

## Viable but Nonculturable *Vibrio cholerae* O1 in the Aquatic Environment of Argentina

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**In Argentina, as in other countries of Latin America, cholera has occurred in an epidemic pattern. *Vibrio cholerae* O1 is native to the aquatic environment, and it occurs in both culturable and viable but nonculturable (VNC) forms, the latter during interepidemic periods. This is the first report of the presence of VNC *V. cholerae* O1 in the estuarine and marine waters of the Río de la Plata and the Argentine shelf of the Atlantic Ocean, respectively. Employing immunofluorescence and PCR methods, we were able to detect reservoirs of *V. cholerae* O1 carrying the virulence-associated genes *ctxA* and *tcpA*. The VNC forms of *V. cholerae* O1 were identified in samples of water, phytoplankton, and zooplankton; the latter organisms were mainly the copepods *Acartia tonsa*, *Diaptomus* sp., *Paracalanus crassirostris*, and *Paracalanus parvus*. We found that under favorable conditions, the VNC form of *V. cholerae* can revert to the pathogenic, transmissible state. We concluded that *V. cholerae* O1 is a resident of Argentinean waters, as has been shown to be the case in other geographic regions of the world.**

Cholera reemerged in Latin America in 1991 after being absent from the continent for nearly a century. In Argentina, there have been seven epidemics since 1992, all of which were caused by El Tor biotype *Vibrio cholerae* O1. These cholera outbreaks occurred mainly during the summer months, and, consistent with reports from other geographic regions of the world, strains of *V. cholerae* O1 were isolated from water samples collected from rivers during epidemic periods. However, the microorganism could not be recovered from the environment during interepidemic periods (12).

*V. cholerae* is a natural inhabitant of aquatic environments, and it has been found to survive for extended periods in estuarine and brackish waters (12, 13, 15). Furthermore, in response to extreme environmental conditions, the bacteria may enter a dormant state (7, 30), which has been designated the viable but nonculturable (VNC) state, since the organism cannot be recovered by using traditional culture media. It has been hypothesized that it is in the VNC state that *V. cholerae* survives in the environment during interepidemic periods. Also, interaction with plankton appears to play an important role in the ecology of the microorganism and to facilitate persistence, mainly in response to low temperatures and reduced nutrient concentrations (12, 13, 14, 19, 21).

In Argentina, *V. cholerae* non-O1 was isolated from samples collected from the Río de la Plata in previous studies (5). Several facts, such as the seasonality of the outbreaks most likely associated with temperature shifts and plankton blooms,

suggested that there are environmental reservoirs of *V. cholerae*. Additionally, in a recent study in which the genetic diversity of *V. cholerae* O1 strains isolated during the seven outbreaks that occurred in this country was analyzed, most of the cholera toxin (CT)-producing strains were found to belong to a single clonal group already recognized as the Latin American epidemic strain (22). Also, a new variant of isolates (the Tucumán variant [22]) from a northern district of Argentina which did not produce CT and was genetically related was identified. Both the Latin American epidemic clone and the Tucumán variant were repeatedly isolated in different outbreaks after long interepidemic intervals, and they produced exactly the same pulsed-field gel electrophoresis pattern as isolates from previous outbreaks. This finding strongly suggested that no new lineages were introduced into this region but instead the strains belonging to the two clones were present in the environment and sporadically reemerged as culturable, pathogenic agents, probably in response to favorable environmental conditions.

The aims of this study were to determine the environmental reservoir(s) of *V. cholerae* O1 in Argentina and to evaluate the association of this pathogen with plankton species. For these purposes, two different geographic areas were selected; these areas were a marine station in the Atlantic Ocean, at which there was little anthropogenic influence, and four stations in the Río de la Plata estuary, which serves as a vast funnel that collects most of the continental waters of Argentina.

