Genomic and Phenotypic Characterization of \textit{Vibrio cholerae} Non-O1 Isolates from a US Gulf Coast Cholera Outbreak

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Abstract

Between November 2010, and May 2011, eleven cases of cholera, unrelated to a concurrent outbreak on the island of Hispaniola, were recorded, and the causative agent, \textit{Vibrio cholerae} serogroup O75, was traced to oysters harvested from Apalachicola Bay, Florida. From the 11 diagnosed cases, eight isolates of \textit{V. cholerae} were isolated and their genomes were sequenced. Genomic analysis demonstrated the presence of a suite of mobile elements previously shown to be involved in the disease process of cholera (ctxAB, VPI-1 and -2, and a VSP-II like variant) and a phylogenomic analysis showed the isolates to be sister taxa to toxigenic \textit{V. cholerae} V51 serogroup O141, a clinical strain isolated 23 years earlier. Toxigenic \textit{V. cholerae} O75 has been repeatedly isolated from clinical cases in the southeastern United States and toxigenic \textit{V. cholerae} O141 isolates have been isolated globally from clinical cases over several decades. Comparative genomics, phenotypic analyses, and a \textit{Caenorhabditis elegans} model of infection for the isolates were conducted. This analysis coupled with isolation data of \textit{V. cholerae} O75 and O141 suggests these strains may represent an underappreciated clade of cholera-causing strains responsible for significant disease burden globally.

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Introduction

\textit{Vibrio cholerae} non-O1/non-O139 are the causative agents of sporadic, yet significant, gastrointestinal and extraintestinal infections globally, and it is well established that all strains of this species are capable of causing human infections that represent a significant global health burden [1,2,3,4,5,6,7]. Infection and subsequent illness caused by these organisms are linked to the presence of virulence factors in the core backbone of \textit{V. cholerae} (hemolysins, lipases) or mobile pathogenicity islands (VPIs-1 and -2, and CTX\textsuperscript{D}) that are frequently found in clinical isolates from cholera patients suffering severe rice water diarrhea [8,9,10].

Epidemic cholera is typically ascribed to \textit{V. cholerae} serogroup O1 or O139; however, it is now understood that, similar to pathogenic \textit{Escherichia coli}, a constellation of virulence factors along with host immune and nutritional status, are responsible for the severity and characteristic infections caused by these organisms [8,9,10,11,12]. It is established that those \textit{V. cholerae} which acquire and express genes carried on mobile elements (O-antigens, VPI-1, VPI-2, CTX\textsuperscript{D}, NAG-ST, etc.) are linked to epidemics of cholera. The scenario of mobile genetic element acquisition has been shown to have occurred within the 7\textsuperscript{th} pandemic and PG-1 and -2 clades (12), but occurrence and persistence of such genetic constellations...
Table 1. Genomes/strains used in this study.

