RESEARCH ARTICLE

Hybrid *Vibrio cholerae* El Tor Lacking SXT Identified as the Cause of a Cholera Outbreak in the Philippines

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ABSTRACT **Cholera continues to be a global threat, with high rates of morbidity and mortality. In 2011, a cholera outbreak occurred in Palawan, Philippines, affecting more than 500 people, and 20 individuals died. *Vibrio cholerae* O1 was confirmed as the etiological agent. Source attribution is critical in cholera outbreaks for proper management of the disease, as well as to control spread. In this study, three *V. cholerae* O1 isolates from a Philippines cholera outbreak were sequenced and their genomes analyzed to determine phylogenetic relatedness to *V. cholerae* O1 isolates from recent outbreaks of cholera elsewhere. The Philippines *V. cholerae* O1 isolates were determined to be *V. cholerae* O1 hybrid El Tor belonging to the seventh-pandemic clade. They clustered tightly, forming a monophyletic clade closely related to *V. cholerae* O1 hybrid El Tor from Asia and Africa. The isolates possess a unique multilocus variable-number tandem repeat analysis (MLVA) genotype (12-7-9-18-25 and 12-7-10-14-21) and lack SXT. In addition, they possess a novel 15-kb genomic island (GI-119) containing a predicted type I restriction-modification system. The CTXΦ-RS1 array of the Philippines isolates was similar to that of *V. cholerae* O1 MG116926, a hybrid El Tor strain isolated in Bangladesh in 1991. Overall, the data indicate that the Philippines *V. cholerae* O1 isolates are unique, differing from recent *V. cholerae* O1 isolates from Asia, Africa, and Haiti. Furthermore, the results of this study support the hypothesis that the Philippines isolates of *V. cholerae* O1 are indigenous and exist locally in the aquatic ecosystem of the Philippines.**

IMPORTANCE **Genetic characterization and phylogenomics analysis of outbreak strains have proven to be critical for probing clonal relatedness to strains isolated in different geographical regions and over time. Recently, extensive genetic analyses of *V. cholerae* O1 strains isolated in different countries have been done. However, genome sequences of *V. cholerae* O1 isolates from the Philippines have not been available for epidemiological investigation. In this study, molecular typing and phylogenetic analysis of *Vibrio cholerae* isolated from both clinical and environmental samples in 2011 confirmed unique genetic features of the Philippines isolates, which are helpful to understand the global epidemiology of cholera.**

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Cholera is a life-threatening diarrheal disease caused by *Vibrio cholerae*, a bacterium autochthonous to the aquatic environment. The disease is endemic in many developing countries of Asia, Africa, and South America. Furthermore, cholera poses a serious health risk for those residents of countries where cholera is endemic and also non-endemic countries where the public health infrastructure is compromised (1). The recent cholera epidemic in Haiti has drawn the attention of epidemiologists interested in identifying the origin and transmission of *V. cholerae*, since cholera had been reported to have been absent in that region previous to the current epidemic (2–4). Asiatic cholera has been reported to be endemic in the Ganges delta of Bangladesh and India, whereas the occurrence of cholera in countries where it is not endemic is usually attributed to imported cases, i.e., travel-associated dissemination of the bacteria. Molecular typing, genomic analysis, and epidemiological data enable identification of a probable source of an organism causing a given outbreak.

Traditionally, *V. cholerae* isolates have been classified serologically, based on the somatic O antigen, with >200 serogroups identified to date (5). However, only *V. cholerae* serogroups O1

and O139 have been linked to cholera pandemics. *V. cholerae* serogroup O1 has two biotypes, El Tor and classical, each showing biotype-specific phenotypic and genetic traits. Allelic variation is evident between classical and El Tor biotypes for genes encoding the major protein subunit of toxin coregulated pilus (*tcpA*), cholera toxin subunit B (*ctxB*), regulatory region for phage lysogeny (*rstR*), and hemolysin (*hlyA*). Moreover, the classical biotype lacks *Vibrio* seventh-pandemic islands (VSP-I and -II) and RS1 satellite phage (5, 6).

