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Environmental Surveillance for Toxigenic *Vibrio cholerae* in Surface Waters of Haiti

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Abstract. Epidemic cholera was reported in Haiti in 2010, with no information available on the occurrence or geographic distribution of toxigenic *Vibrio cholerae* in Haitian waters. In a series of field visits conducted in Haiti between 2011 and 2013, water and plankton samples were collected at 19 sites. *Vibrio cholerae* was detected using culture, polymerase chain reaction, and direct viable count methods (DFA-DVC). Cholera toxin genes were detected by polymerase chain reaction in broth enrichments of samples collected in all visits except March 2012. Toxigenic *V. cholerae* was isolated from river water in 2011 and 2013. Whole genome sequencing revealed that these isolates were a match to the outbreak strain. The DFA-DVC tests were positive for *V. cholerae* O1 in plankton samples collected from multiple sites. Results of this survey show that toxigenic *V. cholerae* could be recovered from surface waters in Haiti more than 2 years after the onset of the epidemic.

INTRODUCTION

Toxigenic *Vibrio cholerae* serogroup O1 and O139 are etiologic agents associated with epidemic cholera. Water contaminated with toxigenic *V. cholerae* is considered the primary source of the causative agent of cholera, which can spread rapidly in poverty-stricken areas with poor sanitation and limited access to safe drinking water.[1] Haiti is the poorest country in the Western Hemisphere, and it is estimated that in 2011 only 64% of the population had access to improved drinking water sources and 26% had access to improved sanitation.[2] However, epidemic cholera had not been diagnosed in Haiti until the onset of the outbreak in October 2010.[3] During the first 2 years of the outbreak, more than 600,000 cases of cholera were reported, with more than 7,000 deaths.[4] Because of the absence of reported cholera outbreaks in Haiti before 2010, there is little information on the potential presence of toxigenic *V. cholerae* in surface waters in Haiti beyond detections reported during the acute phase of the 2010 outbreak and an environmental surveillance study performed in the Ouest Department in Haiti.[5,6]

Vibrio cholerae is present in the environment both as free-living cells and attached to chitinous exoskeletons of zooplankton as a commensal of copepods.[7,8] Detection of toxigenic *V. cholerae* can be difficult because of an abundance of non-toxigenic *V. cholerae* in natural water systems, and the inability to isolate viable but non-culturable bacteria using traditional culture methods.[9] When *V. cholerae* cannot be detected by traditional culture methods, viable cells can be detected in environmental samples using fluorescent antibody and direct viable count methods (DFA-DVC).[10,11] Because DFA-DVC allows for the detection of plankton-associated *V. cholerae*, data obtained from DFA-DVC can be used to evaluate whether *V. cholerae* may be present in an environmental niche. Additionally, gene sequences specific to toxigenic *V. cholerae* can be used to determine the presence of viable *V. cholerae* cells in enrichment cultures.[5,12]

To assess the presence of toxigenic *V. cholerae* in Haitian water resources, water samples from 19 fresh and marine water sites were collected during four field visits between October 2011 and January 2013. Multiple water sample collection and detection methods were used to detect and isolate *V. cholerae* in Haiti. The DFA-DVC was used to obtain a direct count of viable free-living and plankton-associated *V. cholerae* O1 and O139 cells. Traditional culture methods and polymerase chain reaction (PCR) were used to detect culturable cells of *V. cholerae* using *omp*W, *tox*R, *ctx*A, *tcp*A, and serogroup O1 and O139 gene targets. Molecular characterization was performed on recovered *V. cholerae* isolates for comparison to the epidemic strain. The goal of this surveillance study was to obtain data on the environmental presence of epidemic *V. cholerae* that could be used in conjunction with epidemiological data to guide decisions about cholera prevention efforts in Haiti.

METHODS

Sample collection. Field visits were conducted in October 2011, March and August 2012, and January 2013. During each field visit, 16 freshwater and 3 marine water samples were collected from 19 sites in the Artibonite, Center, Ouest, and Sud-Est Departments (Figure 1). Six of the sites were sampled at the beginning of the cholera outbreak in October and November 2010.[5] The remaining sites were chosen to represent various water types and geographic locations, with several tributaries of the Artibonite River included. At each site, 100 L of water was concentrated using dead-end hollow-fiber ultrafiltration (DEUF).[13,14] A 1-L grab sample was also collected to determine whether this simple, small-volume collection method could be as effective as a more labor-intensive large-volume method such as DEUF. Plankton samples (PLK) containing zooplankton and phytoplankton were collected by filtering 100 L through a 20-μm mesh plankton net fitted with a polyvinyl chloride (PVC) cod end bucket (Aquatic Research Instruments, Lehmi, ID) to obtain a final volume of < 1 L. A plankton net-filtered water (PFW) fraction containing free-living bacteria and plankton < 20 μm was obtained by collecting 1 L of the flow-through from the plankton net. Turbidity, specific conductance, salinity, temperature, and pH were measured in the field. Turbidity was measured with a Hach 2100P portable turbidimeter (Hach, Loveland, CO). Specific conductance, salinity, and temperature were