### MATERIALS AND METHODS

**Sample collection sites.** Samples were collected from five sites. One of the sites was located at a fixed station on the Argentine continental shelf at 38°28'S, 57°41'W and where the water depth was 48 m. The remaining four sites were located along a transect at the border between the Río de la Plata and

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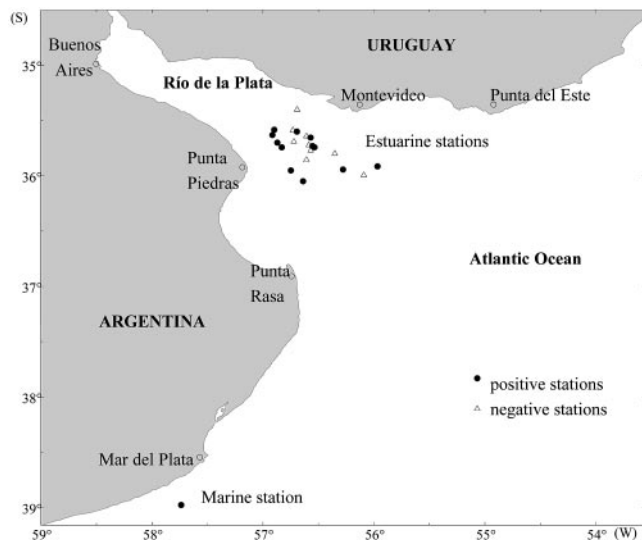


FIG. 1. Map showing the sampling stations for VNC *V. cholerae* O1.

its estuary (ecotone) (that is, from Punta Piedras in Argentina to Montevideo in Uruguay).

Between February 1999 and May 2000, a total of 13 cruises were conducted by R/V *Captain Oca Balda*, R/V *E. L. Holmberg*, and R/V *Captain C'anepa* of the Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP); these cruises included 7 cruises to the marine sampling station in the Argentine shelf and 6 cruises to the estuary of the Río de la Plata (Fig. 1).

**Sample collection and processing.** Plankton samples were collected with 25- and 200- $\mu$ m mesh nets. For each concentrated plankton sample (final volume, 20 ml), 10 ml was divided into three portions, and these portions were placed into three vials immediately after sampling. One of these vials, which contained 0.025% yeast extract and 0.002% nalidixic acid, was incubated for 6 to 12 h at 35°C and fixed with formaldehyde at a final concentration of 3% for analysis by the direct fluorescent antibody (DFA)-direct viable count (DVC) technique (9). The second vial was preserved with formaldehyde at a final concentration of 4% and used for identification of zooplankton and phytoplankton species. The sample in the last vial was kept at -20°C for PCR analysis. The remaining 10 ml of the plankton sample was enriched by addition of alkaline peptone broth for isolation of *V. cholerae* by conventional culture methods.

Water samples were collected in sterile 5-liter bottles and were filtered through 0.22- $\mu$ m-pore-size membranes. Subsequently, the membranes were washed with 4 ml of phosphate buffer, and this buffer was fractionated for analysis by DFA-DVC,

PCR, and direct culturing. Physical and chemical parameters (temperature and salinity) were measured in situ at all sites at the time of sampling.

**DFA-DVC.** For detection of *V. cholerae* O1, formaldehyde-preserved samples were processed by using cholera DFA kits (3, 9) generously provided by New Horizons Diagnostics Corporation. Briefly, samples were incubated in the dark at room temperature for 6 to 8 h in the presence of yeast extract and nalidixic acid. Under these conditions, viable but nonculturable bacteria elongate from a coccoid shape to rod-like cells, yet they do not multiply due to the inhibitory effect of nalidixic acid (a DNA gyrase inhibitor). After incubation, samples were fixed with 3% formaldehyde and stained with monoclonal fluorescein isothiocyanate-conjugated anti-O1 antibodies. Stained preparations were observed by using an epifluorescence microscope (model IX 70; Olympus) at a maximum excitation wavelength of 490 nm.