| Organism     | Strain ID | Serogroup/Serotype | Biotype   | Geographical origin                         | Source of isolation | Year of isolation | Accession nos. |
|--------------|-----------|--------------------|-----------|---------------------------------------------|---------------------|-------------------|----------------|
| Vibrio cholera | NCTC 8457 | O1                 | El Tor    | Saudi Arabia                                | Clinical            | 1910              | NZ_AAWD01000000 |
| Vibrio cholera | M66-2     | O1                 | El Tor    | Makassar, Indonesia                         | Clinical            | 1937              | NC_012578/NC_012580 |
| Vibrio cholera | MAK757    | O1                 | El Tor    | Sulawesi, Indonesia (Celebes Islands)       | Clinical            | 1937              | NZ_AAUS00000000 |
| Vibrio cholera | O395      | O1                 | Classical | India                                       | Clinical            | 1965              | NC_009456/NC_009457 |
| Vibrio cholera | V52       | O37                | Sudan     |                                             | Clinical            | 1968              | NZ_AAK02000000 |
| Vibrio cholera | N16961    | O1                 | El Tor    | Bangladesh                                  | Clinical            | 1975              | NC_002505/NC_002506 |
| Vibrio cholera | E7946     | O1                 | El Tor    | Bahrain                                     | Clinical            | 1978              | Not Sequenced    |
| Vibrio cholera | 2740-80   | O1                 | El Tor    | Gulf Coast, USA                             | Water               | 1980              | NZ_ACHW00000000 |
| Vibrio cholera | TM 11079-80| O1                | El Tor    | Brazil                                      | Sewage              | 1980              | NZ_ADAL00000000 |
| Vibrio cholera | CT 5369-93| O1                 | El Tor    | Brazil                                      | Sewage              | 1980              | NZ_ADAL00000000 |
| Vibrio cholera | TMA21     | non-O1/non-O139    | Brazil    | Water                                       |                     | 1982              | NZ_ACHY00000000 |
| Vibrio cholera | 12129(1)  | O1                 | El Tor    | Australia                                   | Water               | 1985              | NZ_ACF00000000 |
| Vibrio cholera | RC9       | O1                 | El Tor    | Kenya                                       | Clinical            | 1985              | NZ_ACHX00000000 |
| Vibrio cholera | BX 330286 | O1                 | El Tor    | Australia                                   | Water               | 1986              | NZ_ACA00000000 |
| Vibrio cholera | V51       | O141               | USA       |                                             | Clinical            | 1987              | NZ_AAK02000000 |
| Vibrio cholera | RC27      | O1                 | Classical | Indonesia                                   | Clinical            | 1991              | NZ_ADAM00000000 |
| Vibrio cholera | INDRE 91/1| O1                 | El Tor    | Mexico                                      | Clinical            | 1991              | NZ_ADAK00000000 |
| Vibrio cholera | C6706     | O1                 | El Tor    | Peru                                        | Clinical            | 1991              | NZ_AHGO00000000 |
| Vibrio cholera | CPI032(5)| O1                 | El Tor    | Mexico                                      | Clinical            | 1991              | NZ_ALDA00000000 |
| Vibrio cholera | MO10      | O139               | Madras, India |                                         | Clinical            | 1992              | NZ_AAKF00000000 |
| Vibrio cholera | Amazonia  | O1                 | Amazonia  | Amazonas, Brazil                            | Clinical            | 1992              | NZ_AFSL00000000 |
| Vibrio cholera | MJ-1236   | O1                 | El Tor    | Matlab, Bangladesh                          | Clinical            | 1994              | NC_012668/NC_012667 |
| Vibrio cholera | IEC224    | O1                 | El Tor    | Belém, Brazil                               | Clinical            | 1994              | NC_016994/NC_016945 |
| Vibrio cholera | 1587      | O12                | Lima, Peru |                                             | Clinical            | 1994              | NZ_AAUO00000000 |
| Vibrio cholera | RC385     | O135               | Chesapeake Bay, USA                         | Plankton           | Clinical            | 1998              | NZ_AAKH02000000 |
| Vibrio cholera | CPI033(6)| O1                 | El Tor    | Mexico                                      | Clinical            | 2000              | NZ_AJRL00000000 |
| Vibrio cholera | AM-19226  | O39                | Bangladesh |                                             | Clinical            | 2001              | NZ_AAAY01000000 |
| Vibrio cholera | MZO-3     | O37                | Bangladesh |                                             | Clinical            | 2001              | NZ_AAUF01000000 |
| Vibrio cholera | MZO-2     | O14                | Bangladesh |                                             | Clinical            | 2001              | NZ_AAWF01000000 |
| Vibrio cholera | 623-39    | non-O1/non-O139    | Bangladesh |                                             | Water               | 2002              | NZ_AWG00000000 |
| Vibrio cholera | CIRS101   | altered El Tor    | Dhaka, Bangladesh                           | Clinical            | 2002              | NZ_ACW00000000 |
| Vibrio cholera | CPI038    | O1                 | El Tor    | Zimbabwe                                    | Clinical            | 2003              | NZ_ALDC00000000 |
| Vibrio cholera | B33       | O1                 | El Tor    | Beira, Mozambique                           | Clinical            | 2004              | NZ_ACH20000000 |
| Vibrio cholera | CPI040(13)| O1                 | El Tor    | Zambia                                      | Clinical            | 2004              | NZ_ALDD00000000 |
| Organism       | Strain ID     | Serogroup/Serotype | Biotype     | Geographical origin | Source of isolation | Year of isolation | Accession nos.       |
|---------------|---------------|--------------------|-------------|---------------------|---------------------|-------------------|----------------------|
| *Vibrio cholerae* | CP1041(14)    | O1                 | El Tor     | Zambia              | Clinical           | 2004              | NZ_ALDE00000000      |
| *Vibrio cholerae* | VC35          | O1                 | El Tor     | Kedah, Malaysia    | Clinical           | 2004              | NZ_AMBR00000000      |
| *Vibrio cholerae* | 3500-05       | O1                 | El Tor     | India              | Clinical           | 2005              | NZ_AHGL00000000      |
| *Vibrio cholerae* | 3582-05       | O1                 | El Tor     | Pakistan           | Clinical           | 2005              | NZ_AHGP00000000      |
| *Vibrio cholerae* | 3546-06       | O1                 | El Tor     | India              | Clinical           | 2006              | NZ_AHGM00000000      |
| *Vibrio cholerae* | LMA3994-4     | O1                 | El Tor     | Belém, Brazil      | River Water        | 2007              | CP002555/CP002556    |
| *Vibrio cholerae* | 3554-08       | O1                 | El Tor     | Nepal              | Clinical           | 2008              | NZ_AHGN00000000      |
| *Vibrio cholerae* | 3569-08       | O1                 | El Tor     | Gulf Coast, USA    | Environmental      | 2009              | NZ_AHGO00000000      |
| *Vibrio cholerae* | 2009V-1046    | O1                 | El Tor     | Pakistan           | Clinical           | 2009              | NZ_AHFX01000000      |
| *Vibrio cholerae* | 2009V-1085    | O1                 | El Tor     | Sri Lanka/India    | Clinical           | 2009              | NZ_AHFO00000000      |
| *Vibrio cholerae* | 2009V-1096    | O1                 | El Tor     | India              | Clinical           | 2009              | NZ_AHFP00000000      |
| *Vibrio cholerae* | 2009V-1116    | O1                 | El Tor     | Pakistan           | Clinical           | 2009              | NZ_AHG90000000       |
| *Vibrio cholerae* | 2009V-1131    | O1                 | El Tor     | India              | Clinical           | 2009              | NZ_AHGB00000000      |
| *Vibrio cholerae* | 2010V-1014    | O1                 | El Tor     | Pakistan           | Clinical           | 2009              | NZ_AHGD00000000      |
