APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2003, p. 3676–3680 Vol. 69, No. 6
0099-2240/03/$08.00+0 DOI: 10.1128/AEM.69.6.3676–3680.2003

Direct Detection of *Vibrio cholerae* and *ctxA* in Peruvian Coastal Water and Plankton by PCR

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Received 18 November 2002/Accepted 20 March 2003

Seawater and plankton samples were collected over a period of 17 months from November 1998 to March 2000 along the coast of Peru. Total DNA was extracted from water and from plankton grouped by size into two fractions (64 μm to 202 μm and >202 μm). All samples were assayed for *Vibrio cholerae*, *V. cholerae* O1, *V. cholerae* O139, and *ctxA* by PCR. Of 50 samples collected and tested, 33 (66.0%) were positive for *V. cholerae* in at least one of the three fractions. Of these, 62.5% ($n = 32$) contained *V. cholerae* O1; *ctxA* was detected in 25% ($n = 20$) of the *V. cholerae* O1-positive samples. None were positive for *V. cholerae* O139. Thus, PCR was successfully employed in detecting toxigenic *V. cholerae* directly in seawater and plankton samples and provides evidence for an environmental reservoir for this pathogen in Peruvian coastal waters.

Cholera continues to be an important and devastating disease, especially in those regions of the world where it is endemic. Before its reemergence in Peru and throughout Latin America in 1991, the disease had been absent from the Americas for nearly 100 years. There has been much speculation as to the cause of this reemergence and whether there has always been an environmental reservoir for *Vibrio cholerae* in Latin America. Since 1991, seasonal patterns of cholera outbreaks have been well documented in Central and South America, with the largest numbers of cases occurring during the warm, summer months (January to March) (7, 21).

In 1977, Colwell et al. (3) first hypothesized that coastal waters were an important reservoir of *V. cholerae*. *V. cholerae* has since been detected in seawater and other environmental sources around the world, both in areas where cholera is endemic and in cholera-free areas (4, 12, 13, 14, 16). Despite its ubiquity, the ability to determine the presence of this bacterial species in the environment with a degree of efficiency has been hindered by the culture techniques relied upon for detection. Under certain environmental conditions, *V. cholerae* has been shown to enter a viable but nonculturable state that can result in significant underestimation of the total *V. cholerae* population (23). Techniques employing microscopy, with either direct or indirect fluorescent-antibody staining, have been developed and provide important data on the occurrence of viable but nonculturable *V. cholerae* O1 and O139. However, it is obvious that the labeled antibody approach to detect all ~200 serogroups of *V. cholerae* is not feasible (24). Furthermore, *V. cholerae* non-O1 and non-O139 strains can acquire genes for toxin production by transduction and, therefore, have been hypothesized to be the source of new epidemic and pandemic clones, the toxigenic O139 serogroup having arisen from recombination with a toxigenic O1 strain(s) (5). Currently, neither traditional culture methods nor the direct fluorescent-antibody assay (DFA) can detect the presence of the cholera toxin directly in the field. While most environmental *V. cholerae* strains lack the genes required to produce cholera toxin (19), the possibility of genetic exchange in the environment and the potential emergence of new toxigenic clones highlight the importance of including *ctxA* in environmental screening.

PCR and other molecular detection methods offer a useful alternative to culturing and microscopy, especially for environmental samples. Recently, species-specific oligonucleotide probes for *V. cholerae* have been used in colony blot hybridization (14, 15, 20). This approach has resulted in higher total counts than traditional methods because nonselective media can be used. However, colony blot probing is limited to culturable cells. PCR primers have now been developed that allow for specific detection of a range of targets (species, serogroup, toxin, etc.) in any given sample (22). While these results cannot provide direct evidence that such cells are viable, they do make possible rapid assessment of the potential total population. We report here a PCR method for direct detection of *V. cholerae* serogroups O1 and O139 and the gene coding for cholera toxin production (*ctxA*) in environmental samples. Seawater and plankton samples were collected along the coast of Peru for analysis. The objective of the research was to develop an understanding of the ecology of *V. cholerae*, as well as the occurrence and distribution of the toxigenic O1 and O139 subpopulations. This information, based on direct detection of the toxin gene (*ctxA*) in the environment, will allow development of a predictive model for cholera epidemics.