**Direct detection of the cholera toxin gene (*ctxA*).** A heminested PCR method was used to detect a region spanning the *ctxA* and *ctxB* genes in water and plankton samples; this method was based on a method used previously in our laboratory for detection in fecal specimens (29). For this assay, water and plankton samples were boiled for 10 min and centrifuged. The supernatant was used as the template DNA. For the first round of PCR, the primers used were 5' GTGGGAATGCTCCAAGATCAAATCG 3' (forward) and 5' ATTGCGGCA ATCGCATGAGGCGT 3' (reverse). For the second round of PCR, the internal primer 5' GATATGCAATCCTCAGGGTATCC 3' was used along with the forward primer. The amplification reaction consisted of 5 min at 94°C and 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C.

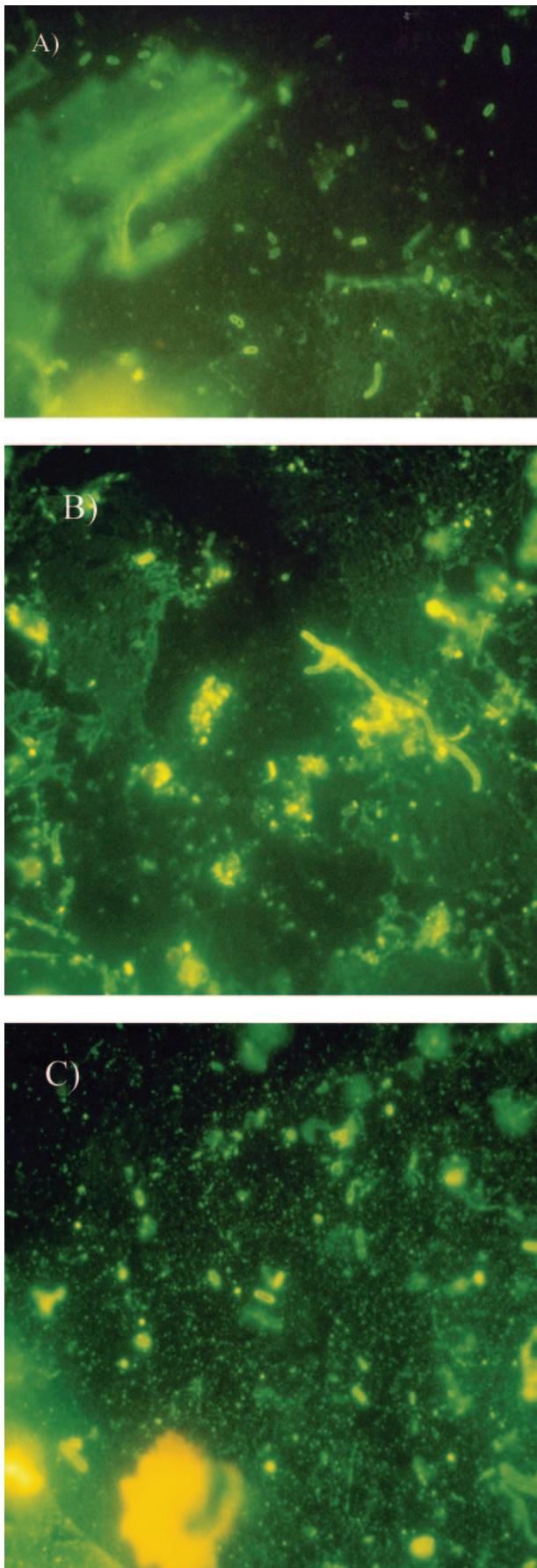
**DNA extraction.** DNA was extracted from estuarine and marine water samples and from plankton samples by a recently described protocol by using cetyltrimethylammonium bromide (25); the method used was based on the method of Somerville (27). Briefly, a 1-ml aliquot of a plankton or water sample was homogenized, centrifuged, and suspended in SET buffer (20% sucrose, 50 mM EDTA, 50 mM Tris HCl; pH 7.6). After treatment with lysozyme, proteinase K, and ammonium dodecyl sulfate, DNA was extracted with cetyltrimethylammonium bromide and phenol-chloroform-isoamyl alcohol, concentrated with isopropanol, and suspended in tridistilled water.

