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Genomic and Phenotypic Characterization of *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, *Vibrio cholerae* serogroup O75, was traced to oysters harvested from Apalachicola Bay, Florida. From the 11 diagnosed cases, eight isolates of *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 *V. cholerae* V51 serogroup O141, a clinical strain isolated 23 years earlier. Toxigenic *V. cholerae* O75 has been repeatedly isolated from clinical cases in the southeastern United States and toxigenic *V. cholerae* O141 isolates have been isolated globally from clinical cases over several decades. Comparative genomics, phenotypic analyses, and a *Caenorhabditis elegans* model of infection for the isolates were conducted. This analysis coupled with isolation data of *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

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 *V. cholerae* (hemolysins, lipases) or mobile pathogenicity islands (VPIs-1 and -2, and CTXΦ) that are frequently found in clinical isolates from cholera patients suffering severe rice water diarrhea [8,9,10]. Epidemic cholera is typically ascribed to *V. cholerae* serogroup O1 or O139; however, it is now understood that, similar to pathogenic *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 *V. cholerae* which acquire and express genes carried on mobile elements (O-antigens, VPI-1, VPI-2, CTXΦ, NAG-ST, etc.) are linked to epidemics of cholera. The scenario of mobile genetic element acquisition has been shown to have occurred within the 7th 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 cholerae	NCTC 8457	O1	El Tor	Saudi Arabia	Clinical	1910	NZ_AAWD01000000
Vibrio cholerae	M66-2	O1	El Tor	Makassar, Indonesia	Clinical	1937	NC_012578/NC_012580
Vibrio cholerae	MAK757	O1	El Tor	Sulawesi, Indonesia (Celebes Islands)	Clinical	1937	NZ_AAUS00000000
Vibrio cholerae	O395	O1	Classical	India	Clinical	1965	NC_009456/NC_009457
Vibrio cholerae	V52	O37		Sudan	Clinical	1968	NZ_AAKJ02000000
Vibrio cholerae	N16961	O1	El Tor	Bangladesh	Clinical	1975	NC_002505/NC_002506
Vibrio cholerae	E7946	O1	El Tor	Bahrain	Clinical	1978	Not Sequenced
Vibrio cholerae	2740-80	O1	El Tor	Gulf Coast, USA	Water	1980	NZ_AAUT01000000
Vibrio cholerae	TM 11079-80	O1	El Tor	Brazil	Sewage	1980	NZ_ACHW00000000
Vibrio cholerae	CT 5369-93	O1	El Tor	Brazil	Sewage	1980	NZ_ADAL00000000
Vibrio cholerae	TMA21	non-O1/non-O139		Brazil	Water	1982	NZ_ACHY00000000
Vibrio cholerae	12129(1)	O1	El Tor	Australia	Water	1985	NZ_ACFQ00000000
Vibrio cholerae	RC9	O1	El Tor	Kenya	Clinical	1985	NZ_ACHX00000000
Vibrio cholerae	BX 330286	O1	El Tor	Australia	Water	1986	NZ_ACIA00000000
Vibrio cholerae	V51	O141		USA	Clinical	1987	NZ_AAKI02000000
Vibrio cholerae	RC27	O1	Classical	Indonesia	Clinical	1991	NZ_ADAI00000000
Vibrio cholerae	INDRE 91/1	O1	El Tor	Mexico	Clinical	1991	NZ_ADAK00000000
Vibrio cholerae	C6706	O1	El Tor	Peru	Clinical	1991	NZ_AHGQ00000000
Vibrio cholerae	CP1032(5)	O1	El Tor	Mexico	Clinical	1991	NZ_ALDA00000000
Vibrio cholerae	MO10	O139		Madras,India	Clinical	1992	NZ_AAKF00000000
Vibrio cholerae	Amazonia	O1	Amazonia	Amazonas, Brazil	Clinical	1992	NZ_AFSV00000000
Vibrio cholerae	MJ-1236	O1	El Tor	Matlab, Bangladesh	Clinical	1994	NC_012668/NC_012667
Vibrio cholerae	IEC224	O1	El Tor	Belém, Brazil	Clinical	1994	NC_016944/NC_016945
Vibrio cholerae	1587	O12		Lima, Peru	Clinical	1994	NZ_AAUR01000000
Vibrio cholerae	RC385	O135		Chesapeake Bay, USA	Plankton	1998	NZ_AAKH02000000
Vibrio cholerae	CP1033(6)	O1	El Tor	Mexico	Clinical	2000	NZ_AJRL00000000
Vibrio cholerae	AM-19226	O39		Bangladesh	Clinical	2001	NZ_AATY01000000
Vibrio cholerae	MZO-3	O37		Bangladesh	Clinical	2001	NZ_AAUU01000000
Vibrio cholerae	MZO-2	O14		Bangladesh	Clinical	2001	NZ_AAWF01000000
Vibrio cholerae	623-39	non-O1/non-O139		Bangladesh	Water	2002	NZ_AAWG00000000
Vibrio cholerae	CIRS101	O1	altered El Tor	Dhaka, Bangladesh	Clinical	2002	NZ_ACVW00000000
Vibrio cholerae	CP1038	O1	El Tor	Zimbabwe	Clinical	2003	NZ_ALDC00000000
Vibrio cholerae	B33	O1	El Tor	Beira, Mozambique	Clinical	2004	NZ_ACHZ00000000
Vibrio cholerae	CP1040(13)	O1	El Tor	Zambia	Clinical	2004	NZ_ALDD00000000

Table 1. Cont.

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	LMA3984-4	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	2008	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_AHFY00000000
Vibrio cholerae	2009V-1096	O1	El Tor	India	Clinical	2009	NZ_AHFZ00000000
Vibrio cholerae	2009V-1116	O1	El Tor	Pakistan	Clinical	2009	NZ_AHGA00000000
Vibrio cholerae	2009V-1131	O1	El Tor	India	Clinical	2009	NZ_AHGB00000000
Vibrio cholerae	2010V-1014	O1	El Tor	Pakistan	Clinical	2009	NZ_AHGG00000000
Vibrio cholerae	2011EL-1137	O1	El Tor	Zimbabwe	Clinical	2009	NZ_AHGJ00000000
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	NC_016445/NC_016446
Vibrio cholerae	EL1798	O1	El Tor	Haiti	Clinical	2010	NZ_AELI00000000
Vibrio cholerae	EL1792	O1	El Tor	Haiti	Clinical	2010	NZ_AELJ00000000
Vibrio cholerae	HE-09	non-O1/non-O139		Haiti	Environmental	2010	NZ_AFOP00000000
Vibrio cholerae	HE-39	non-O1/non-O139		Haiti	Environmental	2010	NZ_AFOQ00000000
Vibrio cholerae	HE-48	non-O1/non-O139		Haiti	Environmental	2010	NZ_AFOR00000000
Vibrio cholerae	HC-02A1	non-O1/non-O139		Haiti	Clinical	2010	NZ_AFOT00000000
Vibrio cholerae	HC-21A1	O1	El Tor	Saint-Marc, Haiti	Clinical	2010	NZ_AGUK00000000
Vibrio cholerae	HC-22A1	O1	El Tor	Saint-Marc, Haiti	Clinical	2010	NZ_AGUL00000000
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_AGUY00000000
Vibrio cholerae	HC-80A1	O1	El Tor	Port-au-Prince, Haiti	Clinical	2010	NZ_AGVB00000000
Vibrio cholerae	2010EL-1961	O1	El Tor	Haiti	Clinical	2010	NZ_AHGD00000000
Vibrio cholerae	2010EL-2010H	O1	El Tor	Haiti	Clinical	2010	NZ_AHGE00000000
Vibrio cholerae	2010EL-2010N	O1	El Tor	Haiti	Clinical	2010	NZ_AHGF00000000
Vibrio cholerae	2011EL-1089	O1	El Tor	Haiti	Clinical	2010	NZ_AHGH00000000
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_AJRX00000000

Table 1. Cont.

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_AJSK00000000
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_ALDO00000000
Vibrio cholerae	HC-47A1	non-O1/non-O139		Haiti	Clinical	2010	NZ_ALDR00000000
Vibrio cholerae	HE-16	non-O1/non-O139		Haiti	Gray Water	2010	NZ_ALEB00000000
Vibrio cholerae	HE-25	non-O1/non-O139		Haiti	Gray Water	2010	NZ_ALEC00000000
Vibrio cholerae	HE-45	non-O1/non-O139		Haiti	Gray Water	2010	NZ_ALED00000000
Vibrio cholerae	CP1042(15)	O1	El Tor	Thailand	Clinical	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	Clinical	2010	NZ_AHGC00000000
Vibrio cholerae	2011EL-1133	O1	El Tor	Haiti	Clinical	2011	NZ_AHGI00000000
Vibrio cholerae	2011V-1021	O1	El Tor	Dominican Republic	Clinical	2011	NZ_AHGK00000000
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_AMWV00000000
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_ANNM00000000
Vibrio cholerae	CP1117	O75		Florida Gulf Coast, USA	Clinical	2010–2011	NZ_AMWW00000000
Vibrio cholerae	VL426	non-O1/non-O139	albensis	Maidstone, Kent, UK	Water		NZ_ACHV00000000
Vibrio anguillarum	96F	O1		Chesapeake Bay, USA	Striped bass (*Morone saxatilis*)		NZ_AEZA00000000
Vibrio anguillarum	RV22	O2β		Atlantic coast of Spain	Turbot (*Scophthalmus maximus*)		NZ_AEZB00000000
Vibrio anguillarum	775	O1		coast of Washington state, USA	Coho salmon (*Oncorhynchus kisutch*)		NC_015633/NC_015637
Vibrio coralliilyticus	ATCC BAA-450			Zanzibar, Tanzania	Coral	1999	NZ_ACZN00000000
Vibrio mimicus	SX-4			China	Clinical	2009	NZ_ADOO00000000
Vibrio mimicus	VM573			United States	Clinical	1990s	NZ_ACYV00000000
Vibrio mimicus	MB-451			Matlab, Bangladesh	Clinical		NZ_ADAF00000000
Vibrio orientalis	CIP 102891			Yellow sea, China	Water		NZ_ACZV00000000

Table 1. Cont.