Sample collection. Three stations (Fig. 1) were sampled monthly from a 1,600-km coastline transect between Trujillo and Arequipa from November 1998 to March 2000. Additional

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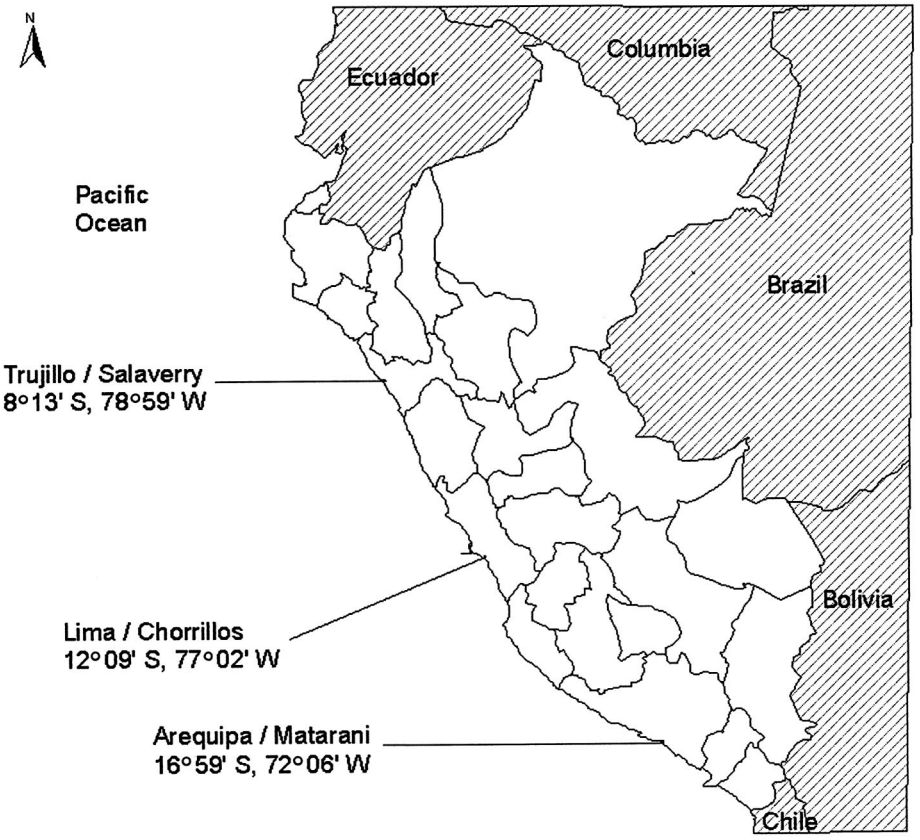

FIG. 1. Map of sampling stations along the coast of Peru.

bimonthly samples were collected from October to December 2000, just prior to the time of the annual outbreaks of cholera in Peru. From the total sample collection, 50 samples were selected, from which three fractions (water, plankton of >202 μm, and plankton of 64 to 202 μm) were obtained. Approximately 100 ml of seawater was filtered onto 0.2-μm-pore-size polycarbonate membranes, and the filters were stored in 1 to 5 ml of distilled water at −20°C. One to five milliliters of each plankton fraction, without diluent, was also stored at −20°C.

DNA extraction. Total genomic DNA was extracted according to the method described by Rivera et al. (19a) using CTAB (cetyltrimethylammonium bromide) and phenol-chloroform-isopropyl alcohol. DNA was precipitated with a 0.6× volume of isoamyl alcohol, and the pellet was washed with 70% ethanol. DNA was resuspended in a total volume of 100 μl, quantitated by UV spectrophotometry, and stored at −20°C.

Plankton samples were thawed and vigorously mixed by vortexing, after which aliquots (~2 ml) were extracted using DNeasy tissue kits (Qiagen, Valencia, Calif.) with a modification of the manufacturer's protocol. To accommodate a larger sample volume, three 700-μl sample aliquots were passed through the column for a total of 2.1 ml. DNA was eluted with two additions of 50 μl of sterile filtered (0.02-μm-pore-size filter) Milli-Q water, quantitated with UV spectrophotometry, and stored at −20°C.

PCR. PCR was performed with the samples sequentially. All 150 samples (three fractions times 50 samples) were tested for the presence of *V. cholerae*. Positive samples were subsequently analyzed for the presence of O1- and/or O139-specific sequences (10, 19a). Finally, those samples positive for O1 or O139 were tested for *ctxA*.