**PCR.** DNA extracted from water and plankton samples was used as the template to detect *V. cholerae*. First, an amplification assay was performed by using universal 16S rRNA primers to confirm the presence of bacterial DNA (1). The samples that showed positive results in the 16S rRNA PCR were subjected to another amplification reaction with primers specific for *V. cholerae* (4). Subsequently, a multiplex PCR assay was performed to detect genes coding for the somatic antigens O1 and O139 (25), and the positive samples were tested for the presence of the virulence-associated genes *ctxA* and *tcpA* (24). The primers used in all assays in this study are listed in Table 1.

**Virulence factors.** In culturable non-O1 *V. cholerae* strains, virulence-associated genes were detected by colony blot hybridization with specific probes. The probes, kindly provided by J. Nataro and J. Kaper, Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, included the ClaI fragment of pCVD628 for *zot* and *ace* gene sequences, the EcoRI fragment of pCVD27 for the *ctxA* gene, and the ClaI-XbaI fragment of pBB241 for the *zot*

TABLE 1. Primers and PCR conditions used in this study

Primer	Sequence (5'-3')	Amplicon size (bp)	Annealing temp. (°C)	Extension time (min)
P16S-F P16S-R	CAGCMGCCGCGGTAATWC ACGGGCGGTGTGTRC	888	55	3
VC-F2 VC-R1	TTAAGCSTTTTCRCCTGAGAATG AGTCACTTAACCATACACCCG	295	60	1
VCO1F2 VCO1R2	CAACAGAATAGACTCAAGAA TATCTTCTGATACTTTTCTAC	647		
VCO139F2 VCO139R2	TTACCAGTCTACATTGCC CGTTTCGGTAGTTTTTCTGG	741	55	3
94F 614R	CGGGCAGATTCTAGACCTCCTG CGATGATCTTGGAGCATTCCCAC	564		
72F 477R	CACGATAAGAAAACCGGTCAAGAG CGAAAGCACCTTCTTTCACGTTG	405	60	1



gene. The probes were labeled with digoxigenin by random priming (Boehringer). The *tcpA* gene, coding for the toxin coregulated pilus (TCP) pilin subunit, was detected by the multiplex PCR assay, which allows identification of both El Tor (471-bp) and Classical (617-bp) *tcpA* sequences (8). The primers used were 5' GAAGAAGTTTGTAAAAGAAGAACAC 3' (*tcpA1* El Tor) and 5' GAAAGGACCTTCTTTTACGTTG 3' (*tcpA2* El Tor); and 5' CACGATAAGAAAACCGTCAAGAG 3' (*tcpA3* Classical) and 5' ACCAAATGCACGCCGAATGGAG 3' (*tcpA4* Classical). The amplification conditions were 5 min at 94°C, 30 cycles of 1.5 min at 94°C, 1.5 min at 60°C, and 1.5 min at 72°C, and a final extension of 10 min at 72°C.

**Statistics.** The relationships between the presence of culturable non-O1 *V. cholerae* or VNC *V. cholerae* O1 and two physicochemical parameters, temperature and salinity, were evaluated by using Pearson  $\chi^2$  and  $\chi^2$  for linear trends (a *P* value of  $\leq 0.05$  for the two-tailed test was considered significant) and logistic regression for multivariate analysis. The dependent variable presence of culturable non-O1 *V. cholerae* was defined with two categories: positive (value, 1), when non-O1 *V. cholerae* was isolated in at least one sample of water or plankton from a single site; and negative (value, 0), when no non-O1 *V. cholerae* was found in any sample from a single site. The same categories were defined for *V. cholerae* O1 as a dependent variable.

The independent variables salinity and temperature were each divided into three categories, as follows: salinity, less than 10 g/ml, 10 to 25 g/ml, and more than 25g/ml; and temperature, less than 15°C, 15 to 20°C, and more than 20°C. A likelihood ratio test was used to assess linear trends in the multivariate analysis. The nonparametric test Spearman coefficient was used to evaluate the relationship between the presence of non-O1 *V. cholerae* or VNC *V. cholerae* O1 and the different kinds of samples collected at a single site. Data analyses were performed by using the Epi Info v6.02 and Stata v7.0 programs.

