

DETECTION OF VIRULENCE GENES IN ENVIRONMENTAL STRAINS OF *Vibrio cholerae* FROM ESTUARIES IN NORTHEASTERN BRAZIL

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SUMMARY

The objectives of this study were to detect the presence of *Vibrio cholerae* in tropical estuaries (Northeastern Brazil) and to search for virulence factors in the environmental isolates. Water and sediment samples were inoculated onto a vibrio-selective medium (TCBS), and colonies with morphological resemblance to *V. cholerae* were isolated. The cultures were identified phenotypically using a dichotomous key based on biochemical characteristics. The total DNA extracted was amplified by PCR to detect *ompW* and by multiplex PCR to detect the virulence genes *ctx*, *tcp*, *zot* and *rfbO1*. The results of the phenotypic and genotypic identification were compared. Nine strains of *V. cholerae* were identified phenotypically, five of which were confirmed by detection of the species-specific gene *ompW*. The dichotomous key was efficient at differentiating environmental strains of *V. cholerae*. Strains of *V. cholerae* were found in all four estuaries, but none possessed virulence genes.

KEYWORDS: Cholera; Estuaries; Pathogenicity; Genes.

INTRODUCTION

The genus *Vibrio* (family: Vibrionaceae) comprises 104 species⁶, some of which are pathogenic to humans. One of the best known of these is the Gram-negative species *Vibrio cholerae*, which is capable of inducing cholera, an acute intestinal infection, when ingested through contaminated water and food¹⁶.

In the early 1960s, Colwell demonstrated the importance of aquatic environments to the ecology and epidemiology of *V. cholerae*³⁵, contrary to the earlier notion that the organism could only be transmitted by a human source and that water served merely to carry it from host to host.

V. cholerae is widely distributed in estuarine, marine and freshwater environments and has been associated with outbreaks of endemic, epidemic and pandemic proportions^{11,12,23}.

Over 200 serogroups of *V. cholerae* are known, but only O1 and O139 have been implicated in epidemic cholera^{12,36}, although local sporadic outbreaks of diarrhea associated with non-O1 and non-O139 strains have been documented. Nevertheless, despite the extensive research done over the past years on the ecology, pathogenicity and epidemiological behavior of the species, many questions remain unanswered^{19,32}.

In Brazil, cholera reemerged in 1991, after a century of absence.

Between 1992 and 2005, the highest incidences of *V. cholerae* in samples of water from aquatic ecosystems and foods were registered in the northeastern region⁴, especially in the state of Pernambuco, making that state one of the most strongly impacted by cholera⁸. In the state of Ceará, outbreaks were reported between December 1991 and September 1993¹⁵.

The pathogenesis of cholera is complex and involves the synergy of a number of genes, such as *ctx*, *tcp*, *zot* and *rfbO1*^{10,12}. The presence of these genes may be used as an indicator of virulence, although the cholera toxin is considered the most important epidemic marker.

The purpose of the present study was to isolate and test environmental strains of *Vibrio cholerae* from estuaries in northeastern Brazil, due to the presence of virulence markers.

MATERIAL AND METHODS

Sampling locations: Sixty-four samples of water (n = 32) and sediments (n = 32) were collected in the estuaries of the rivers Pacoti, Choró, Pirangi and Jaguaribe (east of Fortaleza, Ceará, northeastern Brazil). Sample collections took place on a monthly basis, between January and April 2009. Two points in each river were chosen: one close to and other far away from the river mouth. The coordinates of the sampling locations were registered by GPS (Garmin III Plus) (Fig. 1): Pacoti 1 03°49'16.6"S and 038°24'11.7"W, Pacoti 2 03°48'52.4"S and