| *Vibrio cholerae* | 2011EL-1137   | O1                 | El Tor     | Zimbabwe           | Clinical           | 2010              | NC_016445/NC_016446  |
| *Vibrio cholerae* | CP1048(21)    | O1                 | El Tor     | Bangladesh         | Clinical           | 2010              | NZ_ALDJ00000000      |
| *Vibrio cholerae* | CP1050(23)    | O1                 | El Tor     | Bangladesh         | Clinical           | 2010              | NZ_ALDK00000000      |
| *Vibrio cholerae* | EL1786        | O1                 | El Tor     | Haiti              | Clinical           | 2010              | NZ_AEUG00000000      |
| *Vibrio cholerae* | EL1798        | O1                 | El Tor     | Haiti              | Clinical           | 2010              | NZ_AEVL00000000      |
| *Vibrio cholerae* | EL1792        | O1                 | El Tor     | Haiti              | Clinical           | 2010              | NZ_AELA00000000      |
| *Vibrio cholerae* | HE-09         | non-O1/non-O139    | Haiti      | Environmental      | 2010              | NZ_AFOA00000000     |
| *Vibrio cholerae* | HE-39         | non-O1/non-O139    | Haiti      | Environmental      | 2010              | NZ_AFOA00000000     |
| *Vibrio cholerae* | HE-48         | non-O1/non-O139    | Haiti      | Environmental      | 2010              | NZ_AFOA00000000     |
| *Vibrio cholerae* | HC-02A1       | non-O1/non-O139    | Haiti      | Clinical           | 2010              | NZ_AFOA00000000     |
| *Vibrio cholerae* | HC-21A1       | O1                 | El Tor     | Saint-Marc, Haiti  | Clinical           | 2010              | NZ_AGXI00000000      |
| *Vibrio cholerae* | HC-22A1       | O1                 | El Tor     | Saint-Marc, Haiti  | Clinical           | 2010              | NZ_AGXI00000000      |
| *Vibrio cholerae* | HC-32A1       | O1                 | El Tor     | Port-au-Prince, Haiti | Clinical         | 2010              | NZ_AGUO00000000      |
| *Vibrio cholerae* | HC-72A2       | O1                 | El Tor     | Arcahaie, Haiti    | Clinical           | 2010              | NZ_AGUV00000000      |
| *Vibrio cholerae* | HC-80A1       | O1                 | El Tor     | Port-au-Prince, Haiti | Clinical         | 2010              | NZ_AGV80000000      |
| *Vibrio cholerae* | 2010EL-1961   | O1                 | El Tor     | Haiti              | Clinical           | 2010              | NZ_AHGO00000000      |
| *Vibrio cholerae* | 2010EL-2010H  | O1                 | El Tor     | Haiti              | Clinical           | 2010              | NZ_AHGO00000000      |
| *Vibrio cholerae* | 2010EL-2010N  | O1                 | El Tor     | Haiti              | Clinical           | 2010              | NZ_AHGO00000000      |
| *Vibrio cholerae* | 2011EL-1089   | O1                 | El Tor     | Haiti              | Clinical           | 2010              | NZ_AHGV00000000      |
| *Vibrio cholerae* | HC-41B1       | non-O1/non-O139    | Haiti      | Clinical           | 2010              | NZ_AJRP00000000     |
| *Vibrio cholerae* | HE-40         | non-O1/non-O139    | Haiti      | Hospital Latrine   | 2010              | NZ_AJRI00000000     |
| Organism          | Strain ID | Serogroup/Serotype | Biotype | Geographical origin | Source of isolation | Year of isolation | Accession nos.          |
|------------------|-----------|--------------------|---------|--------------------|--------------------|-------------------|------------------------|
| Vibrio cholerae  | HE-46     | non-O1/non-O139    |         | Haiti              | Gray Water         | 2010              | NZ_AJRY00000000       |
| Vibrio cholerae  | HC-44C1   | non-O1/non-O139    |         | Haiti              | Clinical           | 2010              | NZ_AUSK00000000       |
| Vibrio cholerae  | HC-39A1   | non-O1/non-O139    |         | Haiti              | Clinical           | 2010              | NZ_ALDM00000000       |
| Vibrio cholerae  | HC-41A1   | non-O1/non-O139    |         | Haiti              | Clinical           | 2010              | NZ_ALDN00000000       |
| Vibrio cholerae  | HC-42A1   | non-O1/non-O139    |         | Haiti              | Clinical           | 2010              | NZ_ALDR00000000       |
| Vibrio cholerae  | HC-47A1   | non-O1/non-O139    |         | Haiti              | Clinical           | 2010              | NZ_ALEB00000000       |
| Vibrio cholerae  | HE-16     | non-O1/non-O139    |         | Haiti              | Gray Water         | 2010              | NZ_ALEC00000000       |
| Vibrio cholerae  | HE-25     | non-O1/non-O139    |         | Haiti              | Gray Water         | 2010              | NZ_ALED00000000       |
| Vibrio cholerae  | HE-45     | non-O1/non-O139    |         | Haiti              | Gray Water         | 2010              | NZ_ALED00000000       |
| Vibrio cholerae  | CP1042(15)| O1                 |         | El Tor            | Thailand           | 2010              | NZ_ALDF00000000       |
| Vibrio cholerae  | BJGO1     | non-O1/non-O139    |         | Mississippi Gulf Coast, USA | Clinical | 2010 | NZ_AFou00000000 |
| Vibrio cholerae  | 2010EL-1749| O1               |         | El Tor            | western Africa     | 2010              | NZ_AHGC00000000       |
| Vibrio cholerae  | 2011EL-1133| O1               |         | El Tor            | Haiti              | 2011              | NZ_AHGJ00000000       |
| Vibrio cholerae  | 2011V-1021| O1                 |         | El Tor            | Dominican Republic | 2011              | NZ_AHGR00000000       |
| Vibrio cholerae  | 2011EL-301| non-O1/non-O139   |         | Taganrog, Russia  | Water              | 2011              | NZ_AJFN00000000       |
| Vibrio cholerae  | CP1110    | O75                |         | Florida Gulf Coast, USA | Clinical | 2010-2011 | NZ_AMWF00000000       |
| Vibrio cholerae  | CP1111    | O75                |         | Florida Gulf Coast, USA | Clinical | 2010-2011 | NZ_AMWS00000000       |
| Vibrio cholerae  | CP1112    | O75                |         | Florida Gulf Coast, USA | Clinical | 2010-2011 | NZ_AMWT00000000       |
| Vibrio cholerae  | CP1113    | O75                |         | Florida Gulf Coast, USA | Clinical | 2010-2011 | NZ_AMWU00000000       |
| Vibrio cholerae  | CP1114    | O75                |         | Florida Gulf Coast, USA | Clinical | 2010-2011 | NZ_AMWW00000000       |
| Vibrio cholerae  | CP1115    | O75                |         | Florida Gulf Coast, USA | Clinical | 2010-2011 | NZ_AMWR00000000       |
| Vibrio cholerae  | CP1116    | O75                |         | Florida Gulf Coast, USA | Clinical | 2010-2011 | NZ_AMWN00000000       |
| Vibrio cholerae  | CP1117    | O75                |         | Florida Gulf Coast, USA | Clinical | 2010-2011 | NZ_AMWV00000000       |
| Vibrio cholerae  | VL426     | non-O1/non-O139    |         | Maidstone, Kent, UK | Water              |                  | NZ_ACHV00000000       |
| Vibrio anguillarum| 96F       | O1                 |         | Chesapeake Bay, USA | Striped bass (Morone saxatilis) | 2010 | NZ_AEZA00000000 |
| Vibrio anguillarum| RV22     | O2j                |         | Atlantic coast of Spain | Turbot (Scophthalmus maximus) | 2010 | NZ_AEZB00000000 |
| Vibrio anguillarum| 775      | O1                 |         | coast of Washington, USA | Coho salmon (Oncorhynchus kisutch) | 1999 | NZ_ACZn00000000 |
| Vibrio coralliilyticus| ATCC 88A-450 |          |         | Zanzibar, Tanzania | Coral                  | 1999 | NZ_ACZn00000000 |
| Vibrio mimicus   | SX-4      | O1                 |         | China              | Clinical           | 2009              | NZ_ADoo00000000       |
| Vibrio mimicus   | VM573     | O1                 |         | United States      | Clinical           | 1990             | NZ_ACY00000000        |
| Vibrio mimicus   | MB-451    | O1                 |         | Matlab, Bangladesh | Clinical           |                  | NZ_ADAF00000000       |
| Vibrio orientalis| CIP 102891| O1                 |         | Yellow sea, China  | Water              |                  | NZ_ACZV00000000       |
remains underappreciated in *V. cholerae* non-O1/non-O139 (non-\(PG\)) lineages. These elements, among many others, can be laterally transferred between strains of the same species or distantly related species in the environment [13,14,15] and give rise to virulent strains that potentially can cause epidemics. Further, these elements can be stable in *V. cholerae* non-O1/non-O139 isolates, as in strains of the 7th pandemic clade and persist in these conformations over time, ultimately conserved in the environment.