Organism	Strain ID	Serogroup/Serotype	Biotype	Geographical origin	Source of isolation	Year of isolation	Accession nos.
Vibrio parahaemolyticus	RIMD 2210633	O3:K6		Japan	Clinical	1996	NC_004603/NC_004605
Vibrio splendidus	12B01			Plum Island Estuary, Massachusetts, USA	Water		NZ_AAMR00000000
Vibrio vulnificus	YJ016		biotype 1	Taiwan	Clinical		NC_005139/NC_005140
Vibrio sp. RC341	RC341	O153		Chesapeake Bay, USA	Water	1998	NZ_ACZT00000000
Grimontia hollisae	CIP 101886			Maryland, USA	Clinical		NZ_ADAQ00000000

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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 vibrioses 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 (*rstR* alleles, *nanH*, and *ctxB* biotype) were done using the methods of Choi et al. [24], Vora et al. [25], and Nusrin et al. [26]. Phenotypic assays (proteolysis, hemolysis, biofilm formation, and motility) were conducted following methods standardized for *V. cholerae* [27]. Hemolysis, biofilm formation, motility, and proteolysis 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

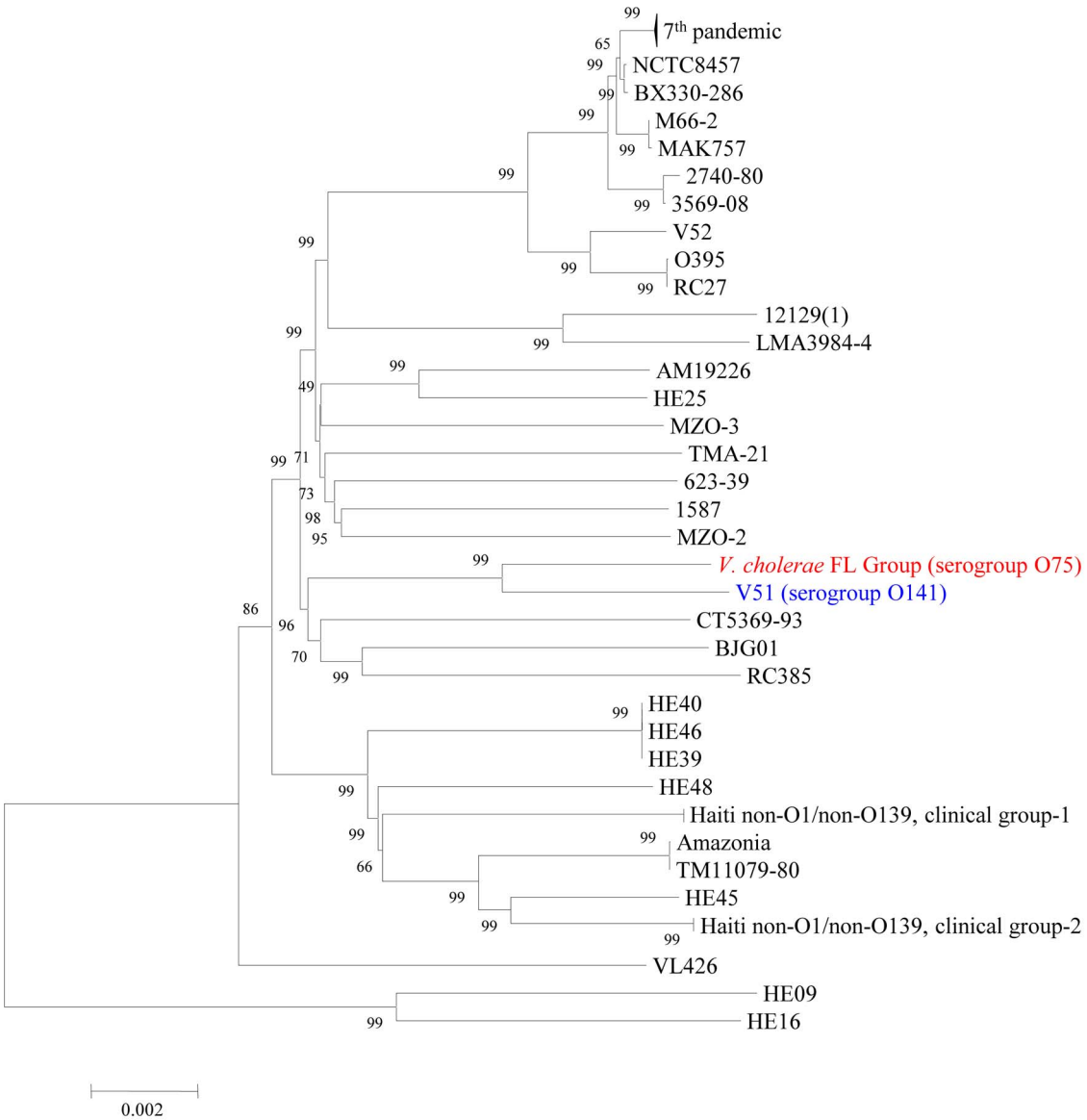


Figure 1. Neighbour-joining tree inferring phylogenetic relationships of 84 *V. cholerae* genomes based on 995 orthologous protein-coding genes (954,646 bp). *V. cholerae* FL Group is labelled in red and *V. cholerae* V51 is labelled in blue. Haiti non-O1/non-O139 clinical groups-1 and -2 are further defined by Hasan et al. [6]. Numbers at nodes represent bootstrap values. Nucleotide substitution model is the Kimura-2-parameter. Bar length = 0.002 nucleotide substitutions per site.
doi:10.1371/journal.pone.0086264.g001

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.

N16961 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	C	C	T	C	C	C	hypothetical protein
VC0028	C	C	C	C	C	C	T	C	Dihydroxy-acid dehydratase
VC0031	C	C	C	C	C	C	C	A	Acetolactate synthase large subunit
VC1359	T	T	T	T	C	C	T	T	ABC-type polar amino acid transport system ATPase component
GI-26*	G	G	T	G	G	G	G	G	putative transcriptional activator ToxR
	A	A	T	A	A	A	A	A	
VCA1063	G	G	G	G	T	T	G	G	Ornithine decarboxylase

* = Not found in *V. cholerae* N16961.
doi:10.1371/journal.pone.0086264.t002

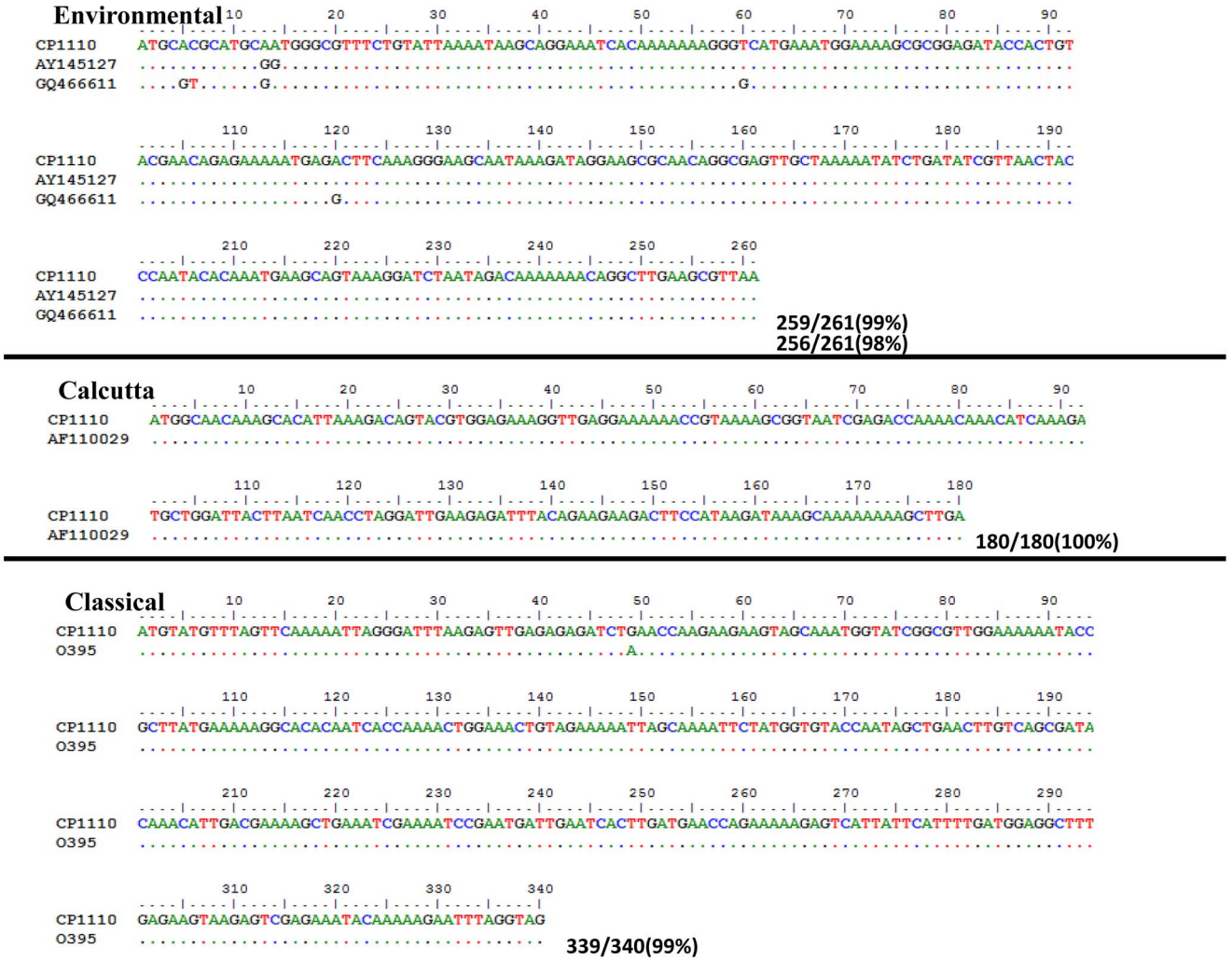


Figure 2. *rstR* sequences from *V. cholerae* CP1110. Sequences are aligned to their most similar homologs extracted from NCBI Genbank database. Nulceotide sequence identity is shown to the right of the last nucleotide aligned for each allele.
doi:10.1371/journal.pone.0086264.g002