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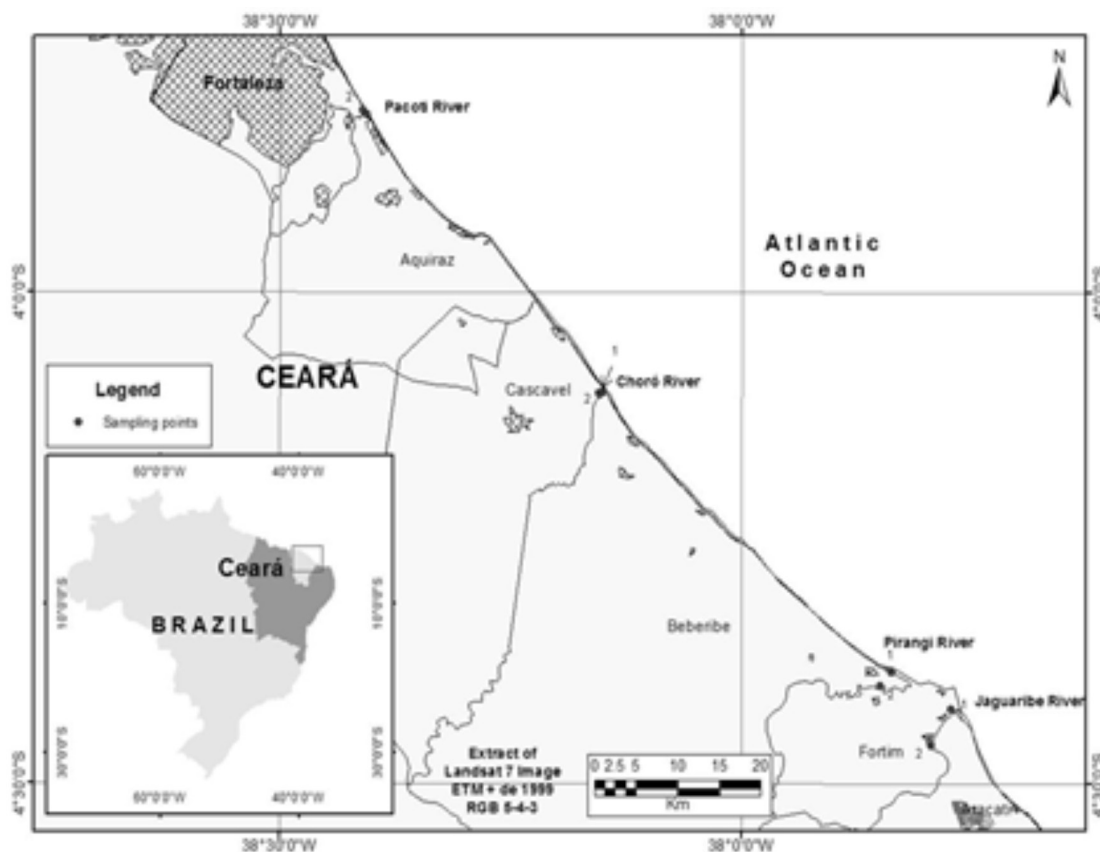


Fig. 1 - Map of the four estuaries Pacoti, Choró, Pirangi and Jaguaribe (Ceará, Brazil) from where water and sediment were sampled between January and April 2009.

038°24'38.1"W, Choró 1 04°06'07.2"S and 038°09'01.8"W, Choró 2 04°06'13.2"S and 038°09'13.8"W, Pirangi 1 04°23'11.6"S and 037°50'18.4"W, Pirangi 2 04°24'03.8"S and 037°51'00.3"W, Jaguaribe 1 04°25'28.7"S and 037°46'22.5"W, Jaguaribe 2 04°27'39.9"S and 037°47'39.5"W.

Collection of samples: Water samples were collected from a depth of 50 cm and stored in 1-L sterilized amber vials. Core-surface sediment samples were collected using a soil sampler. The samples were transported in isothermal boxes to the Laboratory of Seafood and Environmental Microbiology (LABOMAR/UFC) for immediate analysis. The temperature of the water was registered (thermometer, Incoterm) at the time of sampling.