Historically, *V. cholerae* O1 is linked to seven distinct pandemics, of which the sixth, and presumably earlier ones, was caused by the classical biotype, while the ongoing seventh pandemic, which started in 1961 in Indonesia, is attributed to the El Tor biotype (1). Recently, genetic analysis of an archival intestinal specimen of a victim who died of cholera in 1849 during an outbreak in Philadelphia confirmed association of the classical biotype with the second cholera pandemic (7). Over the past two decades, *V. cholerae* O1 El Tor strains have undergone substantial genetic change, and new variants, including altered El Tor, with the potential to cause a more severe cholera have emerged (6, 8, 9). El Tor cholera occurred in the Philippines during late September 1961, with large outbreaks in several different provinces (10). In the following years, until 1969, cholera occurred frequently in the Philippines during the rainy season (11). During the last decade, outbreaks of cholera in the Philippines have occurred immediately after water-related disasters, i.e., floods and typhoons. Although cholera is not considered endemic to the Philippines, sudden increases in cholera cases were reported at times when sanitation and hygienic practices were disturbed due to a natural calamity. Recently, extensive genetic analyses of *V. cholerae* O1 strains isolated in different geographical locations were done, but molecular analysis of *V. cholerae* O1 isolates from the Philippines has not yet been accomplished.

In April 2011, a diarrheal outbreak occurred in Palawan, Philippines, and an epidemiological investigation determined *V. cholerae* to be the causative agent. In the study reported here, *V. cholerae* O1 clinical and environmental isolates from the region of the Philippines affected by cholera were subjected to serological, bacteriological, and whole-genome sequencing to determine the source of the outbreak. Comparative genomics was done to determine the phylogenetic relationship of these isolates with *V. cholerae* O1 strains currently circulating in different regions of the world.

RESULTS AND DISCUSSION

MLVA. Multilocus variable-number tandem repeat analysis (MLVA) was used to discriminate *V. cholerae* isolates from various geographic locations and distinct populations within a single geographic cluster (12, 13). MLVA of the Philippines isolates revealed two genotypes: 12-7-9-18-25 and 12-7-10-14-21. Genotype 12-7-9-18-25 comprised the environmental isolate (PhRBD_VcEnv) and one of the clinical isolates (PhRBD_Vc311), while the other clinical isolate (PhRBD_Vc326) displayed the 12-7-10-14-21 genotype. The MLVA genotypes of the Philippines isolates did not match previously published MLVA genotypes of *V. cholerae* from different countries (3, 12, 14–16). However, MLVA is suitable mainly for outbreak investigations and spatiotemporal analysis of *V. cholerae* strains because of the relatively higher rate of mutation of the small-chromosome (Chr II) loci, which encompass the last two loci in the MLVA nomenclature. If only the three stable MLVA loci of the large chromosome (Chr I) are considered and the two variable loci of the small chromosome are ignored, the genotypes 12-7-9-X-X and 12-7-10-X-X continue to be unique for *V. cholerae* O1, compared with the profiles of *V. cholerae* O1 isolates from other countries. Interestingly, genotype 12-7-9-X-X matched that of a *V. cholerae* O139 strain isolated in 1992 in India. Overall, the MLVA genotypes of the Philippines isolates suggest that a distinct subpopulation of indigenous *V. cholerae* O1 most likely caused the outbreak of interest in this study.

Toxin gene cluster analysis. Pathogenicity of *V. cholerae* has been associated with production of cholera toxin, an enterotoxin encoded by genes carried by lysogenic CTXΦ. CTXΦ consists of two components, a "core" region (with cholera toxin subunit genes *ctxA* and *ctxB* and phage morphogenesis proteins) and an RS2 cluster (containing *rstA*, *rstB*, and *rstR*). Satellite phage RS1 is often found in toxigenic *V. cholerae* carrying an additional *rstC* gene and the entire RS2. The location of CTX prophage and its orientation can vary among strains. Therefore, it can be used as an indicator of both relatedness and evolution of *V. cholerae* strains. PCR assays, using a combination of phage-related and chromosome-specific primers, were performed to determine the presence of phage elements and RS1-CTX organization of the Philippines *V. cholerae* genomes. The three isolates were found to carry the *rstC* gene, indicating the presence of an RS1 element. In addition, further analysis by PCR confirmed the RS1 element to be present on Chr I (Fig. 1) and CTXΦ on Chr II (Fig. 1). The RS1 element contained *V. cholerae* El Tor biotype $rstR^{ET}$, but CTXΦ possessed *V. cholerae* classical-biotype *ctxB* and *rstR*, suggesting hybrid El Tor. Moreover, the Philippines isolates carried two copies of the RS1 element and TLC (toxin-linked cryptic plasmid) on Chr I and two copies of CTXΦ on Chr II. A similar RS1-CTXΦ array was identified previously in *V. cholerae* O1 MG116926 isolated in Bangladesh during 1991 (12, 17). Similar *V. cholerae* hybrid El Tor isolates have been associated with cholera in Bangladesh, India, Thailand, Vietnam, and Mozambique (17–20).