Annotation	*V. cholerae* CP1110 Locus	*V. cholerae* N16961 Locus	Strand	Length	*V. cholerae* V51	*V. cholerae* N16961	*V. cholerae* O395	*V. cholerae* 3569-08	*V. mimicus* SX-4	*V. mimicus* VM573
Aldehyde dehydrogenase	1489...3009 A	VC0819	-	1521						
inner membrane protein putative	3995...7315	VC0822	+	3321						
inner membrane protein putative	7333...8586	VC0822	+	1254						
Putative zinc metalloprotease	8583...9521	VC0823	+	939						
Thiol peroxidase	9630...10124	VC0824	+	495						
TcpI	10423...12285	VC0825	-	1863						
TcpP	12724...13389	VC0826	+	666						
TcpH	13482...13784	VC0827	+	303						
TcpA	14325...14999	VC0828	+	675						
TcpB	15080...16372	VC0829	+	1293						
TcpQ	16444...16824	VC0830	+	381						
TcpC	16827...18296	VC0831	+	1470						
TcpR	18466...18744	VC0832	+	279						
TcpD	18747...19568	VC0833	+	822						
TcpS	19584...20042	VC0834	+	459						
TcpT	20032...21543	VC0835	+	1512						
TcpE	21683...22540	VC0836	+	858						
TcpF	22550...23566	VC0837	+	1017						
ToxT	23773...24603	VC0838	+	831						
TCP pilin signal peptidase	24603...25367	VC0839	+	765						
AcfB	25369...27249	VC0840	+	1881						
AcfC	27264...28025	VC0841	+	762						
TagE protein	28523...29431	VC0843	+	909						
AcfA	29434...30081	VC0844	-	648						
AcfD precursor	30060...34832	VC0845	+	4773						

A = location in sequence: ref|NZ_AMWF01000022.1|

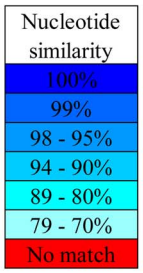


Figure 3. Comparative genomic analysis of *Vibrio* pathogenicity island 1 (VPI-1). VPI-1 of the *V. cholerae* FL Group is the reference sequence in a BLAST alignment with homologs of other *Vibrionaceae* genomes. Colored squares show degree of similarity.
doi:10.1371/journal.pone.0086264.g003

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

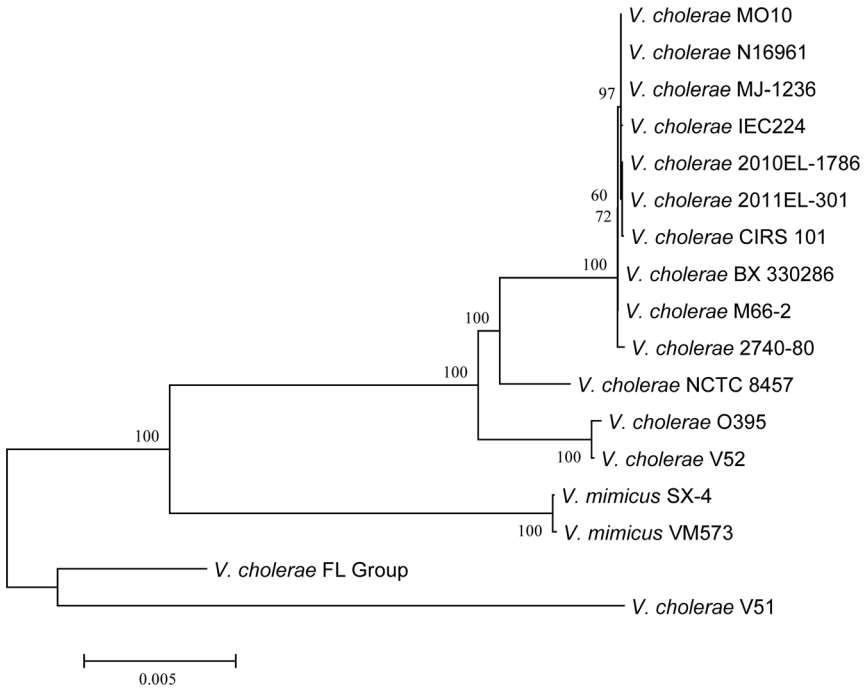


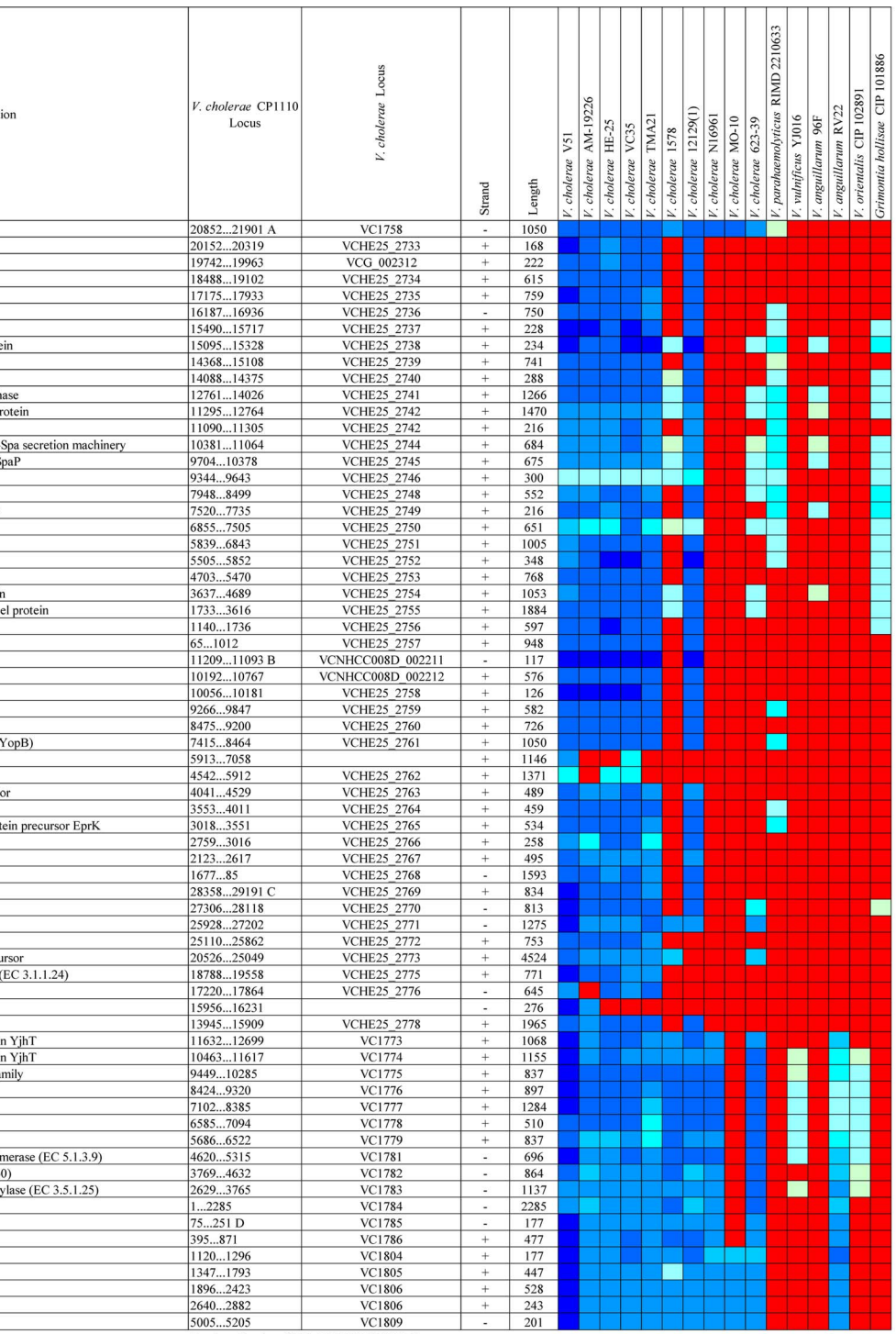
Figure 4. Phylogenetic analysis of *Vibrio* pathogenicity island 1 (VPI-1). Neighbor-joining tree showing evolutionary relationships of VPI-1. The calculation was based on aligned fragments of 25 orthologous genes (VC0819 to VC0845) comprising ca. 26.9 kb. Bar length = 0.005 substitutions per site.
doi:10.1371/journal.pone.0086264.g004