## RESULTS

**Isolation of *V. cholerae*.** A total of 21 samples were obtained from water collected at the marine sampling station on the Argentine continental shelf, and 63 samples were collected from the Río de la Plata estuary; these samples included water and plankton samples. Based on the net mesh size, the plankton were divided into two categories: microplankton (~25 to 200  $\mu\text{m}$ ), which included mainly phytoplankton and protozooplankton; and mesoplankton (>200  $\mu\text{m}$ ), which included mainly multicellular zooplankton. While no isolates of *V. cholerae* O1 were recovered by conventional culture methods, 97 strains of *V. cholerae* non-O1 were isolated. None of these strains carried the virulence-associated genes of *V. cholerae* O1 (i.e., *ctxA*, *zot*, *ace*, and *tcpA*), as determined by colony blot hybridization and PCR. The rate of isolation of non-O1 *V. cholerae* increased as the temperature increased ( $\chi^2$  for trend [2 df] = 10.26; *P* = 0.001) and decreased as the salinity increased ( $\chi^2$  for trend [1 df] = 8.72; *P* = 0.003). The relationship was statistically significant even when the two variables were considered together; the  $\beta$  coefficients were 1.40 (*P* = 0.024) for temperature and -1.37 (*P* = 0.040) for salinity (multivariate analysis; linear model; LR test  $\chi^2$  = 1, 42; *P* > 0.05). Thus, culturable non-O1 *V. cholerae* strains were recovered from the estuarine stations only during the warmer months (November through March).

There was a positive correlation between isolation of non-O1 *V. cholerae* strains from water and isolation of non-O1 *V. cholerae* strains from mesoplankton (diameter, >200  $\mu\text{m}$ ) at a single site, indicating that plankton samples positive for *V. cholerae* were frequently accompanied by positive water samples (Spearman rho = 0.69; *P* < 0.05).

FIG. 2. Fluorescent photomicrographs of *V. cholerae* O1 attached to microplankton (A), in a water sample (B), and attached to zooplankton (C).

**Viable but nonculturable *V. cholerae*.** Despite the fact that no strains of *V. cholerae* O1 were obtained by conventional culture, DFA-DVC revealed the presence of VNC *V. cholerae* O1, which appeared as rod-shaped bacteria after incubation with yeast extract and nalidixic acid (Fig. 2). The results obtained by DFA-DVC were confirmed by heminested PCR and multiplex PCR. The amplification reactions showed the presence of *V. cholerae* O1-specific genes, as well as genes coding for CT and TCP (Fig. 3, 4, and 5).

There was good correlation between the results of PCR and the results of DFA-DVC (Table 2); a total of 19 of 84 samples yielded positive results with both PCR and DFA-DVC. This group included nine water, five mesoplankton, and five microplankton samples. Additionally, one microplankton sample was positive as determined by PCR and negative as determined by DFA-DVC; this sample was considered positive for VNC *V. cholerae* O1, since all the PCRs were positive, which proved that *V. cholerae* O1 CT and TCP genes were present. A total of 59 of the remaining samples were negative as determined by both methods, while six samples having different origins showed positive results when fluorescent staining was used but were negative as determined by PCR. These samples were considered negative, since the observations were confounded by high background fluorescence. Also, two PCR-positive zooplankton samples that gave confusing, weak results when DFA-DVC was used were clearly positive as determined by this technique after trituration; this was most likely a case of the bacteria being attached to unexposed areas of the plankton.