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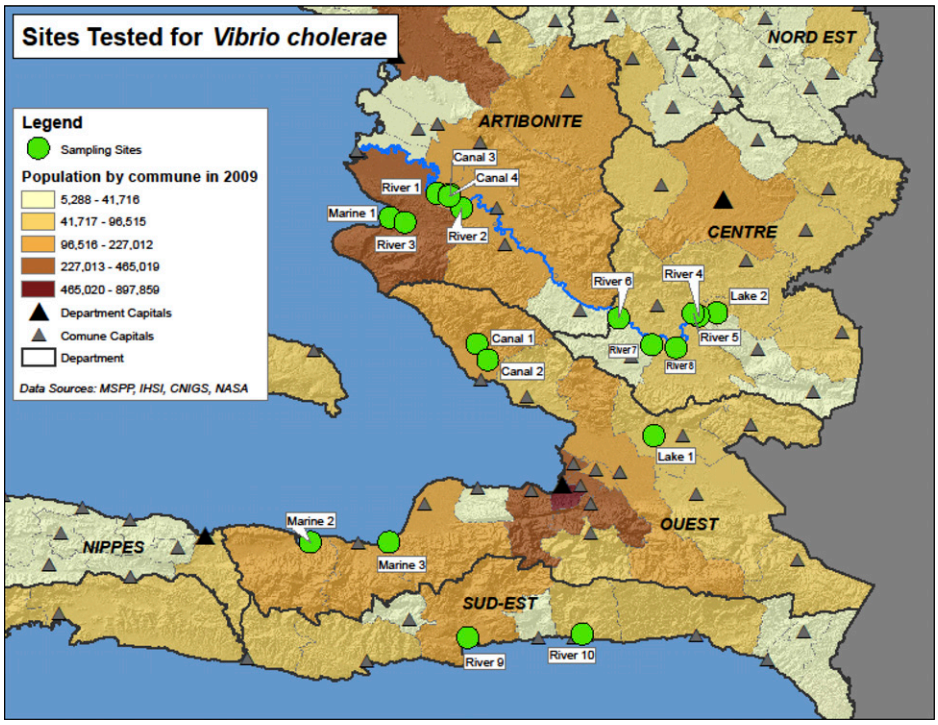

FIGURE 1. Name and locations of sites sampled during the study.

measured with a YSI 556MPS multi-parameter instrument (YSI Inc., Yellow Springs, OH). Water pH was measured with a Hanna HI9812 portable meter (Hanna Instruments, Woonsocket, RI). Fecal indicator bacteria (*Escherichia coli* for freshwater and enterococci for marine water) were quantified from a 100-mL grab sample using the USEPA-approved IDEXX Quanti-Tray/2000 method. *Escherichia coli* and enterococci analyses were conducted within 6–34 hours of sample collection. Water samples for *V. cholerae* testing were held in a cooler at ambient temperature for 24 hours to improve detection by culture.[15] Some samples were held for 48 hours before processing as a result of logistical constraints.

Sample processing and culture. At the Haiti National Public Health Laboratory (LNSP), *V. cholerae* were recovered from ultrafilters by backflushing with 400 mL of a surfactant solution to generate a final backflush volume of ~500 mL. This backflush was added to an equal volume of 2× alkaline peptone water (APW). The PLK samples were passed through a 20-μm mesh filter to obtain a final volume of 10–50 mL, depending on water quality.[16] The samples were vortexed and the plankton were homogenized with a tissue grinder. Two milliliters of the homogenized plankton sample were added to 18 mL of 1× APW, whereas 100 μL was spread plated directly onto thiosulfate citrate bile salts sucrose agar (TCBS) and CHROMagar *Vibrio* (CV) and incubated overnight at 37°C. Because of the logistics of plating on multiple agar media, only TCBS agar culture was performed for the August and January sampling events. The PFW samples (1-L) were filtered through 0.2-μm polycarbonate membrane filters. The filters were placed in 10–20 mL of 0.01 M PBS (1× strength) and vortexed to release the cell material from the surface of the filter. Two milliliters of the resulting solution were added to 18 mL of 1× APW. Nine hundred milliliters from the 1-L grab sample were added to 100 mL of 10× APW. Alkaline peptone water cultures were incubated overnight at 37°C. To extract DNA from APW cultures, duplicate 0.8-mL aliquots were removed from the broth surface and added to an equal volume of lysis buffer[17] and stored at 4°C. In addition, a 10-μL inoculating loop was used to remove the pellicle at the surface and the enrichment culture was streaked for isolation onto TCBS and CV. Agar plates were incubated overnight at 37°C. Five or more presumptive *V. cholerae* colonies from each agar plate were placed into tryptic soy agar (TSA) transport tubes and incubated overnight at 37°C. For DFA-DVC testing, 10 μL each of 2.5% yeast extract and 0.2% nalidixic acid were added to 1-mL aliquots of homogenized PLK and PFW samples.[11] After overnight incubation in the dark at 37°C, samples were fixed with 112 μL of formaldehyde (37% by weight), and stored at 4°C. Alkaline peptone water enrichments in lysis buffer, culture isolates in TSA transport vials, and DFA samples were shipped to the Centers for Disease Control and Prevention (CDC) in Atlanta, GA for subsequent analysis. One replicate set of the APW enrichments in lysis buffer and the DFA-DVC samples were shipped to the University of Maryland (UMD).