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_A1060 and VC0395_A1067 of *V. cholerae* O395) of the CTX phage (*ctxB*, *ctxA*, *zot*, *ace*, *orfU*,*cep*, *rstB*, *rstA*, and $rstR^{Classical}$). To infer the biotype of the cholera toxin, PCR targeting the *ctxB* gene was employed and resulted in an amplicon for primers of targeting $ctxB^{Classical}$. These PCR results are consistent with profiles of other clinically isolated *V. cholerae* strains on a global scale that suggest this cholera toxin biotype is the predominant biotype currently causing the majority of disease [34,35]. Based on the genome sequence data, the CTX phage of the *V. cholerae* FL Group genomes were lacking the *rstR* gene of *V. cholerae* N16961 El Tor (VC1464), but did encode the *rstR* gene homologous to the one encoded in *V. cholerae* O395 Classical. To further investigate and confirm these *in silico* results, PCR targeting the *rstR* region was done and resulted in amplicons for the Calcutta, Environmental, and Classical biotypes, but not the El Tor biotype, an as-to-date uncommon combination. The *rstR* amplicons of CP1110 were subjected to Sanger sequencing and the resulting sequences were compared by BLASTN to the NCBI Genbank database for better interpretation of these results and each showed ≥99% nucleotide sequence similarity to Calcutta, Environmental, and Classical sequences (Figure 2). These amplicon sequences were compared with *V. cholerae* CP1110 reads by BLASTN to re-confirm their presence in the genome sequences. The *rstR* sequences from the *V. cholerae* FL Group were confirmed as Calcutta, Environmental, and Classical biotypes (Figure 2). The prototypical *V. cholerae* O1 El Tor strains encode $rstR^{El\ Tor}$ and $ctxB^{El\ Tor}$ while Classical strains encode $rstR^{Classical}$ and $ctxB^{Classical}$. Altered *V. cholerae* O1 El Tor strains which differ from prototypical El Tor strains in their *rstR*/*ctxB* types have recently been identified [24]. Data from this study further 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 CTXΦ 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

Annotation	*V. cholerae* CP1110 Locus	*V. cholerae* Locus	Strand	Length
Phage integrase	20852...21901 A	VC1758	-	1050
ISSod132C transposase	20152...20319	VCHE25_2733	+	168
Accessory colonization factor AcfA	19742...19963	VCG_002312	+	222
Hypothetical protein	18488...19102	VCHE25_2734	+	615
Hypothetical protein	17175...17933	VCHE25_2735	+	759
putative transcriptional regulator ToxR	16187...16936	VCHE25_2736	-	750
Hypothetical protein	15490...15717	VCHE25_2737	+	228
putative type III secretion apparatus protein	15095...15328	VCHE25_2738	+	234
Hypothetical protein	14368...15108	VCHE25_2739	+	741
Hypothetical protein	14088...14375	VCHE25_2740	+	288
Type III secretion cytoplasmic ATP synthase	12761...14026	VCHE25_2741	+	1266
putative type III secretion system EscC protein	11295...12764	VCHE25_2742	+	1470
Hypothetical protein	11090...11305	VCHE25_2742	+	216
putative Spa292C component of the Mxi-Spa secretion machinery	10381...11064	VCHE25_2744	+	684
Surface presentation of antigens protein SpaP	9704...10378	VCHE25_2745	+	675
Hypothetical protein	9344...9643	VCHE25_2746	+	300
putative transcriptional activator ToxR	7948...8499	VCHE25_2748	+	552
putative Type III secretion protein Spa33	7520...7735	VCHE25_2749	+	216
Hypothetical protein	6855...7505	VCHE25_2750	+	651
Hypothetical protein	5839...6843	VCHE25_2751	+	1005
Hypothetical protein	5505...5852	VCHE25_2752	+	348
putative outer membrane protein	4703...5470	VCHE25_2753	+	768
Type III secretion inner membrane protein	3637...4689	VCHE25_2754	+	1053
Type III secretion inner membrane channel protein	1733...3616	VCHE25_2755	+	1884
Hypothetical protein	1140...1736	VCHE25_2756	+	597
Lipoprotein VsaC	65...1012	VCHE25_2757	+	948
Hypothetical protein	11209...11093 B	VCNHCC008D_002211	-	117
putative dimethyladenosine transferase	10192...10767	VCNHCC008D_002212	+	576
Hypothetical protein	10056...10181	VCHE25_2758	+	126
Hypothetical protein	9266...9847	VCHE25_2759	+	582
Hypothetical protein	8475...9200	VCHE25_2760	+	726
Type III secretion host injection protein (YopB)	7415...8464	VCHE25_2761	+	1050
putative chaperone	5913...7058		+	1146
putative chaperone	4542...5912	VCHE25_2762	+	1371
putative two-component response regulator	4041...4529	VCHE25_2763	+	489
Hypothetical protein	3553...4011	VCHE25_2764	+	459
putative type III secretion system lipoprotein precursor EprK	3018...3551	VCHE25_2765	+	534
Hypothetical protein	2759...3016	VCHE25_2766	+	258
Hypothetical protein	2123...2617	VCHE25_2767	+	495
Hypothetical protein	1677...85	VCHE25_2768	-	1593
Hypothetical protein	28358...29191 C	VCHE25_2769	+	834
Hypothetical protein	27306...28118	VCHE25_2770	-	813
Hypothetical protein	25928...27202	VCHE25_2771	-	1275
FG-GAP/YD repeat domain protein	25110...25862	VCHE25_2772	+	753
Accessory colonization factor AcfD precursor	20526...25049	VCHE25_2773	+	4524
Beta-ketoadipate enol-lactone hydrolase (EC 3.1.1.24)	18788...19558	VCHE25_2775	+	771
Hypothetical protein	17220...17864	VCHE25_2776	-	645
Hypothetical protein	15956...16231		-	276
Surface protein Lk90-like protein	13945...15909	VCHE25_2778	+	1965
Sialic acid-induced transmembrane protein YjhT	11632...12699	VC1773	+	1068
Sialic acid-induced transmembrane protein YjhT	10463...11617	VC1774	+	1155
Sialic acid utilization regulator2C RpiR family	9449...10285	VC1775	+	837
N-acetylneuraminate lyase (EC 4.1.3.3)	8424...9320	VC1776	+	897
TRAP-type transport system	7102...8385	VC1777	+	1284
TRAP-type transport system	6585...7094	VC1778	+	510
TRAP-type transport system	5686...6522	VC1779	+	837
N-acetylmannosamine-6-phosphate 2-epimerase (EC 5.1.3.9)	4620...5315	VC1781	-	696
N-acetylmannosamine kinase (EC 2.7.1.60)	3769...4632	VC1782	-	864
N-acetylglucosamine-6-phosphate deacetylase (EC 3.5.1.25)	2629...3765	VC1783	-	1137
Neuraminidase (sialidase)	1...2285	VC1784	-	2285
Predicted transcriptional regulator	75...251 D	VC1785	-	177
DNA repair protein RadC	395...871	VC1786	+	477
Hypothetical protein	1120...1296	VC1804	+	177
Hypothetical protein	1347...1793	VC1805	+	447
Hypothetical protein	1896...2423	VC1806	+	528
Hypothetical protein	2640...2882	VC1806	+	243
Transcriptional regulator AlpA like	5005...5205	VC1809	-	201



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B = Location in ref|NZ_AMWF01000130.1|
C = Location in ref|NZ_AMWF01000173.1|
D = Location in ref|NZ_AMWF01000168.1|

Figure 5. Comparative genomic analysis of *Vibrio* pathogenicity island 2 (VPI-2). VPI-2 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.g005

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].

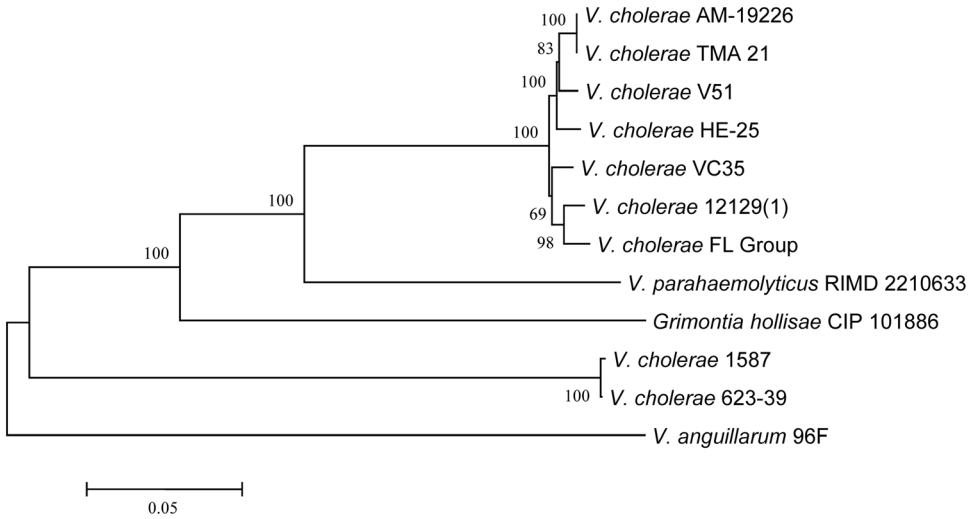

Figure 6. Phylogenetic analysis of ORFs conserved among all T3SS-positive *V. cholerae* and closely related species. Neighbor-joining tree inferred from an alignment of 7 orthologous genes (VCHE25_2738, VCHE25_2741, VCHE25_2742, VCHE25_2744, VCHE25_2745, VCHE25_2749, VCHE25_2754) comprising ca. 5.2 kb.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

VPI-2 of the *V. cholerae* FL Group also encodes a complete sialic acid catabolism operon (homologs of VC1773 to VC1784 in the canonical VPI-2 of *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 [10]. A phylogeny of the sialic acid metabolism region demonstrated this operon in the *V. cholerae* FL Group is closely related to that of *V. cholerae* V51, *V. cholerae* 1587, and *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 *V. cholerae* FL Group and *V. cholerae* V51 than that of the T3SS. Further, when the phylogenies of T3SS and sialic acid metabolism operons of seven *V. cholerae* strains with homologous ORFs are inferred (*V. cholerae* strains AM-19226, TMA 21, HE-25, V51, 12129(1), FL Group, and VC35), sister taxa of the *V. cholerae* FL Group T3SS remains *V. cholerae* VC35 and sister taxa of the sialic acid metabolism region of the *V. cholerae* FL Group remains *V. cholerae* V51 (data not shown). These data suggest the two regions in the *V. cholerae* FL Group originated from different sources. Morita et al. [40] demonstrated these two regions of VPI-2 could be of separate origin and the insertion locus of *V. cholerae* T3SS is exclusively in VPI-2. Interestingly, the 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 *V. cholerae* FL Group isolates with varying levels of similarity and conservation with other homologous sequences in the *Vibrionaceae* (Figure 9). This island was previously identified as GI-123, but was not well characterized [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 *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 GIs-32, 52, 68, 96, 98, 107 are inserted in other *V. cholerae* strains [12,41]. When compared to the prototypical VSP-II island in *V. cholerae* N16961, the *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