VNC *V. cholerae* O1 was found during all seasons examined at both the marine and estuarine stations (Table 3). In the Río de la Plata estuary, VNC *V. cholerae* was identified in samples collected during the six cruises conducted in the summer, winter, and spring (there were no cruises in the autumn), and at the continental shelf marine station the samples were positive for five of the seven cruises in the summer and autumn (there were no cruises in the spring). There was not a significant correlation between the presence of *V. cholerae* O1 and temperature (Pearson  $\chi^2$  [2 df] = 0.399;  $P$  = 0.819) or salinity (Pearson  $\chi^2$  [2 df] = 0.9604;  $P$  = 0.619) at the sampling site (the  $\beta$  coefficients were 0.49 [ $P$  = 0.334] for salinity and 0.31 [ $P$  = 0.525] for temperature; multivariate). Also, there was no correlation between the types of samples collected at a single site; i.e., a positive microplankton sample was not frequently accompanied by a positive water and/or mesoplankton sample (Spearman rho [water-mesoplankton] = 0.16; Spearman rho [water-microplankton] = -0.03; Spearman rho [mesoplankton-microplankton] = -0.06;  $P$  > 0.05).

**Association with plankton.** Several zooplankton species collected with a 200- $\mu$ m-mesh net were associated with *V. cholerae* O1, including *Acartia tonsa*, *Diaptomus* sp., *Paracalanus crassirostris*, and *Paracalanus parvus* at the estuarine stations and *Corycaeus amazonicus*, *Centropages furcatus*, and *Ctenocalanus vanus* at the marine station. All plankton species were separated, identified, and individually confirmed to be associated with *V. cholerae* O1 by DFA-DVC.

For the microplankton, one marine and four estuarine samples were positive for VNC *V. cholerae* O1 as determined by PCR and DFA-DVC; there was also one estuarine sample that was positive as determined by PCR and negative as determined by DFA-DVC (Table 2). In these samples, in spite of isolation

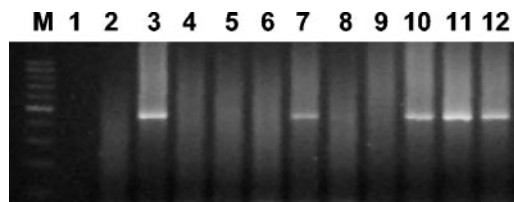


FIG. 3. Heminested PCR for *ctxAB* detection. Lane M, 100-bp ladder; lane 1, negative control (PCR master mixture); lanes 2, 4, 5, 6, 8, and 9, negative samples; lanes 3, 7, 10, and 11, positive samples; lane 12 positive control (*V. cholerae* O1 strain O425).

and staining of the more frequent species, none of the microplankton could be unquestionably observed as carrying *V. cholerae* by microscopy. The major species present in positive samples from the Río de la Plata estuary were the diatoms *Thalassionema nitzschioides*, *Pleurosigma cf. normanii*, and species of the genera *Thalassiosira* and *Coscinodiscus*, together with the dinoflagellates *Ceratium furca*, *Noctiluca scintillans*, *Prorocentrum scutellum*, *Diplopelta asymmetrica*, and species of *Protoperidinium*. At the continental shelf marine station, the most abundant species identified in the sample that was positive for VNC *V. cholerae* O1 were the diatoms *Meunieria membranacea*, *Cerataulina pelagica*, *Leptocylindrus minimus*, *Hemiaulus sinensis*, *Thalassionema nitzschioides*, and *Guinardia flaccida* and species of *Protoperidinium*. Members of other taxonomic groups, such as silicoflagellates, tintinnids, and ebriaceans, were too infrequent in samples associated with *V. cholerae* to be relevant.

VNC *V. cholerae* O1 was identified in microplankton samples in the spring and summer at the estuary stations and in the autumn at the marine station, whereas VNC *V. cholerae* O1 was associated with zooplankton in the summer, winter, and spring in the estuary and in the autumn at the marine station.