In developed nations, the leading cause of human disease caused by vibrios is consumption of raw or undercooked seafood, namely shellfish. In the United States, seafood-borne vibrios have been traced to shellfish harvested from coastal (Atlantic and Pacific) regions, as far north as Alaska, but by far the majority of infections occur in the Gulf of Mexico, where the water temperature is warm, a parameter associated with increased *Vibrio* spp. densities as well as increased risk of vibriosis [16,17,18,19,20]. Recent cases of cholera traced to seafood consumption, and many *V. parahaemolyticus* infections and deaths caused by *V. vulnificus* have been reported in this region.

*V. cholerae* O75 serogroup strains have been reported to cause sporadic shellfish-borne cholera cases in the southeastern United States [21,22]. Outbreaks caused by these strains are not continuous as outbreaks in developing nations because sanitation in the United States is such that untreated human waste is not typically discharged into water used for drinking, recreation, or harvesting of seafood and water used for consumption or for household use is typically treated to remove bacterial pathogens. Further, *V. cholerae* O75 strains have been isolated from environmental waters in the southeastern United States in the absence of reported cholera cases [21]. Here we present results of analysis of eight clinically recovered *V. cholerae* O75 isolates from an indigenous US Gulf Coast cholera outbreak that occurred in, 2010, and during March and April, 2011 [22].

### Materials and Methods

Clinical *V. cholerae* isolates that were epidemiologically linked to consumption of oysters harvested from the Apalachicola Bay, FL were obtained from the Florida Department of Health Bureau of Public Health Laboratories in Jacksonville, FL. The genomes described in this study were either obtained from the NCBI Genbank database or, in the case of strains CP1110, 1111, 1112, 1113, 1114, 1115, 1116 and 1117, were sequenced using the Genome Analyzer IIx system (Illumina, Inc., San Diego, CA) according to the manufacturer’s methods. Raw reads of these genomes were assembled with CLC Genomics Workbench. Genome-to-genome comparisons, identification and characterization of molecular genetic elements (MGEs), as well as core genome phylogenetics were performed by using methods described previously [12]. Genomes of *V. cholerae* strains CP1110 to CP1117 were annotated using Rapid Annotation using Subsystem Technology [23]. For *in silico* genomic island BLASTN and phylogenetic analyses the RAST-annotated ORFs of *V. cholerae* CP1110 were used as a reference. PCR analyses of virulence factors not resolved by genome sequencing (\(\text{rstR}\) alleles, \(\text{nanH}\), and \(\text{ctxB}\) biotype) were done using the methods of Choi et al. [24], Vora et al. [25], and Nusrin et al. [26]. Phenotypic assays (protoxolysis, hemolysis, biofilm formation, and motility) were conducted following methods standardized for *V. cholerae* [27]. Hemolysis, biofilm formation, motility, and protoxolysis assays were done in nine replicates. BiOLOG phenotypic microarrays (PM1, PM2A, PM9, and PM10) were conducted in duplicate following the manufacturer’s instructions (BiOLOG, Hayward, CA). Substrate metabolism was scored by dividing the area under the curve
by the background values. Scores >2 were considered positive for metabolism of that substrate.

For the *Caenorhabditis elegans* model, SS104 glp-4 (bn2) temperature sensitive sterile strain was acquired from the *Caenorhabditis* Genetics Center (CGC). SS104 worms were maintained at 16°C, and experiments were performed at 25°C. Worms were cultured in *C. elegans* habitation media (CeHM) in tissue culture flasks on a platform shaker [28]. Adult nematodes were bleached (0.5 M NaOH, 1% Hypochlorite) to collect eggs, which were incubated in M9 media for 24 hours to bring them to synchronized L1 stage, and then transferred to CeHM. L4 stage worms were transferred to assay plates for survival experiments. Pathogen lawns for survival assays along with control bacteria *E. coli* OP50 were prepared by inoculating Nematode Growth Medium (NGM), in 6-cm Petri dishes, with 50 µl of an overnight *V. cholerae* culture. Plates were incubated overnight at room temperature before worms were added. Temperature sensitive sterile worms (SS104 *glp-4*(bn2)) strain, obtained from *Caenorhabditis* Genetics Center were transferred to NGM plates containing *V. cholerae* wild type strains E7946, CP1112, CP1114, CP1115 or *E. coli* OP 50 bacterial lawns and incubated at 25°C with ~20–30 L4 stage worms added to each plate. Animals were scored every 24 h for survival. Animals were considered dead when they no longer responded to a gentle prod with a platinum wire. *C. elegans* survival was plotted using Kaplan-Meier survival curves and analyzed by log rank test using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Survival curves resulting in *p* values of <0.05 relative to control were considered significantly different [29]. Strains and genomes used in this study are listed in Table 1.
### Table 2. ORFs with polymorphisms within the *V. cholerae* FL group.

| Locus  | CP1110 | CP1111 | CP1112 | CP1113 | CP1114 | CP1115 | CP1116 | CP1117 | Annotation                                                                 |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|-----------------------------------------------------------------------------|
| VC0315 | A      | A      | A      | A      | G      | G      | A      | A      | CDP-diacylglycerol–serine O-phosphatidyltransferase                         |
| VC1899 | C      | C      | T      | C      | C      | C      | T      | C      | hypothetical protein                                                        |
| VC0028 | C      | C      | C      | C      | C      | T      | C      | T      | Dihydroxy-acid dehydratase                                                 |
| VC0031 | C      | C      | C      | C      | C      | C      | A      | Acetyl-CoA synthetase large subunit                                         |
| VC1359 | T      | T      | T      | T      | T      | C      | T      | ABC-type polar amino acid transport system ATPase component                |
| Gl-26  | G      | G      | G      | G      | G      | G      | G      | putative transcriptional activator ToxR                                    |
| VCA1063| A      | A      | A      | A      | A      | A      | A      | Ornithine decarboxylase                                                    |

* = Not found in *V. cholerae* N16961.