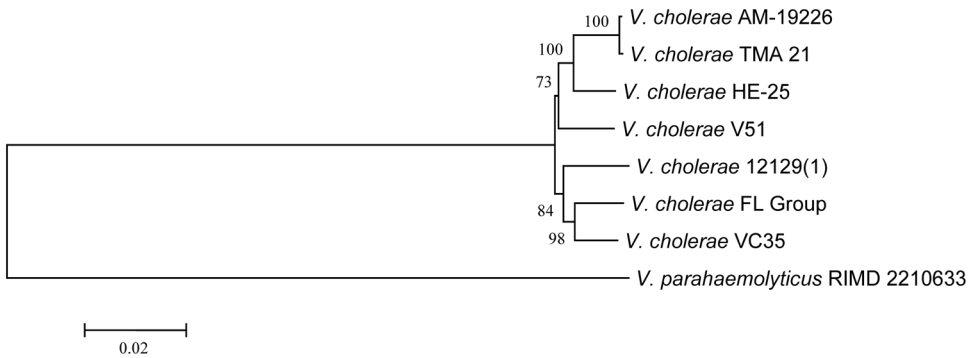

Figure 7. Phylogenetic analysis of most closely related T3SS. Neighbor-joining tree inferred from an alignment of 17 orthologous genes (VCHE25_2737 to VCHE25_2742, VCHE25_2745 to VCHE25_2752, and VCHE25_2754) comprising ca. 10.2 kb. Bar length = 0.02 substitutions per site.
doi:10.1371/journal.pone.0086264.g007

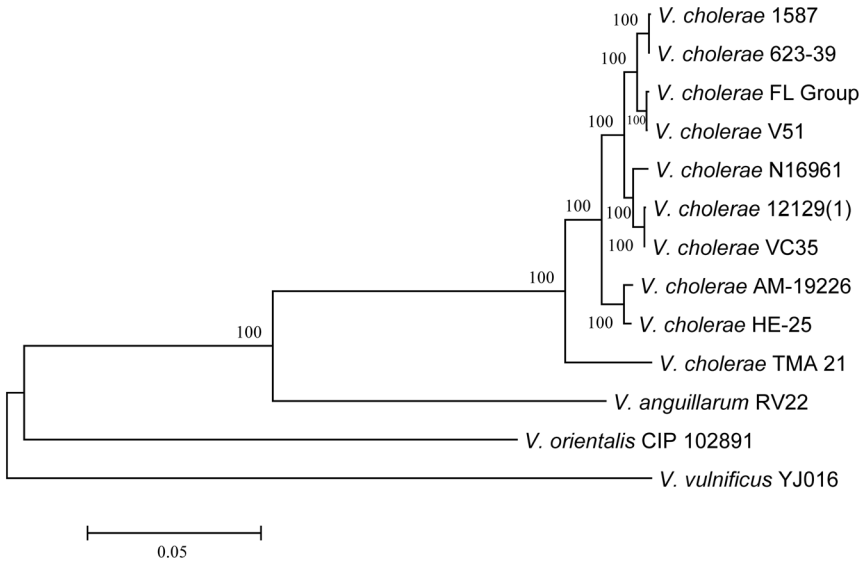

Figure 8. Phylogenetic analysis of sialic acid metabolism region of VPI-2. Neighbor-joining tree inferred from an alignment of 7 orthologous genes (VC1781 to VC1774) comprising ca. 5.7 kb. Bar length = 0.05 substitutions per site.
doi:10.1371/journal.pone.0086264.g008

Annotation	*V. cholerae* CP1110 Locus	*V. cholerae* N16961 Locus	Strand	Length	*V. cholerae* V51	*V. cholerae* N16961	*V. cholerae* TMA21	*V. cholerae* CIRS101	*V. cholerae* MZO3	*V. cholerae* RC385	*V. coralliilyticus* ATCC BAA-450	*V. anguillarum* 775
Hypothetical protein	35012...35896 A		-	885								
Hypothetical protein	36449...37129		+	681								
Hypothetical protein	37089...37865	VC0495	+	777								
Hypothetical protein	38024...38518	VC0496	+	495								
Transcriptional regulator	38656...38787	VC0497	+	132								
Ribonuclease HI2C *Vibrio* paralog	38869...39309	VC0498	+	441								
Hypothetical protein	39519...41903		+	2385								
bacteriocin immunity protein	42203...42454		+	252								
bacteriocin immunity protein	42984...43265		+	282								
Hypothetical protein	43294...43515		+	222								
Hypothetical protein	44974...45201	VC0504	-	228								
Hypothetical protein	45249...45617	VC0505	-	369								
Hypothetical protein	45648...46382	VC0506	-	735								
Hypothetical protein	46521...46646	VC0507	-	126								
Hypothetical protein	46701...47144	VC0508	-	444								
Hypothetical protein	47195...47632	VC0509	-	438								
DNA repair protein RadC	47629...48102	VC0510	-	474								
Hypothetical protein	48326...49903		+	1578								
Modification methylase DdeI	50420...51904		-	1485								
Integrase	52430...53650		-	1221								

A = Location in sequence: ref|NZ_AMWF01000009.1|

Nucleotide similarity
100%
99%
98 - 95%
94 - 90%
89 - 80%
79 - 70%
< 70%
No match

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

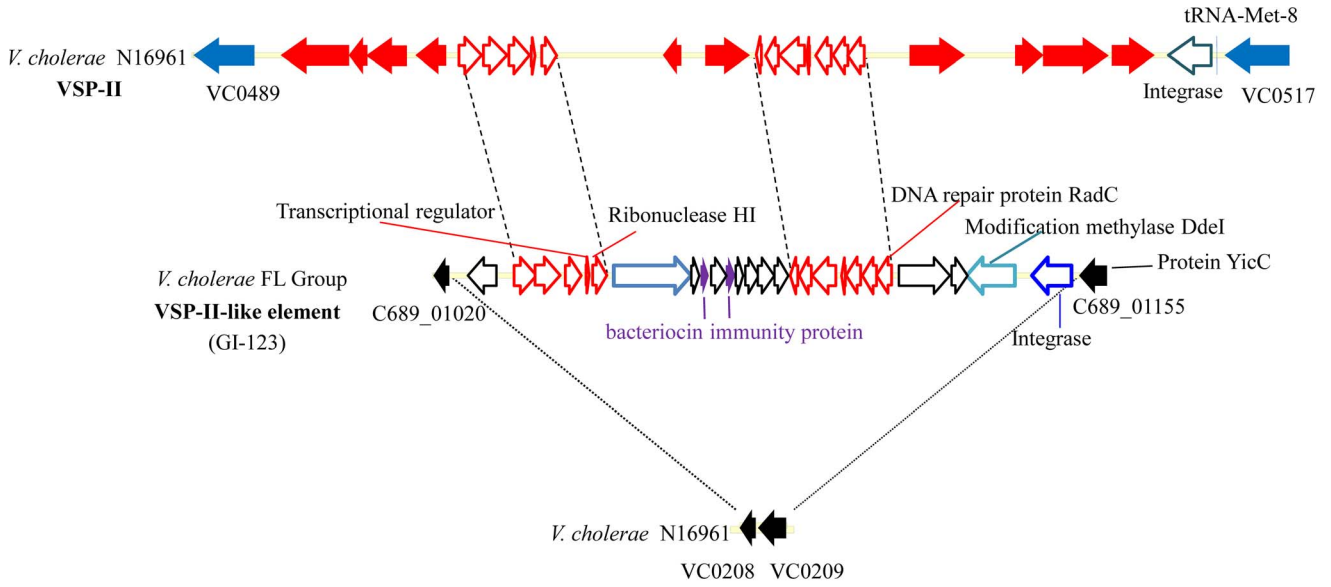

Figure 10. Structure of the canonical *Vibrio* Seventh Pandemic island II (VSP-II) and the VSP-II-like element of the *V. cholerae* FL Group. Structure of the canonical VSP-II element of *V. cholerae* N16961 (top), the VSP-II-like element found in the *V. cholerae* FL Group (middle) based on the annotation in NCBI Genbank, and the homologous locus of insertion of this VSP-II-like element in *V. cholerae* N16961 (bottom). Sequences homologous to VC0208 and VC0209 in *V. cholerae* V51 are found on contig NZ_DS179740 at positions 34343 to 34831 and 34897 to 35763. ORFs conserved between the two elements are outlined in red.
doi:10.1371/journal.pone.0086264.g010

794 amino acids, with cytoxic 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.