For each PCR, 1 to 5 μl of sample DNA was added to a 20- to 24-μl master mix prepared for each target by using a TaqMaster kit from Eppendorf (Hamburg, Germany). All reaction mixtures included 0.2 mM (each) deoxynucleoside triphosphates, 1.25 μM (each) primers, 1× PCR buffer providing 1.5 mM $MgCl_2$, 0.5× TaqMaster additive, and 0.625 U of *Taq* polymerase. For each sample, at least three dilutions were tested (undiluted, 1:20, and 1:100) because of the occasional occurrence of inhibitors. Primers for *V. cholerae* were those described by Chun et al. (2) and targeted to a ~300-bp region of the 16S-23S intergenic spacer region (Table 1). Two separate primer sets were used to target both *V. cholerae* O1 and *V. cholerae* O139 (*wbeO* and *rfb* genes) (10, 19a, 25) (Table 1). Two separate primer sets were also used to target the *ctxA* gene of the CTX element (6, 10) (Table 1). For O1, O139, and *ctxA* targets, where two primer sets were employed, when either set resulted in a positive signal the sample was considered positive for the respective target. Universal primers for the 16S rRNA gene were used as a control test for inhibition in all samples (1).

Reaction conditions for each target are described in Table 1. PCR products were run on 1.4% agarose gels and stained with ethidium bromide, and the bands were visualized with a UV

TABLE 1. Primers and PCR conditions for specific amplification of *V. cholerae, V. cholerae* O1, *V. cholerae* O139, and the *ctxA* gene[a]

Target	Primer sequence	Amplicon length (bp)	Annealing temp (°C)	Extension time (min)	Reference
16S rDNA	5′ CAG CMG CCG CGG TAA TWC 3′ 5′ ACG GGC GGT GTG TRC 3′	888	60	0.5	1
V. cholerae ITS	5′ TTA AGC STT TTC RCT GAG AAT G 3′ 5′ AGT CAC TTA ACC ATA CAA CCC G 3′	~300	60	0.5	4
V. cholerae O1	5′ CAA CAG AAT AGA CTC AAG AA 3′ 5′ TAT CTT CTG ATA CTT TTC TAC 3′	647	50	1.0	19a
V. cholerae O1	5′ GTT TCA CTG AAC AGA TGG G 3′ 5′ GGT CAT CTG TAA GTA CAA C 3′	192	55	1.0	10
V. cholerae O139	5′ TTA CCA GTC TAC ATT GCC 3′ 5′ CGT TTC GGT AGT TTT TCT GG 3′	741	55	1.5	19a
V. cholerae O139	5′ AGC CTC TTT ATT ACG GGT GG 3′ 5′ GTC AAA CCC GAT CGT AAA GG 3′	449	55	1.0	10
ctxA	5′ CGG GCA GAT TCT AGA CCT CCT G 3′ 5′ CGA TGA TCT TGG AGC ATT CCC AC 3′	564	60	1.0	6
ctxA	5′ ACA GAG TGA GTA CTT TGA CC 3′ 5′ ATA CCA TCC ATA TAT TTG GGA G 3′	308	55	1.0	10

[a] All reactions included a 1-min initial denaturation step at 94°C, followed by 35 cycles of denaturation (94°C for 45 s), annealing for 45 s, and extension at 72°C. A final extension at 72°C for 5 min was included in all reactions. Specific annealing temperatures and extension times are listed. rDNA, ribosomal DNA; ITS, intergenic spacer region.

transilluminator (UV Products Inc., Upland, Calif.). Images were captured with a Kodak EDAS 290 digital imaging system (Eastman Kodak Co., New Haven, Conn.).

Analysis. Samples were scored by the presence or absence of specific targets and statistically analyzed by using Fisher's exact or Cochran's Q test for matched samples. In all cases, significance was determined at P values of ≤ 0.05.