Isolation of *Vibrio cholerae* from environmental samples

Water and sediment samples: The sampled water was used to make serial decimal dilutions (from 10^{-1} to 10^{-4}) in alkaline peptone water (APW) at pH 7.5-8.5. In order to prepare the sediment for analysis, 25-g aliquots were homogenized in 225 mL APW for 30 minutes (10^{-1}). Based on this first dilution, subsequent serial decimal dilutions (from 10^{-2} to 10^{-4}) were prepared using the same diluent.

One hundred microliters of each dilution was inoculated onto Thiosulfate Citrate Bile salts sucrose (TCBS) agar plates. Inoculated plates were incubated for 18 hours at 37 °C. Colonies with morphological

resemblance to *V. cholerae* (2-3 mm diameter, smooth, yellow, slightly flattened with opaque centers and translucent borders) were reseeded in tryptic soy agar (TSA) for purification¹⁹.

Identification of strains of *Vibrio cholerae* and detection of pathogenic potential

Phenotypic identification: Following purification, the cultures were submitted to biochemical identification using the dichotomous key of NOGUEROLA & BLANCH²⁴. The standard strains *V. cholerae* O1 Classic 569B and *V. cholerae* non-O1 IOC 15.177 (supplied by the microbe bank of the Oswaldo Cruz Institute, Rio de Janeiro, Brazil) were used as positive controls. Commercially available antibiotic disks were used to test the susceptibility patterns. Antimicrobial classes used in panel screens included: gentamicin, streptomycin, sulfazotrim, tetracycline, ciprofloxacin, nalidixic acid, penicillin, ampicillin, ceftriaxone, aztreonam, cephalothin, chloramphenicol, florfenicol and oxytetracycline. This assay was carried out according to the CLSI³ guidelines.

Genotypic identification

Extraction of chromosomal DNA: Strains of *V. cholerae* were inoculated in APW + 1% sodium chloride and incubated at 35 °C for 24 hours. Subsequently, 1.0-mL aliquots were submitted to DNA extraction using a commercially available kit (DNeasy Blood & Tissue Kit, Qiagen)¹².

Table 1

Primers and thermocycler conditions used in the molecular study of *V. cholerae* strains isolated from water and sediments collected in four estuaries in Ceará, Brazil, between January and April 2009

Technique	Genes	Primer sequence (5'- 3')	Thermocycling conditions	Amplicons (bp) ^f	Source	
PCR	<i>ompW</i> ^a	F: 5' -cac caa gaa ggt gac ttt att gtg- 3'		304		
		R:5' - ggt ttg tcg aat tag ctt cac c - 3'.				
Multiplex PCR	<i>ctxAB</i> ^b	F:5' -gcc ggg ttg tgg gaa tgc tcc aag - 3' R:5' - gcc ata cta att gcg gca atc gca tg - 3'	94°C/10 min. 30 cycles (94°C/1 min., 59°C/ 1min., 72°C/ 2 min.) 72°C/10 min.	536	GOEL <i>et al.</i> , (2007)	
	<i>tcp</i> ^c	F:5' - cgt tgg cgg tca gtc ttg- 3' R:5' - cgg gct ttc ttc ttg ttc g - 3'				805
	<i>rfbO1</i> ^d	F:5' - tct atg tgc tgc gat tgg tg - 3' R:5' - ccc cga aaa cct aat gtg ag - 3';				638
	<i>zot</i> ^e	F:5' - tgc ctt aac gat ggc gcg ttt t - 3' R:5' - aac ccc gtt tca ctt cta ccc a - 3'				947

ompW^a = *V. cholerae*-specific gene; *ctxAB*^b = cholera toxin; *tcp*^c = toxin coregulated pilus; *rfbO1*^d = serogroup O1 identification gene; *zot*^e = zonula occludens toxin; (bp)^f = base pair.