Shotgun genome sequence data complemented the RS1-CTX finding for the Philippines isolates, confirming a CTXΦ composition that included *rstA*, *rstB*, *cep*, *orfU*, *ace*, *zot*, *ctxA*, and *ctxB*. The deduced amino acid sequences of *ctxB* showed histidine at position 39 and threonine at position 68, identical to classical *ctxB*, while *rstA* contained partial polymorphisms of classical and El Tor hybrid *rstA* (Table 1). Four copies of heptamer repeats (TTTT GAT) were observed in the promoter-binding region of *ctxAB* (between *zot* and *ctxA*) in the Philippines *V. cholerae* genomes. These heptamer repeats (TTTTGAT) directly influence the affinity of ToxR binding and activation of the *ctxAB* promoter (21). The number of heptamer repeats was similar to those in *V. cholerae* El Tor (four) and altered El Tor (three or four). However, they differed from those in classical (seven) and Haitian (five) *V. cholerae* O1 strains (3, 22). The three Philippines strains shared a unique point mutation in *zot* (nonsynonymous; Arg to Cys, C to T at 1057 nucleotides). The genomic data confirmed the CTX prophage of Philippines isolates to be similar to that of *V. cholerae* hybrid El Tor strains, except in *rstA* and *zot*, which showed some unique polymorphisms. Therefore, it is concluded to be a variant of seventh-pandemic *V. cholerae* El Tor.

Genomic islands. *Vibrio* pathogenicity island 1 (VPI-1) is a 41.2-kb genetic element encoding one of the major virulence factors, namely, toxin-coregulated pilus (TCP), that serves as a re-

