+$ *toxR*$^+$ | 0 | 1 | 0 | 4 | 5 |
| *hlyA*$^+$ET *ompU*$^+$ *stn*/*sto*$^+$ *tcpI*$^+$ *toxR*$^+$ | 0 | 0 | 0 | 4 | 4 |
| *hlyA*$^+$ET *ompU*$^+$ *toxR*$^+$ | 0 | 0 | 0 | 4 | 4 |
| *hlyA*$^+$ET *ompU*$^+$ *tcpA*$^+$ET *toxR*$^+$ | 0 | 2 | 0 | 1 | 3 |
| *hlyA*$^+$ET *ompU*$^+$ *stn*/*sto*$^+$ *tcpA*$^+$ET/Class *tcpI*$^+$ *toxR*$^+$ | 0 | 0 | 0 | 2 | 2 |
| *hlyA*$^+$ET *tcpI*$^+$ *toxR*$^+$ | 0 | 0 | 0 | 2 | 2 |
| *hlyA*$^+$ET *ompU*$^+$ *stn*/*sto*$^+$ *tcpA*$^+$Class *toxR*$^+$ | 0 | 0 | 0 | 1 | 1 |
| *hlyA*$^+$ET *ompU*$^+$ *stn*/*sto*$^+$ *tcpA*$^+$ET *toxR*$^+$ | 0 | 0 | 0 | 1 | 1 |
| *hlyA*$^+$ET *tcpA*$^+$ET *tcpI*$^+$ *toxR*$^+$ | 0 | 0 | 0 | 1 | 1 |

[a] 1, i.e., an *stn*/*sto* amplicon with another size.

tiate between the two biotypes of *V. cholerae* O1. The sequence of the Classical biotype has an 11-bp deletion within the *hlyA* structural gene, compared to the El Tor biotype (57). Using information on these genes, we designed primers to differentiate both biotypes and verified the presence of this gene in 98.6% of the *V. cholerae* strains, in agreement with previous reports (4, 68, 71, 82, 83). The majority belonged to the El Tor biotype, regardless of the hemolytic phenotype (67 of 69). The multiplex PCR for hemolysin effectively distinguished the two biotypes in non-O1/non-O139 *V. cholerae* strains compared with *V. cholerae* O1 Classical ATCC 11623 and ATCC 14035 and *V. cholerae* O1 El Tor ATCC 14033. Interestingly, the *hlyA* gene is located on chromosome 2 (26).

*V. cholerae* non-O1/non-O139 strains may also produce a 17-amino-acid heat-stable enterotoxin (NAG-ST) (*stn*) that is closely related to the heat-stable toxins produced by enterotoxigenic *E. coli* and other enteric pathogens (1, 55). Also, a heat-stable enterotoxin in *V. cholerae* O1 strains (*sto*) has been described (22). We designed primers based on the *stn*/*sto* genes associated with heat-stable enterotoxin production and found the genes to be homologous to those of toxigenic *V. cholerae* O1 (2 of 19), *V. cholerae* O139 (1 of 7), and non-O1/non-O139 *V. cholerae* (11 of 39) strains. This is the first report showing higher values than those reported in other studies, using hybridization with a NAG-ST probe, in *V. cholerae* non-O1/non-O139 population (12, 28, 56, 58). Interestingly, the *stn*/*sto* gene occurred more frequently in isolates from seawater (7 of 9) and sediment (4 of 7) and was absent in isolates from sewage (0 of 14) or oysters and mussels (0 of 9). It should be mentioned, however, that Caldini et al. (5) reported the presence of the *sto* gene in 12.7% (19 of 150) of the *V. cholerae* non-O1 isolates from freshwater in the river basin of central Italy, but the incidence of NAG-ST in *V. cholerae* non-O1 was not clearly established. Results of a study involving human volunteers demonstrated that, besides the production of NAG-ST, the virulence of non-O1/non-O139 *V. cholerae* depends on its ability to colonize the intestine (50).