Target genes: The identification of *V. cholerae* was confirmed with the primer for the gene *ompW*, while virulence was evaluated with the primers for the genes *ctxAB* (cholera toxin), *tcp* (toxin co-regulated pilus), *rfbO1* (serogroup O1) and *zot* (zonula occludens toxin)¹². The primers were supplied by Cromax BioTechnologies (Brazil) (Table 1). The standard strains *V. cholerae* O1 Classic 569B and *V. cholerae* non-O1 IOC 15.177 (supplied by the microbe bank of the Oswaldo Cruz Institute, Rio de Janeiro, Brazil) were used as positive controls.

PCR: Control strains were used in all amplifications. The total DNA extracted was amplified by PCR to detect *ompW* (*V. cholerae*-specific gene) and by multiplex PCR to detect the virulence genes *ctxAB* (cholera toxin), *tcp* (toxin co-regulated pilus), *rfbO1* (serogroup O1 identification gene) and *zot* (zonula occludens toxin) using a thermocycler (Techne) (Table 2).

Visualization of extraction products and amplicons: The DNA extraction products and amplicons were submitted to electrophoresis in 1% agarose gel and Gel Red (GelRed Nucleic Acid Gel stain) and viewed under UV light with a Spectroline transilluminator. The runs lasted 60 minutes each and were performed with 7x14 cm agarose gel at 120V and 500mA. The gels were photo-documented with a digital camera (Kodak EDAS290). A 1000-bp DNA ladder (Sigma) was used as molecular size standard.

RESULTS AND DISCUSSION

Overall, 212 strains of *Vibrio* spp. were isolated, 98 of which from water samples and 114 from sediment samples. Nine strains were phenotypically identified as *V. cholerae*, five of which from water samples and four from sediment samples. There was no resistance to

Table 2

Composition and concentrations used in the reactions of the molecular study of *V. cholerae* strains isolated from water and sediments collected in four estuaries in Ceará, Brazil, between January and April 2009

Reagents of the reaction*	PCR		Multiplex PCR		
	Species-specific gene		Virulence genes		
	<i>ompW</i>	<i>ctxAB</i>	<i>tcp</i>	<i>rfbO1</i>	<i>zot</i>
Buffer 10X	20 mM Tris pH 8.4, 50 mM KCl		20 mM Tris pH 8.4, 50 mM KCl		
dNTP's (2.5 mM)	0.25 µM		0.25 µM		
Primer F (10 µM)	0.4 µM	0.4 µM	0.4 µM	0.4 µM	0.4 µM
Primer R (10 µM)	0.4 µM	0.4 µM	0.4 µM	0.4 µM	0.4 µM
MgCl2 (50 mM)	1.5 mM		1.5 mM		
Taq polymerase (500 U)	4 U		4 U		
Sample	20-35 ng**		20-35 ng**		
Final reaction volume	25 µL		25 µL		

* = water q.s. was added to each reaction; ** = sample concentration varied from 20 to 25 ng.

the antimicrobial drugs tested in the nine *Vibrio cholerae* strains found.

On PCR, only five percent (55%) of the strains phenotypically identified were confirmed to be *V. cholerae* (Fig. 2). Phenotypic identification of *Vibrio* is often ambiguous due to intra-species biochemical variation¹⁶, whereas genotypic identification yields a 100% match for *V. cholerae* using an *ompW*-specific primer³¹.

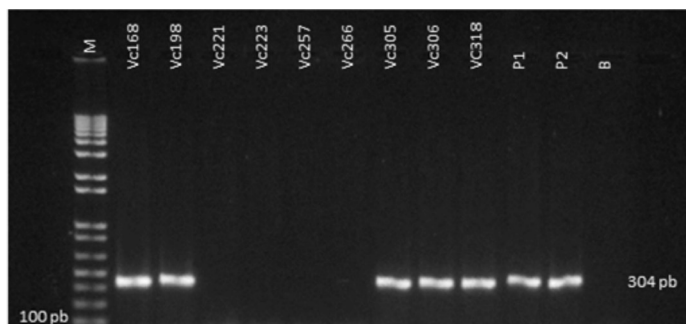


Fig. 2 - Electrophoretic profile of nine strains identified genotypically as *Vibrio cholerae* using primers for the species-specific gene *OmpW* (304 pb) on PCR. P1 = standard strain *V. cholerae* O1 classical 569B, P2 = standard strain *V. cholerae* non-O1 IOC 15.177, *M = marker, **B = negative control (white).