## DISCUSSION

*V. cholerae* is an aquatic microorganism, and several reports have shown that there are environmental reservoirs of this pathogen in different geographical areas of the world (2, 17, 18, 20). In Argentina, as in the rest of Latin America, cholera has occurred since 1991 in an epidemic pattern. It has been suggested that *V. cholerae* O1 remains in the environment during interepidemic periods in a viable but nonculturable state. Prior to this study, many cruises had been conducted in the Río de la Plata and at the marine platform in Argentina in a search for

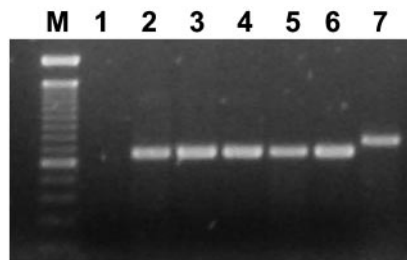


FIG. 4. Multiplex PCR for the detection of O1/O139 genes. Lane M, 100-bp ladder; lane 1, negative control (PCR master mixture); lanes 2 to 5, positive samples; lane 6, O1 positive control strain ATCC 14035; lane 7, O139 positive control strain MO4045.

TABLE 2. Correlation between DFA-DVC and PCR for detection of VNC *V. cholerae* O1

Site	Samples		No. DFA-DVC positive and PCR positive	No. DFA-DVC positive and PCR negative	No. DFA-DVC negative and PCR negative	No. DFA-DVC negative and PCR positive	Total no. of VNC <sup>a</sup>
	Type	No.					
Estuary	Water	21	6	1	14		6
	Mesoplankton (>200 µm) (n = 21)	21	4	0	17		4
	Microplankton (25–200 µm)	21	4	1	15	1	5
Marine station	Water	7	3	1	3		3
	Mesoplankton (>200 µm)	7	1	2	4		1
	Microplankton (25–200 µm)	7	1	1	5		1
Total	84	84	19	6	58	1	20

<sup>a</sup> Total number of samples that were considered positive for VNC *V. cholerae* O1.

culturable forms of *V. cholerae* in which traditional culture methods were used, and only non-O1 strains were recovered. No studies had been carried out to look for VNC *V. cholerae* O1. Thus, this is the first study which proves that VNC *V. cholerae* O1 is present in estuarine and marine waters in the Río de la Plata estuary and the continental shelf of Argentina in the southwest Atlantic Ocean.

Results of previous studies suggested that *V. cholerae* is present in the environment in association with plankton, mainly attached to copepods and some aquatic hydrophytes (6, 15, 16, 28). In 1990, Huq et al. (12) reported that *V. cholerae* O1 cells could be observed by DFA microscopy to be attached to a variety of phytoplankton and zooplankton species, including *Volvox*, *Desmida*, *Rotifera*, *Cladocera*, and *Daphnia* spp. In this study, VNC *V. cholerae* O1 was associated with both microplankton and zooplankton. In the zooplankton fraction collected from the Río de la Plata estuary, species of marine origin (*Acartia tonsa*, *Paracalanus crassirostris*, *Paracalanus parvus*) and species of continental water origin (*Diaptomus* sp.) were associated with VNC *V. cholerae* O1. *Acartia tonsa* is a marine euryhaline species that occurs predominantly in the estuary of the Río de la Plata. *Diaptomus* has been repeatedly found in the continental waters of Argentina (23; F. C. Ramirez, D. R. Soarrain, H. Mianzan, and N. Fernandez Araoz, submitted for publication). Furthermore, at the continental shelf marine station, the species associated with VNC *V. cholerae* O1 (*Corycaeus amazonicus*, *Centropages furcatus*, and *Ctenocalanus vanus*) are common inhabitants of the northern coastal waters of Argentina. It would be useful to examine the plankton in northern Argentina, where most of the cholera cases

occurred during the seven epidemic outbreaks, since the water of the Parano-Platense basin is carried down to the Río de la Plata.

In both the estuarine and marine environments, a larger number of water samples than of plankton fractions were positive for VNC *V. cholerae* O1. The samples that were positive for *V. cholerae* O1 but negative for plankton-attached *V. cholerae* O1 most likely included species that were smaller than the pore size of the plankton net used, as well as organic detritus, exuviae, and phytoplankton and zooplankton remains. Plankton nets with smaller mesh should be used for future studies. It must be considered that during passage through this wide plexus of bodies of water, chitinous remains of copepod exoskeletons disintegrate and are reduced to fine debris. It is well known that copepods, like other crustaceans, undergo a growth cycle that includes a change in the tegument, which due to the action of biological and/or mechanical agents, results in an abundance of microscopic substrates that are suitable for attachment of bacteria. Biofilms also are formed. Thus, the finding that positive water samples are more numerous than positive plankton samples is logical.