Environmental detection. Total DNA extraction provided sufficient template for sequential analysis of the microbial community and permitted a narrowing down to the toxigenic strains of epidemic *V. cholerae* (Table 2). As few as 100 cells of *V. cholerae* in 250 ml of seawater can be detected by using the sample concentration, DNA extraction, and PCR protocols described here (19a). While the presence of nonviable cells cannot be excluded with the use of PCR, this approach offers a rapid assessment of environmental samples with a higher level of sensitivity than traditional methods, notably culture (A. I. Gil, E. K. Lipp, I. N. G. Rivera, V. Louis, A. Huq, C. F. Lanata, D. N. Taylor, E. Russek-Cohen, N. Choopun, R. B. Sack, and R. R. Colwell, submitted for publication).

The *V. cholerae*-specific intergenic spacer region was successfully amplified in 33 of the 50 samples in at least one fraction (66%). PCR for O1 and O139 targets yielded 27 positive amplicons for the *V. cholerae* O1 serogroup and none for O139. Five of the *V. cholerae* O1-positive samples were also positive for *ctxA* (Table 2).

Results from this work showed a higher level of detection of toxigenic *V. cholerae* O1 than those of a concurrent study using traditional detection methods (culture and DFA with direct viable counts) (Gil et al., submitted) (Table 3). Gil et al. (submitted) found that only 5.9 and 14.3% of samples were positive for *V. cholerae* O1 by culture and DFA, respectively. Furthermore, no data were obtained on the presence of *ctxA* in these samples by the methods used (Gil et al., submitted). Despite the difference in detection frequencies, the general trends in both studies followed similar patterns.

Seasonality. *V. cholerae* detection in coastal Peru followed ambient temperature increases and coincided with and/or preceded annual outbreaks of cholera in the summer months (January to March) (Table 4). In particular, detection of DNA from toxigenic *V. cholerae* O1 peaked during this time, when 20% (2 of 10) of all samples were positive for *ctxA* and *V.*

TABLE 2. Occurrence of *V. cholerae*-specific amplicons at stations sampled in this study[a]

Station	No. of *V. cholerae*-positive samples/total (%)	No. of *V. cholerae* O1-positive samples/total (%)	No. of *ctxA*-positive samples/total (%)
Arequipa	6/9 (66.7)	5/6 (83.3)	1/5 (20.0)
Lima	21/29 (72.4)	11/20 (55.0)[b]	3/11 (27.3)
Trujillo	6/12 (50.0)	4/6 (66.7)	1/4 (25.0)

[a] Samples were considered positive if any fraction, i.e., seawater or either of the plankton size fractions, was positive.
[b] One sample missing.

TABLE 3. Method agreement between culture for *V. cholerae* O1 bacterial counts (Gil et al., submitted) and direct PCR for total *V. cholerae* and *V. cholerae* O1 bacterial counts[a]

PCR result	No. of samples negative for *V. cholerae* O1 by culture	No. of samples positive for *V. cholerae* O1 by culture	Total no. of samples
V. cholerae O1			
Negative	8	5	13
Positive	17	3	20
Total	25	8	
V. cholerae			
Negative	17	0	17
Positive	25	8	33
Total	42	8	

[a] A sample was considered positive if any one of the three fractions was positive.

TABLE 4. Seasonal distribution of total *V. cholerae*, *V. cholerae* O1, and *ctxA* among each of the fractions sampled

Target	Season[a]	No. of positive samples/total (%) of:		
		Water	Plankton (64 to 202 μm)	Plankton (>202 μm)
V. cholerae	Summer	7/10 (70.0)	5/10 (50.0)	5/10 (50.0)
	Fall	4/12 (33.3)	2/12 (16.7)	2/12 (16.7)
	Winter	4/11 (36.4)	2/11 (18.2)	4/11 (36.4)
	Spring	10/17 (58.8)	6/17 (35.3)	4/17 (23.5)
V. cholerae O1	Summer	3/7 (42.9)	3/5 (60.0)	4/5 (80.0)
	Fall	1/4 (25.0)	2/2 (100)	1/2 (50.0)
	Winter	1/4 (25.0)	0/1 (0.0)[b]	3/4 (75.0)
	Spring	4/10 (40.0)	2/6 (33.3)	3/4 (75.0)
ctxA	Summer	2/3 (66.7)	0/3 (0.0)	0/4 (0.0)
	Fall	1/1 (100)	0/2 (0.0)	0/1 (0.0)
	Winter	1/1 (100)	0[c]	0/3 (0.0)[c]
	Spring	1/4 (25.0)	0/2 (0.0)	0/33 (0.0)