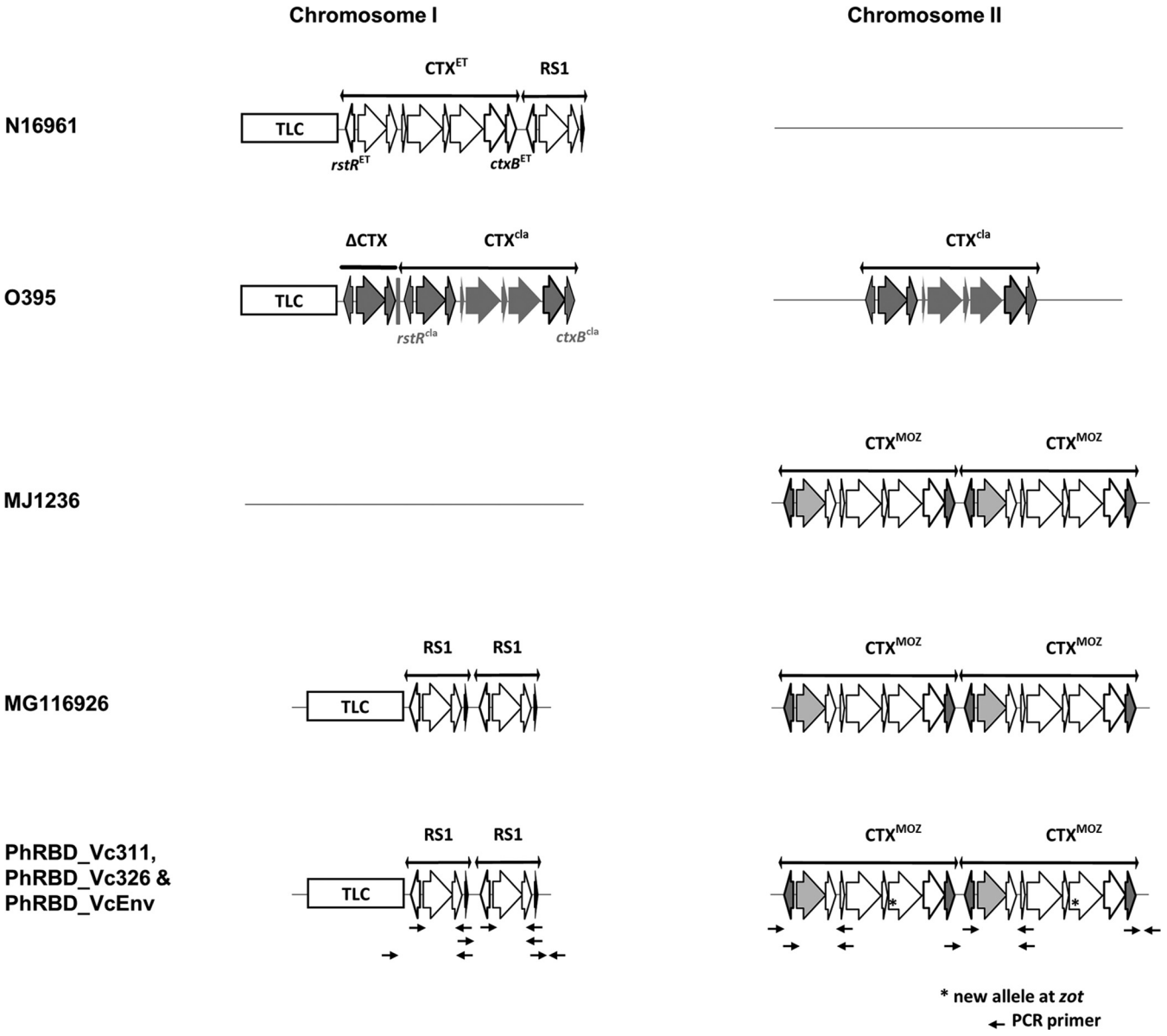

FIG 1 CTXΦ-RS1 array of Philippines *V. cholerae* isolates (PhRBD_Vc311, PhRBD_Vc326, and PhRBD_VcEnv) with El Tor (N16961), classical (O395), and hybrid El Tor (MJ1236 and MG116926) strains. Philippines isolates carry two copies of the RS1 element and TLC (toxin-linked cryptic plasmid) on the large chromosome (Chr I) and two copies of CTXΦ on the small chromosome (Chr II), similar to MG116926.

ceptor for lysogenic CTXΦ and promotes bacterial colonization of the small intestine (23, 24). The Philippines isolates possess an intact VPI-1; however, they also harbor a truncated VPI-2 of about 16.5 kb, similar to several of the *V. cholerae* O139 strains isolated in India and Bangladesh (Fig. 2A) (25). Notably, among genes present on VPI-2, the gene for sialidase/neuraminidase (NanH), which has a putative role in pathogenesis, and the sialic acid catabolism gene cluster, which mediates utilization of sialic acid as the sole carbon source, are both missing in Philippines isolates. Moreover, the Philippines isolates were devoid of SXT, which confers resistance to several antibiotics (e.g., streptomycin, sulfamethoxazole/trimethoprim, and chloramphenicol [26]) and is suggested to have evolutionary significance for *V. cholerae* O139 and altered *V. cholerae* El Tor strains, reported over the last two decades (4). In a previous study, SXT was considered to be a major genetic element providing a selective advantage for altered *V. cholerae* El Tor strains in its global dissemination (4). Thus, the lack of SXT in the Philippines isolates may explain a limited distribution of these strains. The three Philippines isolates contained intact *Vibrio* seventh-pandemic island I (VSP-I; open reading frames [ORFs] VC0175 to VC0185) and *Vibrio* seventh-pandemic island II (VSP-II; ORFs VC0490 to VC0516), as well as several of the well-documented genomic islands of *V. cholerae*, including genomic island 1 (GI-1) to GI-10 and a single copy of GI-12 (8). VSP-I and VSP-II are believed to be conserved in the seventh-pandemic El Tor strains, yet the latter was reported to be polymorphic for ORF distribution (27). The presence of an intact VSP-II in the Philippines isolates is an interesting finding and is in contrast