Culturable *V. cholerae* non-O1 was identified predominantly during warmer months and in the lower-salinity waters of the estuary. This finding is in agreement with reports for other areas (10, 11, 20). A similar relationship was not evident for the presence of nonculturable *V. cholerae* O1, since this organism was found during all seasons at both the marine and estuarine stations. Although the numbers of cruises conducted in the different seasons and in the two geographical areas were not equal, it can be assumed that the VNC state makes it possible for this bacterium to persist at a wide range of salinities and temperatures. Nevertheless, since the methodologies used to

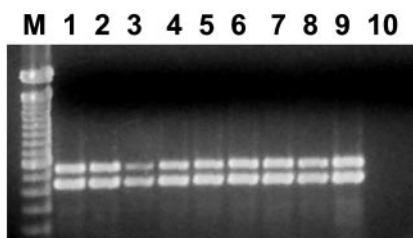


FIG. 5. Multiplex PCR for the detection of *ctxA/ctxB*. Lane M, 100-bp ladder; lanes 1 to 8, positive samples; lane 9, positive control *V. cholerae* O1 strain O425; lane 10, negative control (PCR master mixture).

TABLE 3. Seasonal occurrence of VNC *V. cholerae* O1 in surface water and plankton in the estuarine and marine environments monitored by immunofluorescence and PCR techniques

Season	No. of positive samples/total no. (%)			
	Water	Microplankton (25–200 µm.)	Mesoplankton (>200 µm.)	Total
Summer	2/11 (18.2)	4/11 (36.4)	1/11 (9.1)	7/33 (21.2)
Fall	2/3 (66.7)	1/3 (33.3)	1/3 (33.3)	4/9 (44.4)
Winter	1/5 (20.0)	0/5 (0)	2/5 (40.0)	3/15 (20.0)
Spring	4/8 (50.0)	1/8 (12.5)	1/8 (12.5)	6/24 (25.0)

detect viable but nonculturable forms were specific for *V. cholerae* O1, the possibility that non-O1 strains could also be found in nonculturable forms cannot be ruled out. Furthermore, VNC non-O1 *V. cholerae* has been found in the River Ganga in Varanasi in India (26). If this was the case in the Río de la Plata and in the Argentine marine platform, it could be speculated that non-O1 *V. cholerae* (or at least some strains in this group) could be more readily transformed to the culturable state than the O1 strains in the presence of lower salinity and at higher temperatures.

The heminested PCR technique (29) employed for detection of *V. cholerae* when the concentrations of bacteria were very low (2 to 3 CFU/ml) proved to be a useful tool for analysis of environmental samples. Moreover, the combination of the PCR and DFA-DVC methods used in this study allowed us to confirm not only the viability of the *V. cholerae* O1 identified but also the pathogenic potential of the nonculturable *V. cholerae* population. The DFA-DVC method confirmed the presence of viable forms of *V. cholerae* O1, while PCR allowed detection of virulence-associated genes that could be found not only in *V. cholerae* O1 but also in *V. cholerae* non-O1 strains.

In summary, prior to this study, there was no evidence that *V. cholerae* O1 is part of the normal flora of southwest Atlantic Ocean waters. Therefore, the present study is the first report of the existence of an environmental reservoir of this pathogen in this geographical area, both in coastal water and in the Río de la Plata and its estuary. The VNC *V. cholerae* O1 detected by the methods used in this study possessed genetic information typical for pathogenic *V. cholerae* O1 (e.g., the *ctxAB* and *tcpA* genes). Given that under certain climate conditions VNC *V. cholerae* O1 could revert to a transmissible state, programs for prevention of cholera in South America should not be abandoned. Instead, there is a need for systematic environmental monitoring and strategic surveillance for *V. cholerae* O1 in this region.

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