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. 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 species, 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 food.

In the early 1960s, Colwell demonstrated the importance of aquatic environments to the ecology and epidemiology of V. cholerae, 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.

Over 200 serogroups of V. cholerae are known, but only O1 and O139 have been implicated in epidemic cholera, although local sporadic outbreaks of diarrhoea 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.

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 region, especially in the state of Pernambuco, making that state one of the most strongly impacted by cholera. In the state of Ceará, outbreaks were reported between December 1991 and September 1993.

The pathogenesis of cholera is complex and involves the synergy of a number of genes, such as ctx, tcp, zot and rfbO1. 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 Jaguaripe (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°34’9”’6”S and 038°24’11.7”W, Pacoti 2°03’49’16.6”S and 038°24’11.7”W, Pacoti 2°03’48’52.4”S and
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 purification.

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 & BLANCH. 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, cefotaxime, aztreonam, cephalothin, chloramphenicol, florfenicol and oxytetracycline. This assay was carried out according to the CLSI 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).
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) | Source |
|-----------|-------|-------------------------|--------------------------|----------------|--------|
| PCR       | *ompW* | F: 5'-cac caa gaa ggt gac ttg ttc atg gca ttc c - 3'. R: 5’- ggt ttc tgg gaa ggt gac atg tat ccc a - 3'. | 94°C/10 min. 30 cycles (94°C/1 min., 59°C/ 1 min., 72°C/ 2 min.) | 304 | |
| Multplex PCR | *ctxAB* | F: 5’- gcc ggg tgg tgg gaa ggc ttc aag tgc - 3'. R: 5’- gcc ata tca att ggc atc gca tgc ttc - 3'. | 72°C/10 min. | 536 | GOEL et al., (2007) |
| Multplex PCR | *tcp* | F: 5’- cgt tgg cgg tca gtc ttc - 3'. R: 5’- cgg gct ttc ttc ttg ttc g - 3'. | 805 | |
| Multplex PCR | *rfbO1* | F: 5’- ctc atg tgc tgc gat tgg tgc ttc - 3'. R: 5’- ccc cga aaa cct aat gtg ag - 3'. | 638 | |
| Multplex PCR | *zot* | F: 5’- tcc ctg aat gac ggc ggt ttc ttc ttc ttc - 3'. R: 5’- aac ccc gtt tca ctt cca ccc a - 3'. | 947 | |

*ompW* = *V. cholerae*-specific gene; *ctxAB* = cholera toxin; *tcp* = toxin coregulated pilus; *rfbO1* = serogroup O1 identification gene; *zot* = zonula occludens toxin; (bp) = 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)\(^1\). The primers were supplied by Croma 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 GelRed (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 1

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    | *ompW* | *ctxAB* | *tcp* | *rfbO1* | *zot* |
| 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 cholera* 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 variation\(^2\), whereas genotypic identification yields a 100% match for *V. cholerae* using an *ompW*-specific primer\(^1\).

**Fig. 2** - Electrophoretic profile of nine strains identified genetically 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.\(^{35}\), who identified only eight strains of *V. cholerae* among 80 vibrio strains isolated from water and sediment samples collected in the same estuaries.

**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 absence of virulence genes in environmental strains of *V. cholerae* is explained by DRYSELIUS et al.\(^3\), HEIDELBERG et al.\(^4\) and RASMUSSEN et al.\(^7\). 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 & YILDIZ\(^20\) 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.\(^{29}\), 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 *cholera*, but do not generate epidemics. Many cases of diarrhoeal diseases related to non-O1 and non-O139 *V. cholerae* were reported\(^{2,7,11,26}\). In addition, environmental strains of *V. cholerae* may cause outbreaks in the future\(^{26}\), possibly triggered by environmental changes, or by lateral or horizontal transference of virulence genes mediated by phages, or by other mobile genetic elements encoding cholera toxin\(^{30,34}\), as previously reported for marine populations of *V. cholerae* and *V. parahaemolyticus*\(^22\).

**CONCLUSION**

The dichotomous key of NOGUEROLA & BLANCH\(^20\) was efficient at differentiating environmental strains of *V. cholerae* isolated from the estuaries in Northeastern Brazil. Rev. Inst. Med. Trop. Sao Paulo, 56(5): 427-32, 2014.
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.

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