TABLE 1 Sites of nucleotide polymorphism in CTX prophages

Strain	Prophage[a]	*rstR*[a]	Nucleotide at position in gene[b]																		No. of heptamers in *zot-ctxA* (1197~74)[c]	*ctxB*
			rstA										*rstB*							*zot*		
			27	162	183	258	345	516	540	579	609	774	77-79	90	96	108	192	288	291	1057		
N16961	CTX^{ET}	ET	C	C	C	G	G	G	A	T	T	C	GTA	A	T	G	A	A	C	C	4	*ctxB3*
O395	CTX^{CL}	CL	T	T	A	C	T	A	G	C	C	T	–	T	C	.	.	G	T	.	7	*ctxB1*
MJ1236	CTX^{Hyb}	CL	T	T	A	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4	*ctxB1*
HCO1	CTX^{Hyb}	CL	.	.	.	.	.	.	.	.	.	.	–	.	.	.	.	.	.	.	5	*ctxB7*
PhRBD_Vc326	CTX^{Hyb}	CL	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	4	*ctxB1*
PhRBD_Vc311	CTX^{Hyb}	CL	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	4	*ctxB1*
PhRBD_VcEnv	CTX^{Hyb}	CL	T	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	4	*ctxB1*

[a] ET, El Tor type; CL, classical type; Hyb, hybrid.
[b] Dots indicate sequence identical to that of *V. cholerae* N16961; dashes indicate deletions.
[c] Number of ToxR-binding site repeats.

to the observation of recently isolated altered El Tor strains from Bangladesh, India, and Haiti carrying a variant VSP-II that lacks ORFs VC0495 to VC0512 (3).

Novel type I restriction-modification system. The Philippines *V. cholerae* O1 isolates contained a unique mobile element (GI-119) with a predicted type I restriction-modification system (Fig. 2B). As shown in Fig. 2B, the GI is approximately 15 kb and contains 10 unique genes, including three components of a type I restriction-modification system (R, S, and M subunits), interrupted by inclusion of an anticodon nuclease. The type I restriction-modification system-specific genes in the Philippines strains are unique. However, the presence of an anticodon nuclease with a restriction-modification system has been described in *Escherichia coli* as an anti-T4 phage defense mechanism (28). Although the exact role of a type I restriction-modification system in the Philippines strains is unknown, presumably it could serve as a mechanism protecting against several vibriolytic phages and allowing the bacterium to thrive in an aquatic environment. It should be noted that this novel type I restriction-modification system may have been acquired horizontally by homologous re-