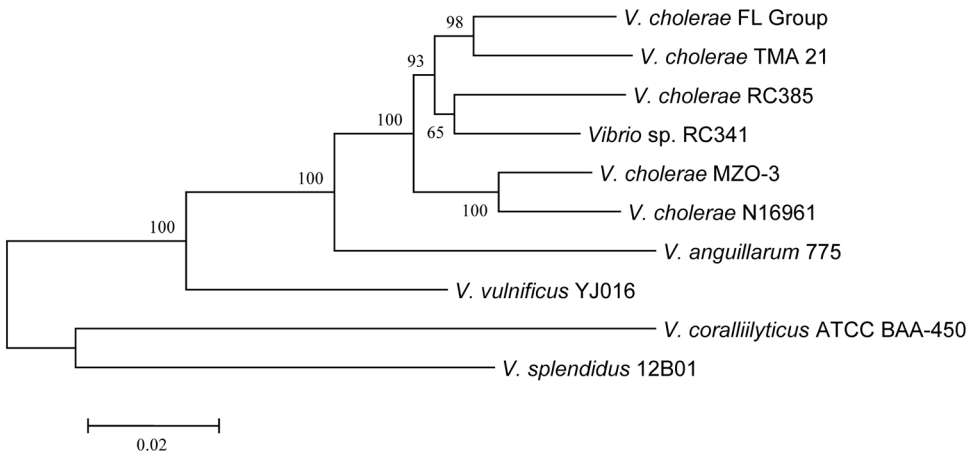

Figure 11. Phylogenetic analysis of *Vibrio* Seventh pandemic island II-like island. Neighbor-joining tree inferred from an alignment 8 orthologous ORFs (VC0495, VC0496, VC0504 to VC0506, VC0508 to VC0510) comprising ca. 3.7 kb. Bar represents 0.02 substitutions per site.
doi:10.1371/journal.pone.0086264.g011

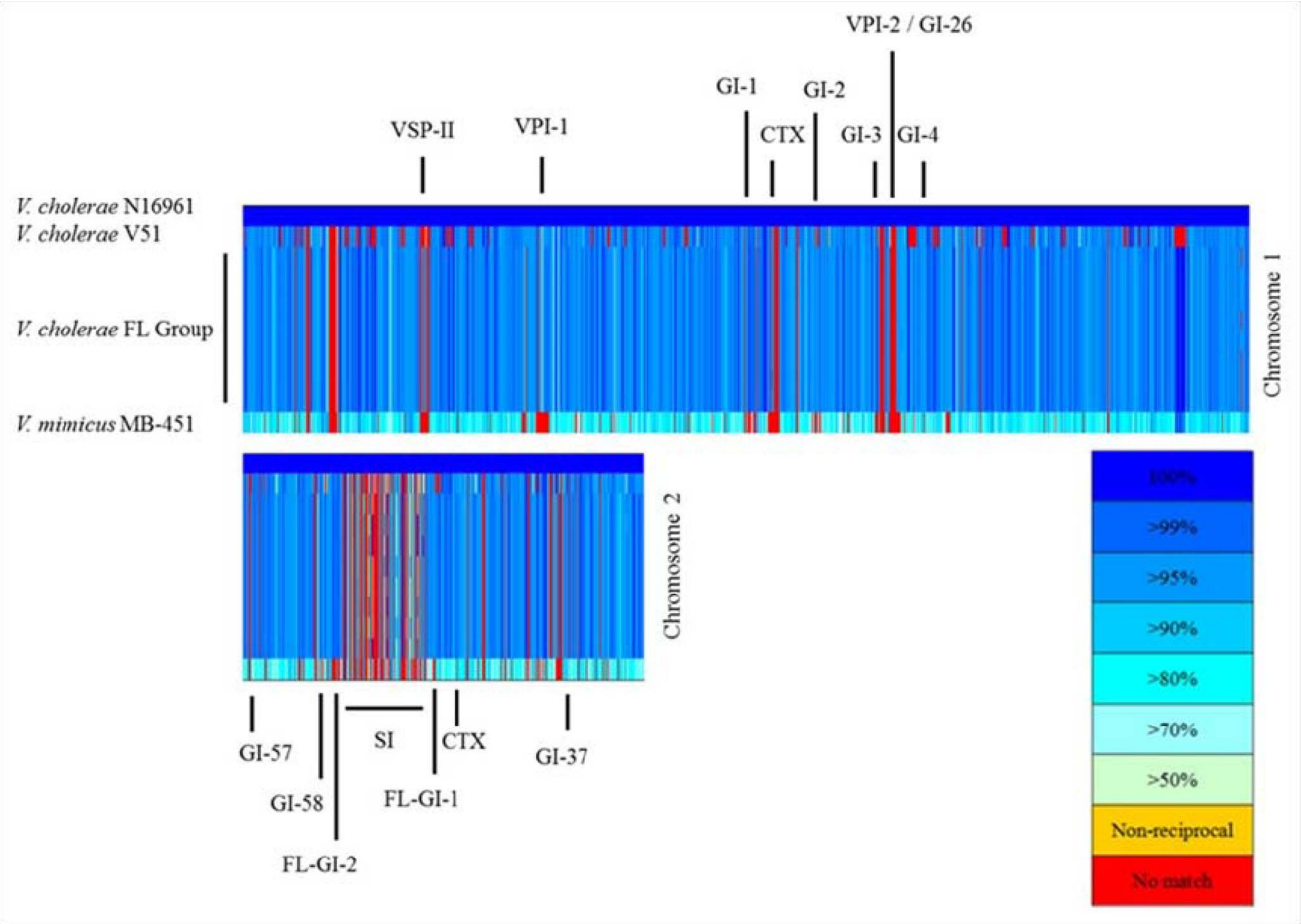

Figure 12. BLAST atlas of *V. cholerae* FL Group, *V. cholerae* V51, and *V. mimicus* MB-451 and *V. cholerae* N16961 genomes. *V. cholerae* N16961 is the reference genome. Genomic islands with the prefix "GI" are described by Chun et al. [12]. SI = superintegron.
doi:10.1371/journal.pone.0086264.g012

A phylogeny of conserved VSP-II ORFs infers these sequences of the *V. cholerae* FL Group to be closely related to *V. cholerae* TMA 21 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

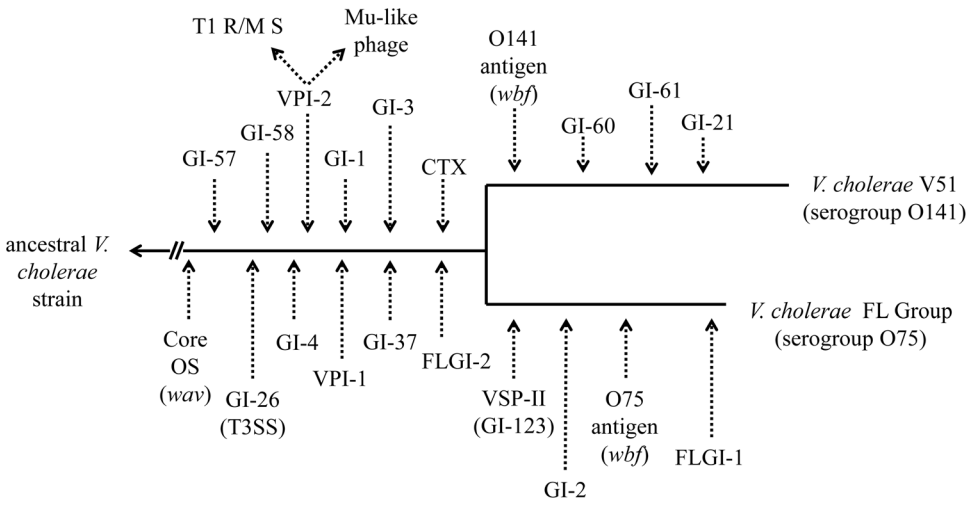

Figure 13. Proposed hypothetical insertions of genomic islands in the *V. cholerae* V51/*V. cholerae* FL Group clade.
doi:10.1371/journal.pone.0086264.g013

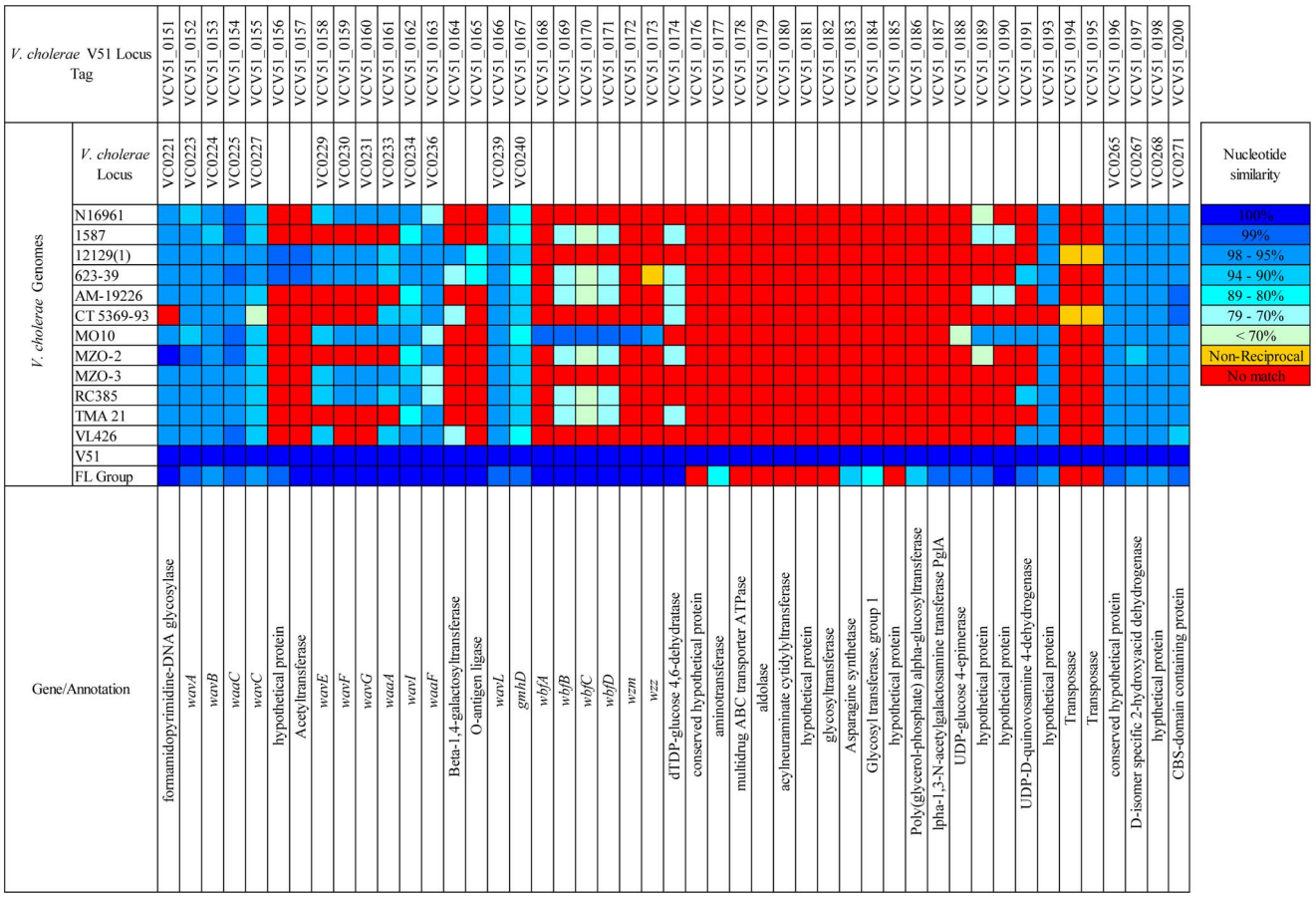

Figure 14. Comparative genomic analysis of LPS coding regions. Reciprocal BLAST analysis of LPS coding region with *V. cholerae* V51 as a reference.
doi:10.1371/journal.pone.0086264.g014

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 *wb** 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 the *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.