Fig. 2 - Electrophoretic profile of nine strains identified genotypically as *Vibrio cholerae* using primers for the species-specific gene *OmpW* (304 pb) on PCR.

According to GOEL *et al.*^{10,12}, *OmpW* acts as an internal control for *V. cholerae*, confirming the biochemical identification of suspected strains. This was recently demonstrated by JAIN *et al.*¹⁷ and by IZUMIYA *et al.*¹⁶ in studies based on clinical and environmental samples.

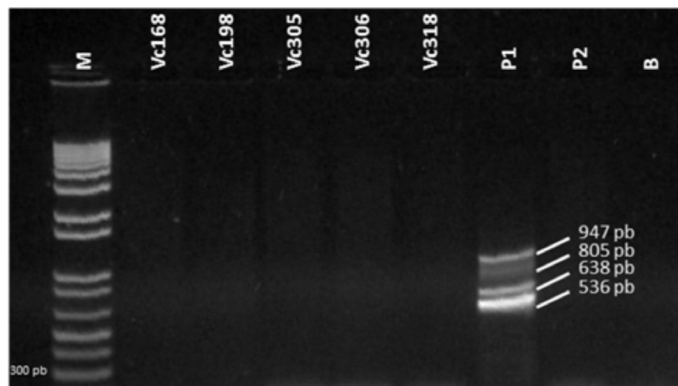
In the present study, the greatest number of *V. cholerae* strains (40%) came from the Pirangi estuary, followed by Jaguaribe, Choró and Pacoti (20% each). The relatively small number of *V. cholerae* isolated (n = 5) matches the findings of SOUSA *et al.*³³, who identified only eight strains of *V. cholerae* among 80 vibrio strains isolated from water and sediment samples collected in the same estuaries.

Figure 3 shows the electrophoretic profile of strains identified as *Vibrio cholerae* using primers for the virulence genes *ctxAB* (536 pb), *tcp* (805 pb), *rfbO1* (638 pb) and *zot* (947 pb) on multiplex PCR. None of the isolates tested positive for these genes.

The absence on PCR of virulence genes in these environmental samples is supported by the findings of GONÇALVES *et al.*¹³: none of their 31 strains of *V. cholerae* isolated from zooplankton, from Baía de São Marcos (São Luis, Maranhão, Brazil), was positive for *ctxA*, *zot* or *ace*.

In an analysis of water collected by LEAL *et al.*²⁰ from four rivers in Pernambuco (northeastern Brazil), strains of non-O1 and non-O139 *V. cholerae* tested negative for *ctxA*, *zot* and *ace*, and only one strain was positive for *tcpA*. In contrast, THEOPHILO *et al.*³⁴ assessed the distribution of virulence markers in Brazilian clinical and environmental strains of non-O1 and non-O139 *V. cholerae* isolated between 1991 and 2000 and found one of two environmental samples to be positive for *ctx*, while *zot*, *ace* and *tcp* were detected in clinical and environmental samples.

Nevertheless, according to SINGH *et al.*³², environmental strains of *V. cholerae* do not carry the toxin TCP, which is responsible for one of



P1 = standard strain *V. cholerae* O1 classical 569B, P2 = standard strain *V. cholerae* non-O1 IOC 15.177, *M = marker, **B = negative control (white).