The regulation and expression of genes for growth and survival depend on the regulon ToxR, coordinately regulated by a cascade mechanism involving three known components: ToxR, ToxS, and ToxT (15). ToxR, a 32-kDa transmembrane protein, is the master regulator, and its expression is dependent upon environmental growth conditions (incubation temperature, pH, osmolarity, bile salts, oxygen tension, hydrostatic pressure, and amino acid composition of the medium) (15, 46). The *toxR* gene encodes a transcriptional activator controlling CT gene

TABLE 5. Distribution of virulence-associated genes among non-O1/non-O139 *V. cholerae* isolates listed by source

| Brazilian source | *n* | Virulence-associated gene-positive isolates[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *ctxA* | *hlyA* | | *stn*/*sto* | *ompU* | *tcpA* | | | *tcpI* | *toxR* | *zot* |
| | | | ET | C | | | ET | C | ETC | | | |
| Mussels | 9 | 0 | 8 | 0 | 0 | 9 | 2 | 0 | 0 | 6 | 9 | 0 |
| Seawater | 9 | 3 | 8 | 1 | 7 | 9 | 1 | 2 | 2 | 7 | 9 | 0 |
| Sediment | 7 | 0 | 7 | 0 | 4 | 7 | 3 | 0 | 1 | 3 | 7 | 0 |
| Wastewater | 14 | 1 | 14 | 0 | 0 | 5 | 3 | 0 | 0 | 6 | 14 | 0 |
| Total | 39 | 4 | 37 | 1 | 11 | 30 | 9 | 2 | 3 | 22 | 39 | 0 |

[a] C, Classical; ETC, both ET and Classical.

expression (*ctxA*), TCP biogenesis (*tcpA*), outer membrane protein expression (*ompU*), and at least 17 distinct genes in O139 and O1 strains (15, 27, 46). In this study, the presence of the *toxR* gene was verified in all *V. cholerae* studied, regardless of serogroup or source of isolation, a finding in agreement with previous reports (21, 71).

The presence of *toxR* (100%), *hlyA* (98.6%), *ompU* (87%), and *tcpI* (65.5%) genes in the *V. cholerae* isolates suggest that they are required for functioning of the organism in the environment and are not solely related to pathogenesis.

Five *V. mimicus* environmental strains were included in our study because of their close relationship to *V. cholerae*. None carried the *toxR* gene. The presence of CT in four of the five *V. mimicus* strains was confirmed, while the *zot* gene was found in only three of the strains, results similar to those reported earlier by Chowdhury et al. (7). The *stn*/*sto* gene was found in three of five environmental strains, as reported elsewhere, using PCR (79). In contrast, Pal et al. (56) reported the presence of NAG-ST gene in 13.7 and 22.6% of *V. mimicus* isolates from environmental and clinical sources, respectively, and Ramamurthy et al. (59) suggested that *V. mimicus* may act as a genetic reservoir for these genes.

Multiplex PCR was found to be sensitive and specific for assessing the pathogenicity of clinical and environmental *V. cholerae* isolates. We tested multiplex PCR for *ctxA*/*tcpA* El Tor genes using four primers—94F, 614R, 72F, and 477R—as a primary screening for *V. cholerae* during epidemiological surveillance with good success (data not shown).

***V. cholerae* genotypes.** A single factor cannot explain enteropathogenicity. In this study eight virulence-associated genes were detected in *V. cholerae* strains isolated from both clinical and environment sources. Previous findings, in an earlier study of 172 *V. cholerae* non-O1/non-O139 environmental isolates from seawater and sediment samples collected in São Paulo, Brazil, showed that 60.4% of the strains were *hlyAC*$^+$, *nanH*$^+$, and *toxR*$^+$ and *ctxAB*, *elt* (33), and *zot* negative as determined by using probes and radioactive hybridization. The frequency of occurrence of the genes among the strains tested was 98.8% for *toxR*, 97.1% for *hlyAC*, 66.9% for *nanH*, 5.8% for *slt*, 1.2% for *zot*, and 0.6% for *ctxAB* (D. E. Alvarado, V. H. Pellizari, T. A. T. Gomes, and I. N. G. Rivera, Abstr. 8th Int. Symp. Microb. Ecol. p. 89, 1998). In this study, we included *V. cholerae* O1 toxigenic and NT strains and *V. cholerae* O139 strains, using multiplex PCR, finding that 100% of the *V. cholerae* O1 and O139 strains carried *ctxA*, *hlyA*ET, *ompU*, *tcpA*ET, *toxR*, *zot*, and *tcpI* and sometimes the *stn*/*sto* gene.