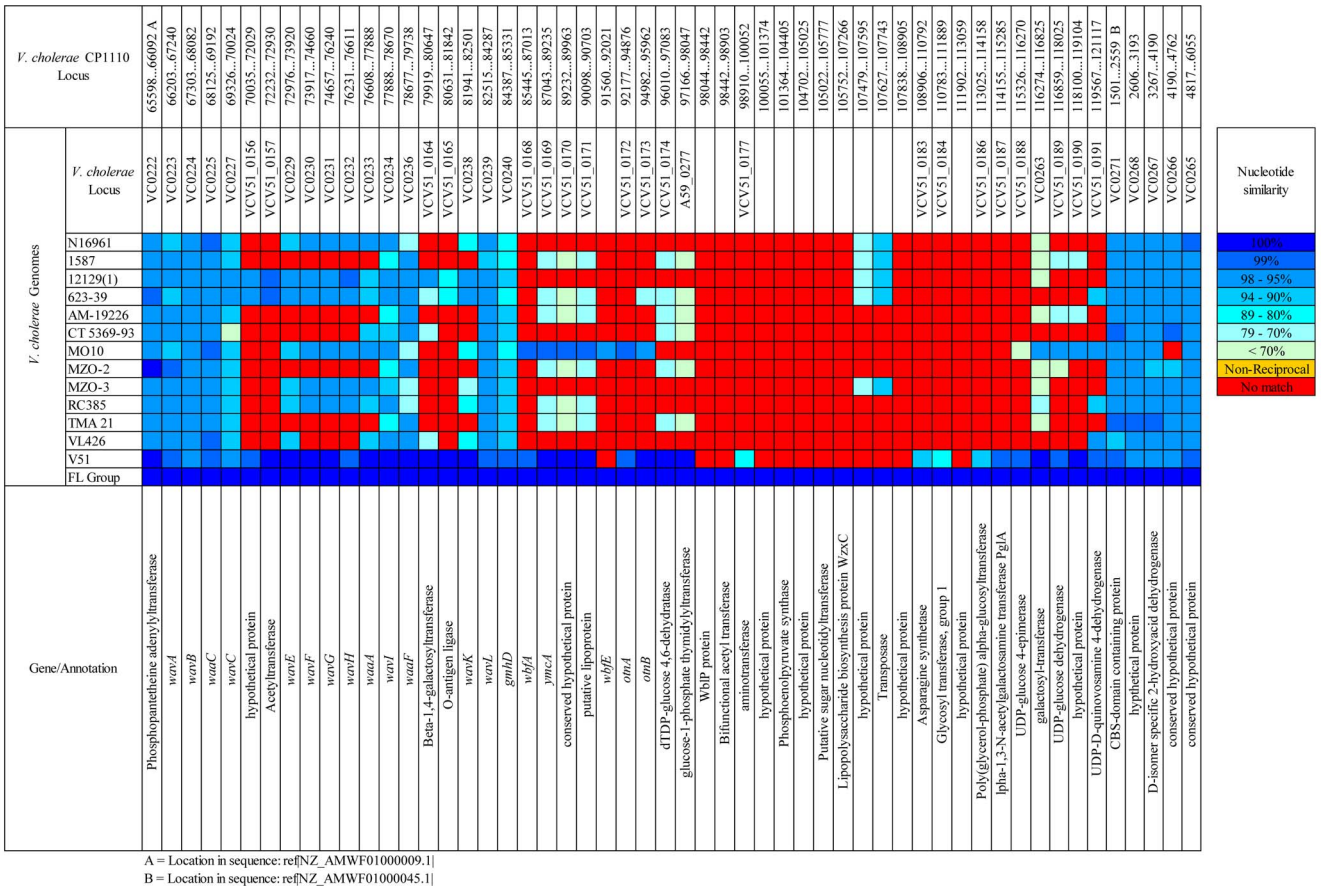

Figure 15. Comparative genomic analysis of LPS coding regions. Reciprocal BLAST analysis of LPS coding region with *V. cholerae* FL Group as a reference.
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Phenotypic Analyses

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].

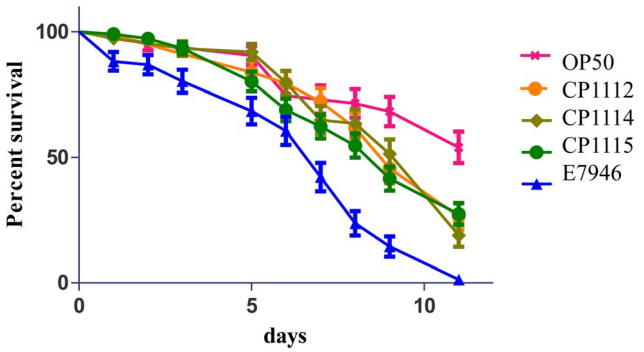

Figure 16. Survival curves of *C. elegans* challenged with *V. cholerae* CP1112, CP1114, CP1115, *V. cholerae* El Tor E7946, *Escherichia coli* OP50.
doi:10.1371/journal.pone.0086264.g016

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, 1114, and 1115). 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

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 caproic 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 caproic 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 [6,7,54,55].

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 $ctxB^{\text{Classical}}$ [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 AHA. Performed the experiments: BJH SYC CJG HNC LC AC ET SNS MHK AHA. Analyzed the data: BJH SYC CJG HNC BDT NAH RRC. Contributed reagents/materials/analysis tools: TJO RB RH CB RRC. Wrote the paper: BJH SYC TAC AH RRC.

References

1. Ko WC, Chuang YC, Huang GC, Hsu SY (1998) Infections due to non-O1 *Vibrio cholerae* in southern Taiwan: predominance in cirrhotic patients. Clin Infect Dis 27: 774–780.
2. Safrin S, Morris JG Jr, Adams M, Pons V, Jacobs R, et al. (1988) Non-O:1 *Vibrio cholerae* bacteremia: case report and review. Rev Infect Dis 10: 1012–1017.
3. Shannon JD, Kimbrough RC (2006) Pulmonary Cholera Due to Infection with a Non-O1 *Vibrio cholerae* Strain. J Clin Microbiol 44: 3459–3460.
4. Lukinmaa S, Mattila K, Lehtinenc V, Hakkinen M, Koskela M, et al. (2006) Territorial waters of the Baltic Sea as a source of infections caused by *Vibrio cholerae* non-O1, non-O139: report of 3 hospitalized cases. Diagn Micr Infec Dis 54: 1–6.
5. Chatterjee S, Ghosh K, Raychoudhuri A, Chowdhury G, Bhattacharya MK, et al. (2009) Incidence, virulence factors, and clonality among clinical strains of non-O1, non-O139 *Vibrio cholerae* isolates from hospitalized diarrheal patients in Kolkata, India. J Clin Microbiol 47: 1087–1095.
6. Hasan NA, Choi SY, Eppinger M, Clark PW, Chen A, et al. (2012) Genomic diversity of 2010 Haitian cholera outbreak strains. Proc Natl Acad Sci USA 109(29): E2010–E2017.
7. Marin MA, Thompson CC, Freitas FS, Fonseca EL, Aboderin AO, et al. (2013) Cholera Outbreaks in Nigeria Are Associated with Multidrug Resistant Atypical El Tor and Non-O1/Non-O139 *Vibrio cholerae*. PLoS Negl Trop Dis 7(2): e2049.
8. Tacket C, Taylor R, Losonsky G, Lim Y, Nataro J, et al. (1998) Investigation of the Roles of Toxin-Coregulated Pili and Mannose-Sensitive Hemagglutinin Pili in the Pathogenesis of *Vibrio cholerae* O139 Infection. Infect Immun 66(2): 692–695.
9. Vanden Broeck D, Horvath C, De Wolf M (2007) *Vibrio cholerae*: Cholera toxin. Int J Biochem Cell Biol 39(10) 1771–1775.
10. Almagro-Moreno S, Boyd EF (2009) Sialic Acid Catabolism Confers a Competitive Advantage to Pathogenic *Vibrio cholerae* in the Mouse Intestine. Infect Immun 77(9): 3807–3816.
11. Manning S, Motiwala A, Springman A, Qi W, Lacher D, et al. (2008) Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. Proc Natl Acad Sci USA (12): 4868–4873.
12. Chun J, Grim CJ, Hasan NA, Lee JH, Choi SY, et al. (2009) Comparative Genomics Reveals Mechanism for Short-term and Long-term Clonal Transitions in Pandemic *Vibrio cholerae*. Proc Natl Acad Sci USA 106: 15442–15447.
13. Meibom K, Li X, Nielsen A, Wu C, Roseman S, et al. (2004) The *Vibrio cholerae* chitin utilization program. Proc Natl Acad Sci USA 101(8):2524–2529.
14. Udden S, Zahid M, Biswas K, Ahmad Q, Cravioto A, et al. (2008) Acquisition of classical CTX prophage from *Vibrio cholerae* O141 by El Tor strains aided by lytic phages and chitin-induced competence. Proc Natl Acad Sci USA 105(33)11951–11956.
15. Boucher Y, Cordero OX, Takemura A, Hunt DE, Schliep K, et al. (2011). Local mobile gene pools rapidly cross species boundaries to create endemicity within global *Vibrio cholerae* populations. mBio 2(2):e00335–10.
16. Hlady W, Klontz K (1996) The Epidemiology of *Vibrio* Infections in Florida, 1981–1993. J Infect Dis 173(5): 1176–1183.
17. Shapiro R, Altekruse S, Hutwagner L, Bishop R, Hammond R, et al. (1998) The Role of Gulf Coast Oysters Harvested in Warmer Months in *Vibrio vulnificus* Infections in the United States, 1988–1996. J Infect Dis 178(3):752–759.
18. Tamplin M (2001) Coastal Vibrios: Identifying Relationships between Environmental Condition and Human Disease. Human and Ecological Risk Assessment: An International Journal 7(5): 1437–1445.
19. Lipp E, Huq A, Colwell R (2002) Effects of Global Climate on Infectious Disease: the Cholera Model. Clin Microbiol Rev 15(4) 757–770.
20. Huq A, Sack R, Nizam S, Longini I, Nair G, et al. (2005) Critical Factors Influencing the Occurrence of *Vibrio cholerae* in the Environment of Bangladesh. Appl Environ Microbiol 71(8): 4645–4654.
21. Tobin-D'Angelo M, Smith A, Bulens S, Thomas S, Hodel M, et al. (2008) Severe Diarrhea Caused by Cholera Toxin–Producing *Vibrio cholerae* Serogroup