Fig. 3 - Electrophoretic profile of strains identified as *Vibrio cholerae* (Vc) using primers for the virulence genes *ctxAB* (536 pb), *tcp* (805 pb), *rfbO1* (638 pb) and *zot* (947 pb) on multiplex PCR.

the factors of pathogenicity in toxigenic strains belonging to serogroups O1 and O139. REEN & BOYD²⁸ have shown the predominant association between *ctx* and *tcp* genes and epidemic isolates of *V. cholerae* O1 and O139 serogroups.

The absence of virulence genes in environmental strains of *V. cholerae* is explained by DRYSELIUS *et al.*⁵, HEIDELBERG *et al.*¹⁴ and RASMUSSEN *et al.*²⁷. According to these authors, all vibrios possess two chromosomes; in the case of *V. cholerae*, a large chromosome of 2.96Mb and a small chromosome of 1.07Mb. SCHOONILK & YILDIZ³⁰ have shown that the large chromosome contains most of the genes required for growth and pathogenicity, while the small chromosome encodes a number of essential metabolic components and regulatory pathways. The duplicity of genetic elements can generate false results in DNA extraction-based gene detection studies. Thus, knowledge of the chromosomal location of target genes can help minimize false-negative results by optimizing the extraction and amplification protocols. However, according to NORIEGA-OROZCO *et al.*²⁵, genotype studies have shown that *V. cholerae*, *V. vulnificus* and *V. alginolyticus* do not express virulence factors in natural environments.

The strains of non-O1 and non-O139 *V. cholerae* isolated from the water and sediments collected in four different estuaries for this study presented no virulence markers. However, there is evidence that, even in the absence of these virulence genes, non-O1 and non-O139 *V. cholerae* can cause diarrhea similar to cholera¹⁸, but do not generate epidemics. Many cases of diarrhoeal diseases related to non-O1 and non-O139 *V. cholerae* were reported^{2,7,21,26}. In addition, environmental strains of *V. cholerae* may cause outbreaks in the future^{1,29}, possibly triggered by environmental changes, or by lateral or horizontal transference of virulence genes mediated by phages, pathogenicity is lets and/or other mobile genetic elements encoding cholera toxin^{20,34}, as previously reported for marine populations of *V. cholerae* and *V. parahaemolyticus*²².

CONCLUSION

The dichotomous key of NOGUEROLA & BLANCH²⁴ was efficient at differentiating environmental strains of *V. cholerae* isolated from

four estuaries, confirming the importance of aquatic ecosystems in the dissemination, evolution and, in some cases, transmission of this pathogen to humans.

Although these strains possessed no virulence genes capable of causing cholera, environmental strains can evolve into epidemic lineages through contact with toxigenic strains.

RESUMO

Detecção de genes de virulência em estirpes de *Vibrio cholerae* isolados de estuários no Nordeste do Brasil

O objetivo deste estudo foi detectar a presença potencial virulência de *Vibrio cholerae* isolado de estuários do Nordeste do Brasil. Amostras de água e sedimento foram coletadas e inoculadas sobre meio seletivo para vibrios (TCBS) e colônias com características morfológicas de *V. cholerae* foram isoladas. A identificação fenotípica seguiu chave dicotômica baseada em características bioquímicas. Foram empregadas as técnicas de amplificação da polimerase em cadeia (PCR) utilizando o gene *ompW* e a de multiplex PCR para detecção de genes de virulência (*ctx*, *tcp*, *zot* e *rfbO1*). Os resultados da identificação das diferentes abordagens foram comparados. Nove cepas de *V. cholerae* foram identificadas fenotipicamente e cinco confirmadas através da detecção do gene *ompW*. A chave dicotômica utilizada foi eficiente para a confirmação da espécie. Os quatro estuários analisados apresentaram estirpes de *V. cholerae*, e nenhuma das cepas isoladas apresentaram genes de virulência.

ACKNOWLEDGMENTS

The authors would like to thank the government research promotion agencies FUNCAP and CAPES for financial support.

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Received: 8 March 2013

Accepted: 25 February 2014