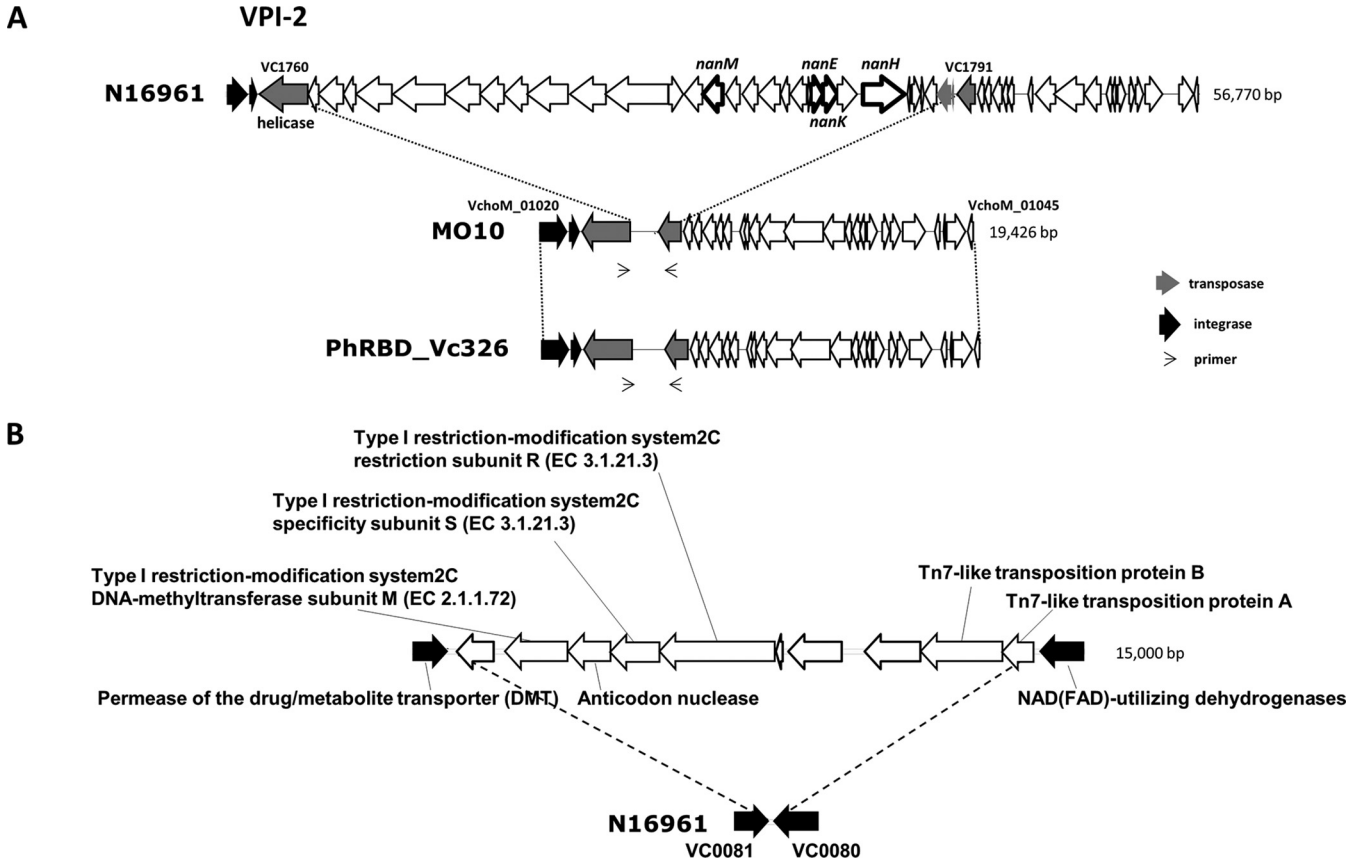

FIG 2 (A) Philippines *V. cholerae* isolates (PhRBD_Vc311, PhRBD_Vc326, and PhRBD_VcEnv) and a *V. cholerae* O139 strain (MO10) possessing a major truncation in the VPI-2 region compared to seventh-pandemic prototype El Tor (N16961). (B) A new genomic island (GI-119) encoding a type I restriction-modification system is present between VC0081 and VC0080 in Philippines *V. cholerae* isolates.