**Figure 2.** *rsf* sequences from *V. cholerae* CP1110. Sequences are aligned to their most similar homologs extracted from NCBI Genbank database. Nucleotide sequence identity is shown to the right of the last nucleotide aligned for each allele.

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Results and Discussion

Phylogenomic Analysis of Florida Outbreak Strains

The eight isolates subjected to analysis in this study have been labeled by number (isolates CP1110, 1111, 1112, 1113, 1114, 1115, 1116 and 1117) and are hereafter collectively referred to as the *V. cholerae* FL Group. The phylogeny of 84 fully and partially sequenced *V. cholerae* strains, including the eight *V. cholerae* FL Group genomes, was inferred (Figure 1). Results of the analysis demonstrate that the *V. cholerae* FL Group are sister taxa with *V. cholerae* V51, a clinical *V. cholerae* O141 serogroup strain isolated from a human clinical case in the United States in 1987, suggesting a common ancestor after it had diverged from other *V. cholerae* lineages. From a public health perspective, the results of the analysis demonstrate the group represents a phyletic lineage of *V. cholerae* non-O1/non-O139 strains that persist in the United States as a cause of morbidity. Although, not added to this analysis due to the absence of their sequenced genomes, results of this analysis coupled with *V. cholerae* isolation data from cholera patients worldwide demonstrate that other *V. cholerae* serogroup O141 and O75 strains result in similar clinical manifestations as the strains in this study, that is symptoms of cholera [30,31]. As with the isolates sequenced in this analysis, other *V. cholerae* O141 and O75 infections in the United States were associated with either seafood consumption or presence of the patient in a coastal state, suggesting infections with strains of these serogroups are transmitted to people in a similar manner as those of the O1 serogroup and therefore they have a similar ecology as serogroup O1 strains in the United States [32,33].

We identified 8 single nucleotide polymorphisms (SNPs) among the *V. cholerae* O75 genomes in this study. Six of these occurred in six separate ORFs and two occurred in one ORF annotated as a "putative transcriptional activator ToxR." It is not clear if these SNPs influence the ecology or virulence potential of these isolates. However, they do demonstrate an appreciable level of genomic diversity between strains of the same outbreak (Table 2). To further estimate the genomic diversity of this lineage, comparisons should be made to other *V. cholerae* O75 isolates from clinical and environmental isolates.

Genomic Islands, Pathogenicity Islands, and Virulence Factors

The *V. cholerae* FL Group isolates were determined to contain the full CTX phage encoding the cholera toxin, but the structure...
of this region was unresolved due to the limitations of assembly since ORFs were found on multiple contigs. For similar reasons, CTX phage copy number could not be resolved. A BLASTN analysis with *V. cholerae* N16961 and O395 as reference demonstrated the presence of regions homologous to VC1456 to VC1463 (VC0395_0512 to VC0395_0505 and VC0395_0506 and VC0395_0507 of *V. cholerae* O395) of the CTX phage (*ctxB*, *ctxA*, *zot*, *ace*, *orfU*, *cep*, *rstB*, *rsdA*, and *rstR*). To infer the biotype of the cholera toxin, PCR targeting the *ctxB* gene was employed and resulted in an amplicon for primers of targeting biotype of the cholera toxin. PCR targeting the *ctxB* gene was employed and resulted in an amplicon for primers of targeting biotype of the cholera toxin.

The calculation was based on aligned fragments of 25 orthologous genes (VC0819 to VC0845) comprising ca. 26.9 kb. Bar length = 0.005 substitutions per site. The calculation was based on aligned fragments of 25 orthologous genes (VC0819 to VC0845) comprising ca. 26.9 kb. Bar length = 0.005 substitutions per site.

Figure 4. Phylogenetic analysis of *Vibrio* pathogenicity island 1 (VPI-1). Neighbor-joining tree showing evolutionary relationships of VPI-1. The calculation was based on aligned fragments of 25 orthologous genes (VC0819 to VC0845) comprising ca. 26.9 kb. Bar length = 0.005 substitutions per site. doi:10.1371/journal.pone.0086264.g004

| V. cholerae MO10 | V. cholerae N16961 | V. cholerae MJ-1236 | V. cholerae IEC224 | V. cholerae 2010EL-1786 | V. cholerae 2011EL-301 | V. cholerae CIRS 101 | V. cholerae BX 330286 | V. cholerae M66-2 | V. cholerae 2740-80 | V. cholerae NCTC 8457 | V. cholerae O395 | V. cholerae V52 | V. mimicus SX-4 | V. mimicus VM573 |
|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|                  |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| V. cholerae FL Group |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| V. cholerae V51 |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |

This figure demonstrates the diversity of the CTX phage outside of the more frequently studied *V. cholerae* O1 strains and suggests many alleles of this phage can be associated with cholera. Cholera toxin expression was not assayed in this study.

The genomes of the eight *V. cholerae* FL Group isolates harbored *Vibrio* pathogenicity island 1 (VPI-1) encoding the toxin co-regulated pilus (TCP) shown to be responsible for biofilm formation in the intestine and a receptor for CTX phage [36,37]. VPI-1 of the *V. cholerae* FL Group is highly similar in structure to those of other clinical and environmental *V. cholerae* and *V. mimicus* (Figure 3). Interestingly, the *tcpA* gene (often used as a marker of *V. cholerae* biotype) of this group has the highest similarity with that of *V. cholerae* O395, a Classical biotype, while showing similarity of 77% with *V. cholerae* V51. However, a phylogeny of concatenated ORFs of this island demonstrates VPI-1 of the *V. cholerae* FL Group and *V. cholerae* V51 are closely related to each other from an evolutionary perspective, and significantly diverged from VPI-1 of other clinical and environmental *V. cholerae* and *V. mimicus* strains (Figure 4).