O75 Infections Acquired in the Southeastern United States. Clin Infect Dis 47(8): 1035–1040.
22. Onifade T, Hutchinson R, Van Zile K, Bodager D, Baker R, et al. (2011) Toxin producing *Vibrio cholerae* O75 outbreak, United States, March to April 2011. Euro Surveill 16(20):19870.
23. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, et al. (2008) The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9:75.
24. Choi SY, Lee JH, Kim EJ, Lee HR, Jeon Y, et al. (2010) Classical RS1 and environmental RS1 elements in *Vibrio cholerae* O1 El Tor strains harbouring a tandem repeat of CTX prophage: revisiting Mozambique in 2005. J Med Microbiol 59: 302–308.
25. Vora G, Meador M, Bird M, Bopp C, Andreadis J, et al. (2005) Microarray-based detection of genetic heterogeneity, antimicrobial resistance, and the viable but nonculturable state in human pathogenic *Vibrio* spp. Proc Natl Acad Sci USA 102(52): 19109–19114.
26. Nusrin S, Khan GY, Bhuiyan NA, Ansaruzzaman M, Hossain MA, et al. (2004) Diverse CTX phages among toxigenic *Vibrio cholerae* O1 and O139 strains isolated between 1994 and 2002 in an area where cholera is endemic in Bangladesh. J Clin Microbiol 42:5854–5856.
27. Son MS, Taylor RK (2011) Genetic Screens and Biochemical Assays to Characterize *Vibrio cholerae* O1 Biotypes: Classical and El Tor. Curr Protoc Microbiol 22:6A.2.1–6A.2.17.
28. Sprando R, Olejnik N, Cinar H, Ferguson M (2009) A method to rank order water soluble compounds according to their toxicity using *Caenorhabditis elegans*, a Complex Object Parametric Analyzer and Sorter, and axenic liquid media. Food and Chemical Toxicology 47(4): 722–728.
29. Cinar HN, Kothary M, Datta AR, Tall BD, Sprando R, et al. (2010) *Vibrio cholerae* Hemolysin Is Required for Lethality, Developmental Delay, and Intestinal Vacuolation in *Caenorhabditis elegans*. PLoS ONE 5(7): e11558.
30. Dalsgaard A, Serichantalergs O, Forslund A, Lin W, Mekalanos J, et al. (2001) Clinical and Environmental Isolates of *Vibrio cholerae* Serogroup O141 Carry the CTX Phage and the Genes Encoding the Toxin-Coregulated Pili. J Clin Microbiol 39(11) 4086–4092.
31. Crump JA, Bopp CA, Greene KD, Kubota KA, Middendorf RL, et al. (2003) Toxigenic *Vibrio cholerae* serogroup O141-associated cholera-like diarrhea and bloodstream infection in the United States. J Infect Dis 187(5): 866–8.
32. Blake PA, Allegra DT, Snyder JD, Barrett TJ, McFarland L, et al. (1980) Cholera–a possible endemic focus in the United States. N Engl J Med 302(6): 305–309.
33. Lin FY, Morris JG Jr, Kaper JB, Gross T, Michalski J, et al. (1986) Persistence of cholera in the United States: isolation of *Vibrio cholerae* O1 from a patient with diarrhea in Maryland. J Clin Microbiol 23(3):624–6.
34. Nair GB, Qadri F, Holmgren J, Svennerholm AM, Safa A, et al. (2006) Cholera due to altered El Tor strains of *Vibrio cholerae* O1 in Bangladesh. J Clin Microbiol 44:4211–3.
35. Safa A, Sultana J, Cam PD, Mwansa JC, Kong RYC (2008) Classical cholera toxin producing *Vibrio cholerae* O1 hybrid El Tor strains in Asia and Africa. Emerg Infect Dis 14:987–8.
36. Waldor MK, Mekalanos JJ (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 272(5270):1910–4.
37. Reguera G, Kolter R (2005) Virulence and the environment: a novel role for *Vibrio cholerae* toxin-coregulated pili in biofilm formation on chitin. J Bacteriol 187(10):3551–5.
38. Murphy RA, Boyd EF (2008) Three pathogenicity islands of *Vibrio cholerae* can excise from the chromosome and form circular intermediates. J Bacteriol 190(2):636–47.
39. Shin OS, Tam VC, Suzuki M, Ritchie JM, Bronson RT, et al. (2011) Type III secretion is essential for the rapidly fatal diarrheal disease caused by non-O1, non-O139 *Vibrio cholerae*. mBio 2(3):e00106–11.
40. Morita M, Yamamoto S, Hiyoshi H, Kodama T, Okura M, et al. (2013) Horizontal gene transfer of a genetic island encoding a type III secretion system distributed in *Vibrio cholerae*. Microbiol Immunol 57(5):334–9.
41. Choi SY, Hasan NA, Chun J, Hoq M, Huq A, et al. (2011) Comparative Genomic MGE finding process streamlines mobile element search. ASM Biodefense and Emerging Pathogens Research Meeting. Washington, DC.
42. Michel-Briand Y, Baysse C (2002) The pyocins of *Pseudomonas aeruginosa*. Biochimie 84(5–6):499–510.
43. Nakai K, Horton P (1999). PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci 24(1) 34–35.
44. Haley BJ, Grim CJ, Hasan NA, Choi SY, Chun J, et al. (2010) Comparative genomic analysis reveals evidence of two novel Vibrio species closely related to *V. cholerae*. BMC Microbiol 10:154.
45. Taviani E, Grim CJ, Choi J, Chun J, Haley B, et al. (2010) Discovery of novel *Vibrio cholerae* VSP-II genomic islands using comparative genomic analysis. FEMS Microbiol Lett 308(2):130–7.
46. Dumontier S, Berche P (1998) *Vibrio cholerae* O22 might be a putative source of exogenous DNA resulting in the emergence of the new strain of *Vibrio cholerae* O139. FEMS Microbiol Lett 164(1) 91–98.
47. Yamasaki S, Shimizu T, Hoshino K, Ho S, Shimada T, et al. (1999) The genes responsible for O-antigen synthesis of *Vibrio cholerae* O139 are closely related to those of *Vibrio cholerae* O22. Gene 237(2)321–332.
48. Halpern M, Gancz H, Broza M, Kashi Y (2003) *Vibrio cholerae* hemagglutinin/protease degrades chironomid egg masses. Appl Environ Microbiol 69(7):4200–4204.
49. Krukonis ES, DiRita VJ (2003) From motility to virulence: sensing and responding to environmental signals in *Vibrio cholerae*. Curr Opin Microbiol 6:186–190.
50. Silva AJ, Leitch GJ, Camilli A, Benitez JA (2006) Contribution of Hemagglutinin/Protease and Motility to the Pathogenesis of El Tor Biotype Cholera. Infect Immun 74(4): 2072–2079.
51. Shinoda S (2011) Proteases Produced by *Vibrio cholerae* and Other Pathogenic Vibrios: Pathogenic Roles and Expression. In Epidemiological and Molecular Aspects on Cholera, Infectious Disease. Ramamurthy, T., Bhattacharya, S.K. (eds.) New York, NY: Springer Science, pp. 245–258.
52. Hall RH, Drasar BS (1990) *Vibrio cholerae* HlyA hemolysin is processed by proteolysis. Infect Immun 58(10): 3375–9.
53. Lång H, Jonson G, Holmgren J, Palva ET (1994) The maltose regulon of *Vibrio cholerae* affects production and secretion of virulence factors. Infect Immun 62(11): 4781–8.
54. Ghosh R, Nair GB, Tang L, Morris JG, Sharma NC, et al. (2008), Epidemiological study of *Vibrio cholerae* using variable number of tandem repeats. FEMS Microbiol Lett 288: 196–201.
55. Mohamed AA, Oundo J, Kariuki SM, Boga HI, Sharif SK, et al. (2012) Molecular epidemiology of geographically dispersed *Vibrio cholerae* O1, Kenya, January 2009–May 2010. Emerg Infect Dis 18(6): 925–931.
56. Octavia S, Salim A, Kurniawan J, Lam C, Leung Q, et al. (2013) Population Structure and Evolution of Non-O1/Non-O139 *Vibrio cholerae* by Multilocus Sequence Typing. PLoS ONE 8(6): e65342.