***Vibrio cholerae* detection and characterization.** Nucleic acid was extracted from replicate APW enrichment broth samples at CDC and UMD. At UMD, samples were tested by two conventional multiplex PCR assays for *tox*R, *ctx*A, and genes specific to the O-antigen biosynthesis region of serogroup O1 (*wbe*T gene) and O139 (*wbf*R gene).[18,19] For the *tox*R multiplex assay, oligonucleotide concentrations were 600 nM for the universal forward primer and 400 nM for each reverse primer. For the *ctx*A/O1/O139 multiplex assay, oligonucleotide concentrations were 170 nM for each *ctx*A primer, 500 nM for each O1 primer, and 270 nM for each O139 primer. Each multiplex PCR assay was performed using PromegaGoTaq Green Master Mix with 1 μL template DNA

in 25-μL reactions with the following thermal cycling conditions: one cycle of denaturation at 94°C for 10 min, followed by 36 cycles of denaturation at 94°C for 30 sec, 55°C annealing for 30 sec, extension at 72°C for 60 sec, with a final extension at 72°C for 10 min.

At CDC, APW enrichments were analyzed by multiplex real-time PCR (qPCR) for *ctx*A and *omp*W genes. The primer and TaqMan probe sequences for these assays are shown in Supplemental Table S1. For both qPCR assays, oligonucleotide concentrations were 625 nM for each primer and 125 nM for each probe. Each qPCR assay was performed using ABI Environmental Master Mix 2.0 and 2 μL template DNA in 20 μL reactions with the following thermal cycling conditions: one cycle of denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, and then annealing, extension, and fluorescence acquisition at 60°C for 1 min.

Presumptive *V. cholerae* culture isolates from TSA transport vials were streaked for isolation on the corresponding agar they were picked from in the field and incubated overnight at 37°C. Isolated colonies were picked and streaked on Luria Bertani (LB) agar and incubated overnight at 37°C. Part of an isolated colony was picked with an inoculating loop and added to 200 μL of TE buffer and heated at 95°C for 10 minutes for multiplex qPCR for *omp*W and *ctx*A genes using Qiagen QuantiTect Probe PCR Master Mix. Isolates positive for *omp*W or *ctx*A were further analyzed by multiplex qPCR for $tcpA_{\text{El Tor}}$ and $tcpA_{\text{Classical}}$. The primer and TaqMan probe sequences for these assays are shown in Supplemental Table S1. Oligonucleotide concentrations and qPCR cycling conditions were the same as for the *omp*W and *ctx*A multiplex assay.

A subset of isolates positive for *ctx*A or *tcp*A were further characterized.[20] Tests included agglutination in serogroup O1 antiserum, sequence determination of *tcp*A and/or *ctx*AB genes, and PCR amplification of *rst*R and the seventh pandemic marker *smp*. Serogroup O1-positive isolates were tested for agglutination in Inaba and Ogawa serotype antisera. Gene sequences were processed using LaserGene software (DNASTAR, Madison, WI) and compared with prototypical *V. cholerae* O1 *ctx*AB and *tcp*A sequences using MEGA5.[21] Toxigenic isolates were also subjected to whole genome sequencing on a MiSeq using the Nextera XT library prep kit following manufacturer's recommendations (Illumina Inc., San Diego, CA). Whole genome assemblies were taken either from National Center for Biotechnology Information (NCBI) or assembled in-house using the Computational Genomics Pipeline (CG-Pipeline) v. 0.3.[22] Genomes without gene predictions were analyzed with the CG-Pipeline module. Genomes were compared with a closed early-outbreak genome 2010EL-1786 (accessions CP003069 and CP003070) using Mauve.[23] Homologous genes were identified using USEARCH at 95% identity[24] and were aligned with MUSCLE,[25] and these individual multiple sequence alignments were concatenated into whole genome multiple sequence alignment. Sites where any genome had a gap or N, or where the site was invariant, were removed. The maximum likelihood tree was constructed with RAxML using rapid bootstrap analysis.[26]