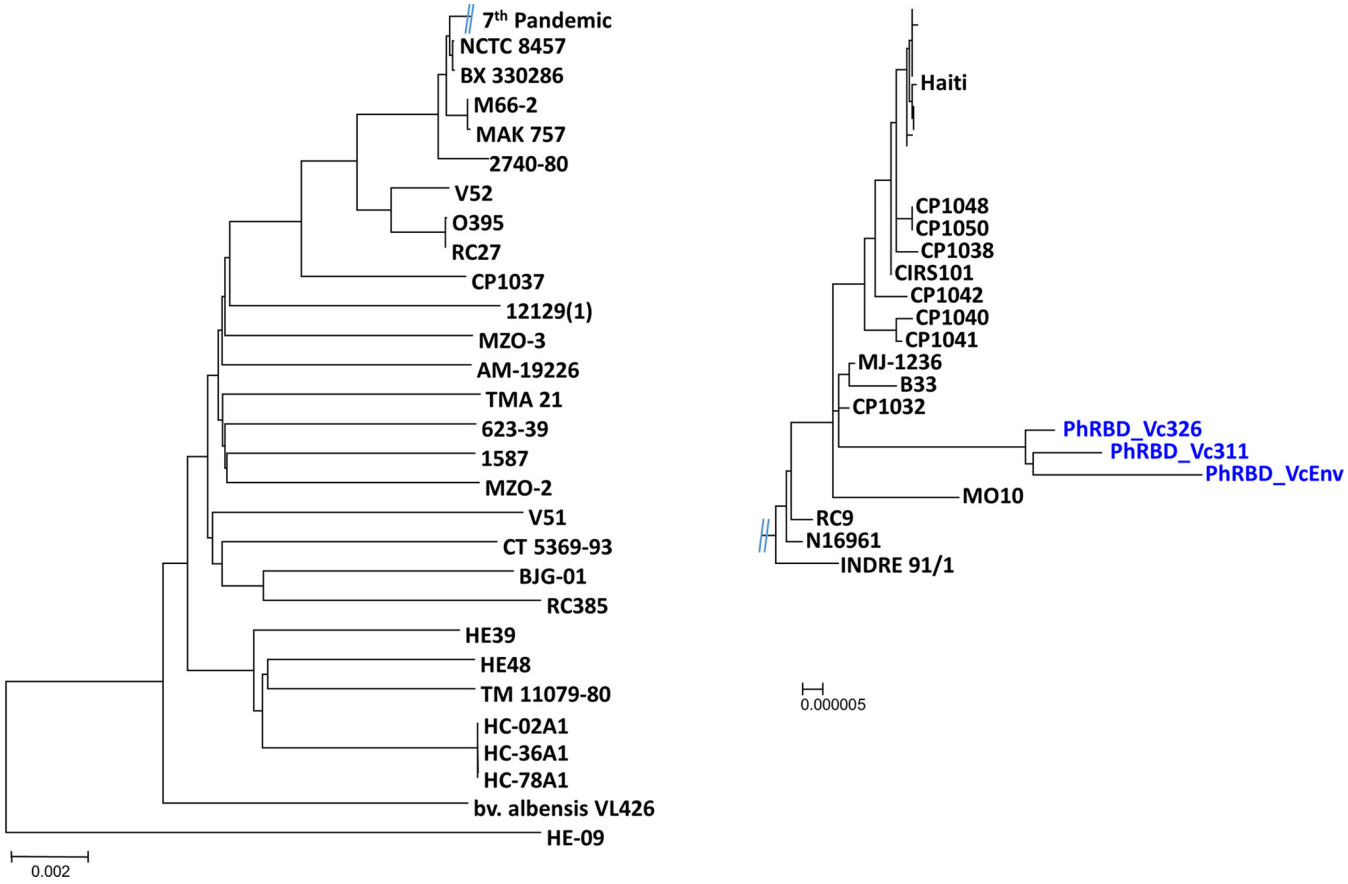

FIG 3 Neighbor-joining trees showing phylogenetic relationships of 78 *V. cholerae* genomes based on 1,051 orthologs of protein-coding genes (~1,054,653 bp). Philippines *V. cholerae* O1 strains are in blue, showing a tight clustering in a monophyletic clade.

combination, a possible indication of an evolution of *V. cholerae* O1 in adapting to the local Philippines niche.

Mutation in housekeeping genes. The Philippines isolates were found to contain a point mutation (Ser83Ile) in the gyrase gene (*gyrA*) but revealed no mutations in the topoisomerase gene (*parC*), as has been shown for *V. cholerae* O1 CIRS101 (Bangladesh, 2002) and CP1041 (Zambia, 2004). However, the same point mutation in *gyrA* and another mutation (Ser85Leu) in *parC* have been reported in *V. cholerae* isolates from Nigeria, Cameroon, Zimbabwe, Thailand, Bangladesh, and Haiti (3). The presence of the wild-type *parC* gene in the Philippines isolates is interesting, considering polymorphisms of currently circulating altered *V. cholerae* El Tor strains from different countries.

Phylogenomics. The phylogeny of the Philippines *V. cholerae* O1 isolates was determined by constructing a genome-relatedness neighbor-joining tree, using homologous alignment of 1,051 orthologs of protein-coding genes (~1,054,653 bp) of 78 *V. cholerae* genomes. The Philippines isolates clustered with seventh-pandemic *V. cholerae* El Tor and *V. cholerae* O139. However, the three isolates (PhRBD_Vc311, PhRBD_Vc326, and PhRBD_VcEnv) formed a distinct monophyletic clade (Fig. 3), distant from chronologically concurrent isolates from Thailand, Bangladesh, Zimbabwe, and Haiti. Moreover, the Philippines *V. cholerae* O1 strains showed close relatedness with hybrid *V. cholerae* El Tor strains from Mexico (CP1032, isolated in 1991), Mozambique (B33, isolated in 2004), and Bangladesh (MJ-1236, isolated in 1994) and a *V. cholerae* O139 strain from India (MO10, isolated in 1992). Interestingly, the time of isolation of these hybrid strains was different from that of the Philippines strains. In the phylogenetic tree, the monophyletic clade of Philippines isolates was positioned between concurrent altered *V. cholerae* El Tor strains and seventh-pandemic prototype *V. cholerae* El Tor strains. The Philippines isolates were not identical, and the environmental strain (PhRBD_VcEnv) showed highest homology with clinical strains (PhRBD_Vc311 and PhRBD_Vc326), confirming the environment as the reservoir of *V. cholerae*. Tight clustering of the Philippines strains in a monophyletic clade suggests that these strains have conserved genetic features and can be assumed to be restricted to the Philippines aquatic ecosystem, the probable source of the outbreak in 2011.