The genomes of all *V. cholerae* FL Group isolates also encoded VPI-2, with a type III secretion system (T3SS) (Figure 5). Two divergent T3SS variants have been identified in *V. cholerae* isolates [38]. T3SS in the *V. cholerae* FL Group genomes are most similar to that of *V. cholerae* V51 and AM-19226, a non-O1 TCP-negative and CTX-negative isolate (Figure 5). The T3SS of *V. cholerae* AM-19226 has been shown to be essential for colonization of the infant rabbit intestine and associated with severe diarrhea in this model, suggesting it plays a significant role in virulence during human infections [39]. This region has been found in environmental and clinical *V. cholerae* on a global scale. For instance, *V. cholerae* HE-25, a gray water isolate from Haiti and *V. cholerae* VC35, a clinical isolate from Malaysia, both encode T3SS that is structurally and phylogenetically similar to the variant in the *V. cholerae* FL Group.
suggesting global distribution of this virulence factor in environmental and clinical isolates (Figure 6). A phylogeny based on conserved ORFs of this variant and of *V. parahaemolyticus* as an outgroup infers the nearest phylogenetic neighbor to T3SS in the *V. cholerae* FL Group is *V. cholerae* VC35 (Figure 7). Although this region has been shown to be part of VPI-2 variants it has been identified as a separate genomic island capable of lateral transfer between *V. cholerae* strains [12,40].
VPI-2 of the \textit{V. cholerae} FL Group also encodes a complete sialic acid catabolism operon (homologs of VC1773 to VC1784 in the canonical VPI-2 of \textit{V. cholerae} N16961), including a neuraminidase (sialidase) which has been shown to unmask the GM1 gangliosides of human intestinal epithelial cells, making them available to the cholera toxin \cite{10}. A phylogeny of the sialic acid metabolism region demonstrated this operon in the \textit{V. cholerae} FL Group is closely related to that of \textit{V. cholerae} V51, \textit{V. cholerae} 1587, and \textit{V. cholerae} 623-39 (Figure 8). The phylogeny of this region is not congruent with that of the T3SS suggesting a more recent ancestral sialic acid metabolism region of the \textit{V. cholerae} FL Group and \textit{V. cholerae} V51 than that of the T3SS. Further, when the phylogenies of T3SS and sialic acid metabolism operons of seven \textit{V. cholerae} strains with homologous ORFs are inferred (\textit{V. cholerae} strains AM-19226, TMA 21, HE-25, V51, 12129(1), FL Group, and VC35), sister taxa of the \textit{V. cholerae} FL Group T3SS remains \textit{V. cholerae} VC35 and sister taxa of the sialic acid metabolism region of the \textit{V. cholerae} FL Group remains \textit{V. cholerae} V51 (data not shown). These data suggest the two regions in the \textit{V. cholerae} FL Group originated from different sources. Morita et al. \cite{40} demonstrated these two regions of VPI-2 could be of separate origin and the insertion locus of \textit{V. cholerae} T3SS is exclusively in VPI-2.

Interestingly, the \textit{mu}-like phage region, the most variable region of the canonical VPI-2, is absent in these genomes. A VSP-II-like island was identified in the \textit{V. cholerae} FL Group isolates with varying levels of similarity and conservation with other homologous sequences in the \textit{Vibrionaceae} (Figure 9). This island was previously identified as GI-123, but was not well characterized \cite{41}. Interestingly, this island does not encode the canonical integrase of VSP-II but rather one that is similar to an integrase of a not yet described genomic island in \textit{V. cholerae} CP1033(6), a serogroup O1 strain isolated from a cholera patient in Mexico in 2000. This VSP-II-like island was not inserted at the tRNA-Met (adjacent to VC0517) where the canonical VSP-II is inserted, but rather at the locus homologous to VC0208 and VC0209, where GI-52, 68, 96, 98, 107 are inserted in other \textit{V. cholerae} strains \cite{12,41}. When compared to the prototypical VSP-II island in \textit{V. cholerae} N16961, the \textit{V. cholerae} FL Group encodes two regions with high similarity: VC0495 to VC0498 and VC0504 to VC0510. A novel region encoding four ORFs annotated as hypothetical protein, bacteriocin immunity protein, bacteriocin immunity protein, and hypothetical protein were inserted between the two regions that are similar to the prototypical VSP-II (Figures 9 and 10). One of these hypothetical proteins comprises

\textbf{Figure 6. Phylogenetic analysis of ORFs conserved among all T3SS-positive \textit{V. cholerae} and closely related species.} Neighbor-joining tree inferred from an alignment of 7 orthologous genes (VCHE25_2738, VCHE25_2741, VCHE25_2742, VCHE25_2744, VCHE25_2745, VCHE25_2749, VCHE25_2754) comprising ca. 5.2 kb. Bar length = 0.05 substitutions per site. doi:10.1371/journal.pone.0086264.g006

\textbf{Figure 7. Phylogenetic analysis of most closely related T3SS.} Neighbor-joining tree inferred from an alignment of 17 orthologous genes (VCHE25_2737 to VCHE25_2742, VCHE25_2745 to VCHE25_2752, and VCHE25_2754) comprising ca. 10.2 kb. Bar length = 0.02 substitutions per site. doi:10.1371/journal.pone.0086264.g007
Figure 8. Phylogenetic analysis of sialic acid metabolism region of VPI-2. Neighbor-joining tree inferred from an alignment of 7 orthologous genes (VC1781 to VC1774) comprising ca. 5.7 kb. Bar length = 0.05 substitutions per site. doi:10.1371/journal.pone.0086264.g008