The DFA-DVC was performed to obtain a direct viable count of *V. cholerae* O1 and O139 from PLK and PFW samples.[11] At UMD, formalin-fixed samples were analyzed by microscopy using DFA kits for *V. cholerae* O1 and O139 (New Horizons Diagnostics, Baltimore, MD).

Statistical analyses. To evaluate the association between measured water quality parameters and *ctx*A gene detection rates in APW enrichments and *V. cholerae* O1 cells by DFA-DVC, a marginal logistic model using the generalized estimating equation (GEE) approach[27,28] was applied. The model accounted for correlations stemming from repeated measures from the same collection site. The water quality analyses were conducted for freshwater only, because of substantial differences in some measured water quality parameters for freshwater and marine water. Separate analyses for marine water could not be performed because of the small sample size.

The performance of TCBS and CV for isolation of *V. cholerae* was evaluated by comparing the number of *V. cholerae* detections out of the total number of presumptive isolates tested. The performances of the large volume DEUF method and small volume grab sample method were evaluated by comparing the number of *V. cholerae* detections out of the total number of presumptive isolates tested and the number of *ctx*A detections from APW enrichments for each collection method. These comparisons were made using the GEE approach. For the TCBS versus CV comparison and DEUF versus grab culture isolation comparison, the GEE model accounted for correlations stemming from repeated measurements for the same collection site by sample type combination. For the DEUF versus grab *ctx*A comparison, the model accounted for collection site by test combination. Independent variables included: sample type (DEUF, PLK, PFW, grab), tests (TCBS or CV), month of collection, and collection site.

The exchangeable working correlation structure was assumed in all GEE models. The GEE analyses were done using a two-sided hypothesis test, and analyses were performed

TABLE 1

Median physical water quality parameters (Min, Max) and geometric mean (SD) of *Escherichia coli* and enterococci for each water type

Parameter	Canal $N = 16$*	Lake $N = 8$†	Marine $N = 12$‡	River $N = 39$§
Temperature (°C)	26.6 (21.5, 29.4)	28.8 (26.3, 32.3)	30.1 (27.1, 32.4)	27.7 (24.1, 34.2)
pH	8.2 (7.8, 8.3)	8.2 (7.9, 8.5)	8.0 (7.1, 8.7)	8.1 (7.6, 8.6)
Turbidity (NTU)	16 (0.89, 120)	30 (1.8, 360)	3.5 (0.92, 31)	9.0 (1.3, 270)
Salinity (mg/L)	0.15 (0.12, 0.18)	0.41 (0.14, 1.6)	36 (23, 39)	0.16 (0.08, 0.26)
Specific conductance (μS/cm)	308 (245, 382)	821 (298, 3,140)	54,200 (36,800, 58,900)	342 (161, 568)
E. coli (MPN/100 mL)	600 (4.2)	22 (6.3)	NA¶	500 (6.0)
Enterococci (MPN/100 mL)	NA	NA	180 (18)	NA

* $N = 12$ for pH.
† $N = 6$ for pH.
‡ $N = 9$ for pH.
§ $N = 29$ for pH.
¶ Not analyzed.

TABLE 2
Detection of *omp*W, *tox*R, and *ctx*A genes in APW enrichments

Site	October 2011			March 2012			August 2012			January 2013		
	*omp*W	*tox*R	*ctx*A	*omp*W	*tox*R	*ctx*A	*omp*W	*tox*R	*ctx*A	*omp*W	*tox*R	*ctx*A
Canal 1	+	+	+	+	+	−	+	+	−	+	−	−
Canal 2	+	+	+	+	+	−	+	+	−	+	−	+
Marine 1	+	+	−	+	+	−	+	−	−	NA*	NA	NA
River 1	+	+	+	+	+	−	+	+	+	+	−	+
River 2	+	+	+	+	+	−	+	+	−	+	−	−
Canal 3†	+	+	+	+	+	−	+	+	−	+	−	+
Canal 4	+	+	+	+	+	−	+	+	+	+	−	−
River 3	+	+	+	+	+	−	+	+	−	+	−	+
Lake 1	+	+	−	+	+	−	+	+	−	+	+	−
Lake 2	+	+	−	+	+	−	+	+	−	+	+	−
River 4	+	+	−	+	+	−	+	+	−	+	+	−
River 5	+	+	−	+	+	−	+	+	−	+	−	+
River 6	+	+	+	+	+	−	+	+	+	+	+	−
River 7	+	+	+	+	+	−	+	+	+	+	+	−
River 8	+	+	+	+	+	−	+	+	−	+	+	+
River 9	+	+	−	+	+	−	+	+	+	+	+	−
River 10	+	+	+	+	+	−	+	+	−	+	−	−
Marine 2	+	+	−	+	−	−	+	−	−	+	−	−
Marine 3	+	−	−	+	−	−	+	−	−	+	−	−