Non-O1/non-O139 *V. cholerae* strains tested revealed the genotype *hlyA*ET$^+$ *ompU*$^+$ *tcpI*$^+$ *toxR*$^+$ and negative for *ctxA, stn*/*sto, tcpAET*, and *zot* for isolates from mussels (4 of 9), wastewater (2 of 14), and seawater (1 of 9). Interestingly, we observed the genotype *hlyA*ET$^+$ *toxR*$^+$ and negative for *ctxA*, *ompU*, *stn*/*sto*, *tcpA*ET, and *zot* in 6 of 14 non-O1/non-O139 *V. cholerae* isolates from wastewater, a finding similar to the virulence pattern obtained for 15 clinical strains associated with an unusual upsurge of cholera-like disease in India (71) and in non-O1/non-O139 *V. cholerae* strains isolated from volunteers in a vaccine trial in Peru (13).

A low frequency of the combination *ctxA*$^+$ *tcpA*Class$^+$ (1 of 39), and *ctxA*$^+$ *tcpA*ET$^+$ (3 of 39) was observed in environmental non-O1/non-O139 *V. cholerae* strains, as reported earlier (21). However, we found 23.1% (9 of 39) of CT-negative, but TcpAET-positive strains, potentially convertible to toxigenic strains by CTXΦ, either inside the host intestine or in the environment.

*V. cholerae* is autochthonous to estuarine and coastal environments (nutrient poor) and also colonize the human intestine (nutrient rich). In the study reported here, we observed a varied incidence of virulence-associated factors in *V. cholerae* isolates, depending on the source or ecosystem (seawater, seafood, wastewater, and clinical specimens). The response to changing conditions occurs by expressing appropriate sets of genes that promote growth in each niche.

Aquatic and marine ecosystems are subjected to large spatial and temporal nutrient fluxes arising from seasonal and geographic variations in temperature, salinity, nutrient input, pH, oxygen tension, etc. (65). The mechanisms by which environmental conditions affect the coordinated expression of virulence factors by *V. cholerae* remain poorly understood. Hase and Mekalanos (25) proposed a model where both ToxR and -S and TcpP and -H are involved in sensing various environmental and internal stimuli and are required for the production of TCP in *V. cholerae*. Furthermore, the *V. cholerae* genome encodes 43 methyl-accepting chemotaxis proteins that regulate swimming behavior in response to aminoacids, sugars, and oxygen (16).

**Non-O1/non-O139 *V. cholerae* and risk assessment.** Non-O1/non-O139 *V. cholerae* strains can no longer be ignored. The rationale for continuous monitoring is based first on the emergence of serogroup O139 (Bengal) in Bangladesh (CT positive) and Argentina (CT negative), each of which clearly evolved independently (73). Second, the sixth pandemic, the seventh pandemic, and U.S. Gulf Coast isolates represent three different clones, each independently evolved from environmental non-O1 *V. cholerae* isolates (34). Third, other epidemic serogroups have emerged, including *V. cholerae* O31 (50), O37 (11), O53 (52), O10, and O12 (13, 71). Finally, the emergence of a new clone of the *V. cholerae* O1 El Tor in Calcutta, India (70), and in Bangladesh (17) has been reported. The possibility exists that additional new strains of toxigenic *V. cholerae* with epidemic potential may emerge in the future.

We reported earlier an ERIC-PCR method for detecting emergent pathogenic *V. cholerae* strains, whereby specific fingerprints for pathogenic strains (CT and ZOT toxin positive) were different from nonpathogenic strains (61). In this study, we report probable parental toxigenic genotypes present in small numbers in the Brazilian environment. While these strains did not cause epidemics, there are environmental factors that may change, enhance multiplication or dominance, or select for genotypes of *V. cholerae*, and the strains themselves need to be tested for potential selective advantage under selected environmental conditions.

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