Figure 9. Comparative genomic analysis of Vibrio Seventh pandemic island II-like island. Vibrio Seventh pandemic II-like island of the V. cholerae FL Group is the reference sequence in a BLAST alignment with homologs of other Vibrionaceae genomes. Colored squares show degree of similarity. doi:10.1371/journal.pone.0086264.g009
794 amino acids, with cytotoxic and S-type Pyocin domains, known toxins active against bacteria [42]. When compared to the NCBI nucleotide database, highest similarity is with an S-type Pyocin domain-containing protein (YP_004564713.1) of *V. anguillarum*, a marine fish pathogen. Two adjacent proteins are bacteriocin immunity proteins, with one 83 amino acids and the other 93 amino acids in length. Both have colicin immunity protein/pyocin immunity protein domains and are predicted by pSort to be in the cytoplasm of the *V. cholerae* [43]. In other species secreted pyocins are known to cause cell death among closely related strains [42]. The presence of a homologous genetic cluster in the *V. cholerae* FL Group may allow it to outcompete other *V. cholerae* strains present in the same local environment which may lead to an increased density of pyocin and pyocin immunity protein-encoding strains in a specific environment such as a single oyster bed. However, further research on pyocins in *V. cholerae* needs to be conducted to further elucidate their potential role in intra-species competition in the environment.

The VSP-II-like element in isolates of the *V. cholerae* FL Group has 12 ORFs with similarity to regions of the *V. coralliilyticus* ATCC BAA-450 and *V. anguillarum* 775 genomes, with percent nucleotide identity between the ORFs ranging from 69 to 99% (Figure 9). These data suggest the suite of VSP-II-like elements is distributed not only among clinical *V. cholerae* isolates, but also environmental isolates including non-cholera vibrios. Further, the presence of similar ORFs in non-pathogenic vibrios strongly indicates a function in the natural environment.
A phylogeny of conserved VSP-II ORFs infers these sequences of the *V. cholerae* FL Group to be closely related to *V. cholerae* TMA21 and significantly divergent from the *V. cholerae* 7th Pandemic strains (Figure 11). The subclade with which VSP-II of the *V. cholerae* FL Group clusters comprises environmental *V. cholerae* strains and *Vibrio* sp. RC341, a novel *Vibrio* species closely related

Figure 12. BLAST atlas of *V. cholerae* FL Group, *V. cholerae* V51, and *V. mimicus* MB-451 and *V. cholerae* N16961 genomes. *V. cholerae* N16961 is the reference genome. Genomic islands with the prefix “GI” are described by Chun et al. [12]. SI = superintegron.
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Figure 13. Proposed hypothetical insertions of genomic islands in the *V. cholerae* V51/*V. cholerae* FL Group clade.
doi:10.1371/journal.pone.0086264.g013
to *V. cholerae* and known to cause sporadic infections in humans \[15,44,45\]. *V. cholerae* V51 does not encode this element.

The presence of genomic islands comprising the *V. cholerae* mobilome described by Chun et al. (12) was evaluated using BLASTN and BLASTP. Including VPI-1 and 2 and a VSP-II-like element, the *V. cholerae* FL Group encoded sequences with high similarity to GIs-1, 2, 3, 4, 26, 37, 57, 58, and two genomic islands not yet described and designated here as FL-GI-1 and FL-GI-2 (Figure 12). All *V. cholerae* FL Group isolates lacked VSP-I genomic island and the site of insertion does not harbor any other genomic island. Figure 13 depicts a proposed arrangement of genomic island insertion and deletion in the *V. cholerae* V51/*V. cholerae* FL Group lineage before and after the two sets of isolates (*V. cholerae* V51 and *V. cholerae* FL Group isolates) would have diverged from a common ancestor.

**Lipopolysaccharide Coding Region**

This region of the *V. cholerae* FL Group is ca. 60.1 kb, with the LPS core region ca. 19.1 kb and *wlb* region ca. 41 kb. Of all *V. cholerae* serogroup data represented in NCBI GenBank, the core oligosaccharide (OS) and the O141-antigen-specific coding regions of *V. cholerae* V51 are most similar to the homologous ORFs of *V. cholerae* FL Group (Figures 14 and 15). Of 54 identified ORFs in this region of the *V. cholerae* FL Group, *V. cholerae* V51 shares 38 with at least 95% nucleotide sequence similarity. When the O-antigen ORFs of *V. cholerae* V51 and *V. cholerae* FL Group are compared the only observed structural differences are seven ORFs absent in the regions homologous to VCV51_0176 to VCV51_0185 in the *V. cholerae* FL Group and 11 ORFs in *V. cholerae* V51 absent in the homologous region (found between positions 98044 and 113059 in contig ref|NZ_AMWF01000009.1|). Eight ORFs were identified in the O75-antigen coding region of the *V. cholerae* FL Group isolates that have not yet been described in the O-antigen coding regions of other *V. cholerae* genomes, and these ORFs may be specific to the O75 antigen (Figures 14 and 15).

Although it is well known that this region is a hot-spot for gene transfer, it can be assumed that O141 and O75 O-antigen coding regions derived from a recent ancestral sequence based on the high level of conservation between the two, and that the difference between the two clusters arises from a substitution of ORFs specific to the O-antigen region. A similar mechanism has been suggested for the relationship between O139 and O22 serogroups [46,47]. This substitution may have involved a ca. 18.2 kb region in the genomes of *V. cholerae* FL Group isolates and a ca. 16.2 kb region in *V. cholerae* V51 flanked by homologs found at nucleotide positions 97166 to 98047 (glucose-1-phosphate thymidylyltransferase) and 116274 to 116825 (lipid carrier:UDP-N-acetylgalactosaminyltransferase). Alternatively, three substitution events involving shorter sequences may have occurred between the flanking regions, indicated by absent ORFs (red squares in Figure 15) in reciprocal comparison. Interestingly, the serogroup with the next highest level of conservation with serogroups O141 and O75 is the epidemic-associated O139 serogroup isolate *V. cholerae* MO10.
Phenotypic Analyses

The eight *V. cholerae* FL Group isolates were evaluated for hemolysis, motility, and proteolysis, following standard methods for testing these methods in *V. cholerae* [27]. Although not responsible for the rice water diarrhea characteristic of cholera, these virulence factors are associated with intestinal and extra-intestinal *V. cholerae* infections, as well as ecological functions in the aquatic environment [48,49,50,51]. All strains are motile, proteolytic, form biofilms and are hemolytic. However, strain CP1114 demonstrated weak or incomplete hemolysis. This isolate was also weakly proteolytic, compared to the other *V. cholerae* FL Group isolates, and incomplete hemolysis may be due to incomplete processing of hemolysin by the hemagglutinin/protease [52].