*NA = not analyzed.
†Detection of *V. cholerae* O1 gene in October 2011.

using SAS version 9.3 (SAS Institute, Inc., Cary, NC). A P value < 0.05 was considered statistically significant.

RESULTS

Water quality. Measured water quality parameters for each of the water sample types are presented in Table 1. Median physical water quality parameters are presented with minimum and maximum values, and the geometric means of bacterial parameters are presented with the SD. Water temperature, pH, salinity, and specific conductance were found to be consistent at each of the sampling sites. However, turbidity and bacterial indicators were more variable. *Escherichia coli* and enterococci data were consistent with substantial fecal contamination at most sites. Of the freshwater sites, 100% of canal samples, 25% of lake samples, and 74% of river samples fell into the high-risk category for *E. coli* levels (> 101 MPN/100 mL), according to WHO guidelines for drinking water.[29]

***Vibrio cholerae* PCR detection.** When pooled, culture data for all sample collection methods showed that *V. cholerae* genes could be detected in APW enrichments at every site during each field visit (Table 2). The *omp*W gene was detected at every site in every month, whereas *tox*R was detected at 18 sites in October 2011, 17 sites in March 2012, 16 sites in August 2012, and 7 sites January 2013. Toxigenic *V. cholerae*, indicated by detection of the *ctx*A gene, was found at 11 sites in October, 5 sites in August, and 6 sites in January. One of the *ctx*A detections in January 2013 was for a PLK sample collected from River 8, which was the only incidence of *ctx*A-positive PLK sample (data not shown). The *ctx*A gene was not detected in samples collected in March 2012. Detections of the *ctx*A gene in APW enrichments were associated with higher concentrations of *E. coli* and lower pH and dissolved oxygen levels (Table 3). The *V. cholerae wbe*T gene (O1) was detected at only one site in October, although the *wbf*R gene (O139) was not detected in any of the samples collected during the study.

Detection rates of *V. cholerae* genes were compared for DEUF and grab sample collection methods. The *omp*W gene was detected in 66 DEUF and 51 grab samples throughout the study. There were 18 *ctx*A detections associated with the DEUF method and 8 *ctx*A detections associated with grab samples (data not shown). This difference in *ctx*A detection for DEUF and grab samples was statistically significant, with *ctx*A detections more likely from DEUF-collected samples (odds ratio [OR] 2.60, 95% confidence interval [CI] 1.10–6.17).

***Vibrio cholerae* culture detection.** A total of 2,353 presumptive colonies of *V. cholerae* were examined by PCR for *omp*W, *ctx*A, and *tcp*A genes (Table 4). Although 936 isolates (40%) were *V. cholerae*, only three isolates from two sites

TABLE 3
Logistic regression analysis for association between water quality parameters and detection rates of the *ctx*A gene in APW enrichments and viable *V. cholerae* O1 cells by DFA-DVC for plankton (PLK) and plankton net-filtered (PFW) water samples

Water quality parameter	Units	APW *ctx*A		DFA-DVC PLK		DFA-DVC PFW	
		Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
E. coli	Log CFU/100 mL	**1.54 (1.24–1.90)***	**< 0.0001**	1.08 (0.76–1.53)	0.67	0.99 (0.81–1.22)	0.95
Salinity	0.1 mg/L	0.89 (0.70–1.13)	0.34	0.26 (0.06–1.23)	0.09	**1.11 (1.06–1.13)**	**< 0.0001**
Temperature	1°C	1.08 (0.86–1.36)	0.52	1.04 (0.85–1.28)	0.69	**1.25 (1.03–1.50)**	**0.02**
Specific conductance	100 μS/cm	0.94 (0.83–1.07)	0.38	0.52 (0.25–1.08)	0.08	**1.06 (1.03–1.08)**	**< 0.0001**
pH	0.1 log[H+]	**0.75 (0.61–0.93)**	**0.01**	1.07 (0.85–1.33)	0.56	1.00 (0.74–1.36)	0.99
Turbidity	50 NTU	1.64 (0.86–3.13)	0.13	1.75 (1.27–2.41)	0.15	**1.75 (1.27–2.41)**	**0.001**

*Bold type indicates a significant association.