[a] Summer, January, February, March; Fall, April, May, June; winter, July, August, September; spring, October, November, December.
[b] One sample missing.
[c] No samples were positive for O1; *ctxA* was not tested.

cholerae O1; the values ranged from 5.9 to 9.1% (1 of 17 to 1 of 11) for the other seasons. In the temperate Chesapeake Bay estuary, where there are no cases of cholera and only ~22% of samples were positive for culturable *V. cholerae* (18), the detection pattern followed a similar seasonal cycle. This pattern of increased frequency of detection in warmer months is consistent with the ecology of *V. cholerae* and other *Vibrio* spp. and has been extensively reported elsewhere (8, 9, 10, 17). Interestingly, salinity, which was a dominant factor in the occurrence of *V. cholerae* in the Chesapeake Bay (18), showed no association with the occurrence of *V. cholerae* in this study, probably due to the lack of variability in the salinity of the coastal geographical areas sampled (Gil et al., submitted). The median and mean salinities were 35 ppt, and salinities ranged between a one-time low of 31 ppt and a one-time high of 37 ppt.

Distribution between plankton and water. Total *V. cholerae* was more consistently isolated from water samples than from plankton (Table 5); however, *V. cholerae* O1 cells were clearly abundant in the large plankton fraction, which included copepods and other zooplankton, relative to those in water and the smaller plankton size fraction. Unexpectedly, samples containing *ctxA* were not detected in the plankton. A possible explanation for this observation and the relatively low detection rate of *V. cholerae*, in general, in the plankton is that extraction of DNA from plankton samples was less efficient than that from water samples, with inhibitors affecting the polymerase activity (19a). This was confirmed by a reduction in amplification of total bacterial DNA (PCR for universal 16S sequences) in the plankton (data not shown).

TABLE 5. Occurrence of *V. cholerae*, *V. cholerae* O1, and *ctxA* in seawater and plankton samples

Sample type	No. of *V. cholerae*-positive samples/total (%)	No. of *V. cholerae* O1-positive samples/total (%)	No. of *ctxA* positive samples/total (%)
Water	25/50 (50.0)	9/25 (36.0)	5/9 (55.6)
Plankton (64 to 202 μm)	15/50 (30.0)	7/14 (50.0)[a]	0/7 (0.0)
Plankton >202 μm	15/50 (30.0)	11/15 (73.3)	0/11 (0.0)

[a] One sample missing.

In this study, the occurrence of *V. cholerae*-positive samples was positively correlated with air temperature ($P = 0.01$), which showed a distinct seasonal trend. Sea surface temperature variations were less definite (with the exception of the warm El Niño temperatures in the summer of 1998), and a statistically significant relationship with the occurrence of *V. cholerae* was not observed. We noted positive associations between the occurrence of *V. cholerae* and both chlorophyll *a* and total bacterial counts, but the relationships were not statistically significant ($P \approx 0.1$) (Gil et al., submitted).

In conclusion, we have shown that direct DNA extraction and application of sequential PCR assays are important tools for the rapid assessment of environmental samples for toxigenic *V. cholerae* and provide a better level of sensitivity than traditional culture and microscopic methods. With these methods, it is clear that *V. cholerae* is present in the coastal waters of Peru throughout the year, with numbers of cells increasing in spring and summer, just prior to the annual outbreak of cholera in the coastal cities. *V. cholerae* O1 cells are especially prevalent in plankton of >202 μm. The data demonstrate that the coastal waters and plankton populations in Peru harbor toxigenic *V. cholerae*, as has been shown for areas of Bangladesh where cholera is endemic (11). It is highly unlikely that cholera in Peru arrived from other cholera-affected regions of the world. A more plausible explanation is that *V. cholerae* is autochthonous to Peruvian coastal, brackish, and riverine waters, and it is reasonable to examine those environmental conditions giving rise to both plankton blooms and increases in *V. cholerae* concentrations. This information will help to develop a predictive model for cholera epidemics and provide an early warning system for conditions conducive to cholera outbreaks.

This research was supported by NIH grant no. RO1NRO4527-01A1, NIH/National Institute of Nursing research grant no. 1RO1A139129-01, NASA grant no. NAG2-1195, and NSF grant no. 01200677.

We thank Redwan Huq for his assistance with DNA extraction during a summer internship at the Center of Marine Biotechnology.

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