The *Caenorhabditis elegans* model of *V. cholerae* infection, which yields data on strength of hemolytic activity (*hlyA*) proved useful [29]. Nematodes were fed three isolates of *V. cholerae* FL Group (*V. cholerae* CP1112, CP1114, and CP1115). CP1115, which showed the largest zone of hemolysis on blood agar, was selected for testing. CP1114 demonstrated incomplete hemolysis and CP1112 showed a moderate zone of clearing when compared to the other isolates of the *V. cholerae* FL Group. The results demonstrated significantly more rapid lethality in nematodes fed the *V. cholerae* FL Group isolates than nematodes fed non-pathogenic *E. coli* as a control, but significantly slower lethality than nematodes fed *V. cholerae* El Tor strain E7946 (*P*<0.05) (Figure 16). It is concluded that all three of the *V. cholerae* FL Group isolates produced in similar *C. elegans* survival patterns. However, median survival time of worms fed isolates *V. cholerae* CP1112 and CP1115 was ca. nine days versus ca. eleven days for worms fed *V. cholerae* CP1114, the isolate with incomplete hemolysis, a consistent result based on previous observations. Interestingly, the three isolates caused a *C. elegans* die-off similar to *V. cholerae* O1 biotype Classical than to El Tor [29], not expected since *hlyA* of the *V. cholerae* FL Group does not have the same 11 bp deletion linked to the decreased hemolytic

**Figure 15. Comparative genomic analysis of LPS coding regions.** Reciprocal BLAST analysis of LPS coding region with *V. cholerae* FL Group as a reference. doi:10.1371/journal.pone.0086264.g015

**Figure 16. Survival curves of *C. elegans* challenged with *V. cholerae* CP1112, CP1114, CP1115, *V. cholerae* El Tor E7946, *Escherichia coli* OP50.** doi:10.1371/journal.pone.0086264.g016
activity of V. cholerae O1 Classical but higher nucleotide sequence similarity with V. cholerae O1 El Tor N16961 than Classical O395.

Based on BiOLOG phenotypic microarray assay, all strains utilized sialic acid three to six times greater than background demonstrating a functional sialic acid catabolism operon of the VPI-2. Almagro-Moreno and Boyd [10] reported the ability to utilize sialic acid confers a competitive advantage to strains encoding this region during infection of the sialic acid-rich environment of the gut. This is due to the ability of V. cholerae encoding a functional sialic acid metabolism region to utilize sialic acid as a carbon source [10]. All strains also utilized maltose, which was shown by Lång et al. [53] to be related to cholera toxin and toxin co-regulated pilus production and translocation across the V. cholerae outer membrane. Results of the study demonstrated that a functional maltose operon is needed for virulence of V. cholerae [53].

The BiOLOG profiles showed similar metabolic profiles among the V. cholerae FL Group strains (data not shown). However, both replicates of V. cholerae CP1110 utilized capric acid as carbon source while all other isolates generally did not, except isolate V. cholerae CP1117 which utilized this substrate in one replicate. Isolates CP1112, CP1113, and CP1116 weakly utilized capric acid in at least one replicate. Isolate V. cholerae CP1115 did not utilize β-methyl-D-glucoside while the other V. cholerae FL Group isolates did.

Conclusions

It is concluded the outbreak was caused by V. cholerae growing to a sufficiently high density in the environment (i.e., not in a single oyster) to cause multiple cases of cholera. Clonality of the isolates, including 67% of all reported cholera cases from this outbreak, demonstrates that there need not be a human vehicle of V. cholerae dispersal into a given geographical region prior to a cholera outbreak, as has been suggested for cholera epidemics. Further, it is concluded that genomic and phenotypic diversity exists among clinical isolates V. cholerae non-O1/non-O139 strains of the same outbreak, supporting a recommendation to investigate the

genomics of cholera epidemics at the population level. Large-scale genomic and molecular analyses accomplished for the cholera epidemics in Haiti and Bangladesh and the recent epidemics in Nigeria and Kenya have revealed distinct V. cholerae populations causing disease [6,7,34,53].

Because the V. cholerae FL Group isolates formed a monophyletic lineage with V. cholerae V51 serogroup O141 (a 1987 clinical isolate), we hypothesize the clade to represent a lineage of cholera-causing isolates, similar to those of the 7th pandemic clade. Although, diverged from recent 7th pandemic strains and older Classical and pre-7th pandemic strains, from an evolutionary perspective, the virulence factors known to be involved in cholera are present in the V. cholerae FL Group and V. cholerae V51. The difference in the constellation of mobile elements and incongruent phylogenies of some elements of V. cholerae V51 and the V. cholerae FL Group suggest that, although these two groups are similar, they have independently acquired various elements from the environment, with some islands globally distributed.

Although the majority of the research on V. cholerae focuses on the O1 serogroup because of the major epidemics associated with these strains, V. cholerae non-O1/non-O139 serogroup strains should be further evaluated for contribution to the global disease burden. V. cholerae serogroup O141 isolates have been shown by other investigators to globally cause significant disease and many encode ctxB [14,30,31,56] as do the V. cholerae FL Group serogroup O75 isolates. Pathogenic V. cholerae causing cholera outbreaks must be characterized in a phylogenomic context and their genomic island constellations as well. It is no longer sufficient to label these V. cholerae strains as serogroups O1, O139, or non-O1/non-O139, without further appropriate genomic analysis.

Author Contributions

Conceived and designed the experiments: BJH SYC NAH CJG HNC BDT NAH RRC. Performed the experiments: BJH SYC CJG HNC BDT NAH RRC. Contributed reagents/materials/analysis tools: TJO RB RH CB RRC. Contributed to the writing of the manuscript: BJH SYC CJG TAC AH RRC. Performed experiments: BJH SYC CJG HNC LC AC ET SNS. Analyzed the data: BJH SYC CJG NAH TAC AH RRC. Wrote the paper: BJH SYC TAC AH RRC.

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