TABLE 4
Characterization of presumptive *V. cholerae* culture isolates

Month	Isolates tested	*omp*W-positive (%)	*ctx*A-positive (%)	*tcp*A-positive (%)*
October	584	186 (32)	1 (0.17)	5 (0.86)
March	911	370 (41)	0	19 (2.1)
August	468	220 (47)	0	10 (2.1)
January	390	160 (41)	2 (0.51)	2 (0.51)
Total	2,353	936 (40)	3 (0.13)	36 (1.5)

*$tcpA_{El\ Tor}$ detected in October, August, January; $tcpA_{Classical}$ detected in March.

were positive for *ctx*A. One toxigenic isolate was recovered from the River 1 sampling site in the Artibonite Department in October 2011 and two others from the River 3 site in the Artibonite Department in January 2013. In October, March, and August, the *tcp*A gene was detected in 5, 19, and 10 nontoxigenic *V. cholerae* isolates, respectively, from the River 3 site. Only $tcpA_{El\ Tor}$ isolates were detected in October and August, and only $tcpA_{Classical}$ isolates were detected in March.

Isolate characterization. Results of genetic characterization of selected *ctx*A and *tcp*A-positive *V. cholerae* isolates from different sample sites are shown in Table 5. The toxigenic isolates matched the profile for the Haiti epidemic strain as characterized in a previous study.[20] The isolates were positive by PCR for El Tor *rst*R and *smp*, and the *ctx*AB and *tcp*A sequences matched 100% to the epidemic strain. The whole genome sequence of two water sample isolates (GenBank accession no. JNEW01000000) clustered with Haiti clinical isolates (Supplemental Figure S1).[30] The *tcp*A gene was sequenced for a total of six isolates that were *tcp*A-positive but *ctx*A-negative by qPCR (Table 5). The *tcp*A sequences did not match prototypical El Tor and classical *tcp*A sequences (Supplemental Figure S2). The allele detected by qPCR as El Tor differed from the prototypical allele at 14 nucleotide sites (2.1%), whereas the allele detected as classical differed from the prototypical allele at two nucleotide sites (0.3%).

DFA-DVC. Viable cells of *V. cholerae* O1 were detected by DFA-DVC at 15 sites in October 2011, 7 sites in August 2012, and 3 sites in January 2013 (Table 6). *Vibrio cholerae* O1 was not detected in March 2012. *Vibrio cholerae* O139 was not detected in any of the samples collected in the study. The DFA-DVC detections of *V. cholerae* O1 from PFW samples were associated with higher salinity, temperature, specific conductance, and turbidity (Table 3). However, no significant correlations were found between water quality parameters and DFA-DVC detection of *V. cholerae* O1 from PLK samples. Statistical analyses using the DFA-DVC concentration data presented in Table 5 were attempted using alternative modeling approaches, but none resulted in a good fit because of data characteristics (e.g., highly skewed, zero-inflated distribution, potential outliers).

Method comparisons. Results from TCBS and CV agar plating in October and March provided a comparison of the effectiveness of each agar for recovery of *V. cholerae* (Table 7). A higher percentage of isolates were recovered from TSA transport vials originally from TCBS (75–94%), compared with CV (58–77%). Additionally, a higher percentage of isolates from TCBS were confirmed as *V. cholerae* (34–48%) compared with CV (28–31%). The GEE analysis comparing TCBS versus CV, adjusting for sample type, found a statistically significant difference in detection rates between these two methods (OR 1.91, 95% CI 1.31–2.77). A comparison of *V. cholerae* detection rates for the DEUF and grab sample collection methods using TCBS agar (data not shown) indicated no significant difference between the two methods for recovering *V. cholerae* (OR 1.37, 95% CI 0.58–3.25).

DISCUSSION

Results of this study show that toxigenic *V. cholerae* O1 can be detected in Haitian freshwater systems, with detections as recently as January 2013. Although toxigenic *V. cholerae* was isolated from only two sites during the course of this surveillance study, *ctx*A genes were found in APW enrichments 22 times at 12 sites, and *V. cholerae* O1 was detected by DFA-DVC 25 times at 17 sites. Detection of *ctx*A genes in APW broths is an indication that culturable toxigenic *V. cholerae* cells were present in the enriched water samples. Therefore, detection of viable toxigenic *V. cholerae* and *V. cholerae* O1 was enhanced by APW qPCR and DFA-DVC compared with culture isolation alone. These findings are not unexpected, as other investigators have found that isolation of *V. cholerae* O1 from the environment is difficult, even when the bacteria can be detected by other methods.[32,33]

Detection of plankton-associated *V. cholerae* O1 by DFA-DVC throughout the study period is evidence of the presence of the bacteria in the environment because *V. cholerae* is a commensal of copepods and cladocerans. The association of *V. cholerae* with zooplankton can protect the bacteria from environmental stresses and low nutrient levels and enhance their distribution in the environment.[7,8,34]

The toxigenic *V. cholerae* detection data from this study are supported by the research results of Alam and others[6] who reported isolating the toxigenic *V. cholerae* O1 El Tor biotype from three water samples collected between April 2012 and March 2013 in the Ouest Department near the towns of Gressier and Leogane. However, other researchers, using only culture methods, did not isolate or detect toxigenic *V. cholerae*

TABLE 5
Characterization of *V. cholerae* isolates

Isolate ID	Location	Month	Serogroup	Serotype	*ctx*A	*tcp*A	*smp*	*rst*R
2012EL-1094	River 3	Oct.	Non-O1/O139	NA	–	El Tor*	–	Classical
2012EL-1098	River 1	Oct.	O1	Ogawa	+	El Tor	+	El Tor
2012EL-1759	River 3	March	Non-O1/O139	NA	–	classical†	–	Calcutta
2012EL-1762	River 3	March	Non-O1/O139	NA	–	classical†	–	Calcutta
2012EL-1765	River 3	March	Non-O1/O139	NA	–	classical†	–	Calcutta
2012EL-1768	River 3	March	Non-O1/O139	NA	–	classical†	–	Calcutta
2012EL-1774	River 3	March	Non-O1/O139	NA	–	classical†	–	Calcutta
2013EL-1241	River 3	January	O1	Ogawa	+	El Tor	+	El Tor

*Additional genetic characterization determined this allele differed from the prototypical El Tor allele by 2.1%.
†Additional genetic characterization determined this allele differed from the prototypical classical allele by 0.3%.

TABLE 6
DFA-DVC counts (cells/liter) of *Vibrio cholerae* O1 from plankton (PLK) and plankton net-filtered water (PFW) fractions

	October 2011		March 2012		August 2012		January 2013	
Site	PLK	PFW	PLK	PFW	PLK	PFW	PLK	PFW
Canal 1	< 20	< 2,000	< 40	< 2,000	72	< 2,000	< 26	< 2,000
Canal 2	80	32,000	< 40	< 2,000	< 40	< 2,000	< 20	< 2,000
Marine 1	< 20	< 2,000	< 70	< 2,000	< 20	< 2,000	ND*	ND
River 1	3,080	112,000	< 40	< 2,000	< 34	< 2,000	40	528,000
River 2	40	312,000	< 40	< 2,000	132	< 2,000	< 24	< 2,000
Canal 3	< 20	8,000	< 40	< 2,000	< 40	< 2,000	< 25	< 2,000
Canal 4	180	210,000	< 40	< 2,000	< 44	< 2,000	< 20	< 2,000
River 3	< 20	< 2,000	< 40	< 2,000	< 34	8,000	< 20	< 2,000
Lake 1	< 20	4,000	< 40	< 4,000	< 180	8,000	< 100	< 2,000
Lake 2	100	10,000	< 40	< 4,000	< 40	< 2,000	< 20	< 2,000
River 4	140	42,000	< 40	< 4,000	40	8,000	72	< 2,000
River 5	< 20	4,000	< 40	< 2,000	< 40	2,000	< 25	< 2,000
River 6	260	134,000	< 40	< 2,000	< 40	4,000	< 20	< 2,000
River 7	< 20	< 2,000	< 40	< 4,000	< 40	< 2,000	< 20	< 2,000
River 8	80	170,000	< 40	< 4,000	< 40	< 2,000	< 20	< 2,000
River 9	200	2,000	< 80	< 2,000	< 40	< 2,000	< 20	4,000
River 10	40	< 2,000	< 40	< 2,000	< 40	< 2,000	< 25	< 2,000
Marine 2	280	< 2,000	< 40	< 2,000	< 40	< 2,000	< 28	< 2,000
Marine 3	260	6,000	< 40	< 2,000	< 40	< 2,000	< 24	< 2,000

*ND = not done.

from water samples collected in July 2012 from 36 aquatic locations in the Ouest and Artibonite Departments.[35]

Non-toxigenic *V. cholerae* non-O1/O139 isolates that were *tcp*A-positive were repeatedly recovered from the River 3 site in the Artibonite Department during the surveillance project. The *tcp*A gene encodes the major subunit of the toxin co-regulated pilus (TCP), the receptor for lysogenic bacteriophage (CTXΦ) carrying cholera toxin genes.[1,36] In other countries, virulence genes, including *tcp*A, have been found in *V. cholerae* non-O1/O139 from the environment, notably in cholera-endemic areas.[37,38] Although initial PCR results suggested these strains carried El Tor and classical *tcp*A alleles, sequence determination of the *tcp*A gene and phylogenetic analysis showed that the alleles were related to (but different from) the prototypical alleles (Supplemental Figure S2). The *ctx*A-negative isolates were also positive by PCR for different alleles of *rst*R, suggesting they could each possess a different variant of pre-CTX prophage (the CTX prophage devoid of the *ctx*AB genes).[39] These results show that at least two non-toxigenic lineages of *tcp*A-positive *V. cholerae* exist in Haitian waters. These strains could conceivably acquire the *ctx*AB genes and become toxigenic, but experimental evidence to support this premise is lacking. Nonetheless, the results highlight the need to characterize environmental *V. cholerae* so that the source of non-toxigenic strains recovered from human cases, or new toxigenic strains should they appear, can be better understood.

Logistical regression indicated a negative association for *ctx*A detections with pH, which appears to contradict the established theories about conditions that support *V. cholerae* survival in the environment. However, the range of pH values for freshwater samples was small (7.6–8.6) and was within the range of favorable pH values for *V. cholerae*. Positive associations between *ctx*A detections and *E. coli* concentrations, and DFA-DVC detections and turbidity, may be indicative of the introduction of *V. cholerae* into surface waters through fecal contamination. The positive associations between DFA-DVC detections and salinity, temperature, and specific conductance are in concordance with the established paradigm of *V. cholerae* as autochthonous to the aquatic environment.[34]

During this study, TCBS agar resulted in a significantly higher percentage of *V. cholerae* positive isolates compared with CV agar. However, these findings warrant further investigation; there are several brands of TCBS on the market and there are differences in performance between brands.[40,41] There was no significant difference in the ability of a large volume sample collection method, such as DEUF, to recover *V. cholerae* from water samples compared with a 1-L grab sample. The DEUF method resulted in a greater number of *ctx*A detections from APW enrichments than the grab sample method, indicating that this large volume sample collection method can be effective for increasing detection rates of toxigenic *V. cholerae* in water samples. However, in three instances the grab sample was *ctx*A positive, whereas the DEUF sample was negative (data not shown). Taken together, these data indicate that the use of large and small volume sample collection methods may improve detection and isolation of *V. cholerae* in water samples.[16]

There were several limitations associated with this study, because only four visits to Haiti could be conducted. As a result of logistical constraints and testing capacity at LNSP, presumptive *V. cholerae* isolates were sent to CDC in Atlanta for confirmation. Only a portion of these isolates could be recovered from the transport media after shipment (Table 7). Additionally, the distance of some collection sites resulted in some samples being held for 48 hours instead of the desired 24 hours before processing. A 24-hour holding time has been shown to be beneficial for *V. cholerae* culture.[15]

TABLE 7
Comparison of TCBS and CHROMagar *Vibrio* for recovery of *V. cholerae* (*omp*W) isolates

	CHROMagar *Vibrio*			TCBS		
Month	Picked	Recovered (%)	Positive (%)	Picked	Recovered (%)	Positive (%)
October	389	226 (58)	63 (28)	480	359 (75)	123 (34)
March	528	405 (77)	125 (31)	541	506 (94)	245 (48)
Total	917	630 (69)	188 (30)	1021	865 (85)	368 (43)

Using multiple sample collection and analytical methods, toxigenic *V. cholerae* O1 was detected in Haitian water sources more than 2 years after the start of the cholera epidemic. The association of *V. cholerae ctx*A gene detections with higher *E. coli* concentrations and turbidity indicate that *V. cholerae* contamination may have been associated with inadequate sanitation and fecal runoff into water bodies. However, detections of plankton-associated *V. cholerae* O1 throughout the study and the detection of the *ctx*A gene in a PLK sample in January 2013 show that *V. cholerae* O1 can be found in a known environmental niche (plankton) in Haitian freshwater. The data from this study suggest that ongoing environmental surveillance of *V. cholerae* could provide valuable data for characterizing the potential for cholera transmission by untreated water consumption. Additional study of the ecology of *V. cholerae* in Haiti would provide useful data for water-related exposure risk management and informed decision making regarding potential public health measures such as vaccination campaigns and distribution of safe drinking water supplies and informational materials. Continued environmental surveillance is important to understand the ongoing ecological dynamics of *V. cholerae* and the contribution of waterborne transmission to cholera in Haiti.

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