Conclusions. The Philippines *V. cholerae* strains isolated during an outbreak of cholera in 2011 belong to the seventh-pandemic clade of *V. cholerae* O1 and O139 serogroups. The genome sequence and organization of CTXΦ and RS1 indicate that the strains are hybrid *V. cholerae* El Tor lacking SXT. Although the Philippines strains showed relatedness to previously isolated hybrid *V. cholerae* El Tor strains from Mexico, Mozambique, and Bangladesh, they possess a novel type I restriction-modification system and truncated VPI-2. Overall, the genomic analyses clearly indicate that the Philippines isolates are novel *V. cholerae* hybrid El Tor strains, perhaps strains that evolved in the Philippines. Evolution of *V. cholerae* is very likely occurring not only in regions

where cholera is endemic but also in areas where it is not, like the Philippines. In conclusion, the data provided here may be helpful in determining the epidemiology of cholera and the evolution of *V. cholerae* in regions where cholera is not endemic.

MATERIALS AND METHODS

Bacterial isolation, identification, and classification. The National Epidemiology Center (NEC), Philippines Department of Health, received a report from the Center for Health Development, Region IV-B, of 90 diarrhea cases, with 15 deaths, from the municipality of Bataraza, Palawan, during the month of March 2011. An NEC team, dispatched to the area in April 2011, identified 562 suspected cholera cases, of which 383 (68%) were among an indigenous tribe of Palawan. Twenty patients suffering from cholera subsequently died (case fatality rate [CFR] = 3.6%). Samples were sent to the Research Institute for Tropical Medicine, Alabang, Philippines, for isolation and identification of the etiological agent. Ten samples from suspected cases were identified as containing *V. cholerae* O1 Ogawa El Tor, and 27 additional samples were identified as containing *Aeromonas* spp. In addition, 37 water samples tested positive for *Escherichia coli*, *Vibrio*, and *Aeromonas* by microbiological and serological procedures.

A single environmental and two clinical isolates of presumptive *V. cholerae* O1 were inoculated into Mueller-Hinton broth and incubated at 37°C overnight (29). Identification was accomplished using the BBL Crystal ID system (Becton, Dickinson) following incubation for 18 to 20 h at 37°C (30). The Crystal ID cartridges were read manually, and the data were entered using Crystal ID software, confirming identification to genus and species levels. DNA was extracted from pure cultures using the Qiagen genomic DNA kit and quantified using a NanoDrop spectrophotometer. The three putative *V. cholerae* strains, PhRBD_Vc311, PhRBD_Vc326, and PhRBD_VcEnv, were confirmed as *V. cholerae* based on results obtained using the BBL Crystal enteric/nonfermenter ID system. Each of the three strains metabolized mannose, sucrose, and mannitol as sole carbon sources.

MLVA. Multilocus variable-number tandem repeat analysis (MLVA) was performed using PCR conditions and primers to amplify five targeted MLVA loci for *V. cholerae*, namely, VC0147, VC0436-7 (intergenic), VC1650, VCA0171, and VCA0283, as described previously (12). The purified PCR products were sequenced in both directions using a BigDye cycle sequencing kit (Applied Biosystems), and sequencing was performed on an ABI 3770 automatic sequencer according to the manufacturer's instructions. The number of repeats was determined for each locus, and the MLVA genotypes were assigned by sequentially combining numbers of repeat units in order for five loci.

Genome sequencing. Isolates were sent to St. Luke's Medical Center for whole-genome sequencing. Shotgun whole-genome sequencing was performed using Roche GS Junior and Illumina MiSeq, and the output from both technologies was combined for downstream bioinformatic analysis. Roche GS Junior sequencing was accomplished employing protocols developed by Roche (31). Briefly, library DNA was prepared from 1 μg of genomic DNA by physically shearing the DNA by nebulization. The ends of the fragments were repaired employing DNA polymerase (Klenow fragment) and Taq DNA polymerase, and terminal adenosine residues were added to fragments using polynucleotide kinase (PNK). Roche RL adapters containing a terminal thymidine were ligated to the fragments, according to the manufacturer's protocol. Library quality was determined using Flashgel (Invitrogen) and quantified with fluorometry (Promega QuantiFluor). DNA fragments were annealed to Roche capture beads, emulsified using a Turrax tube drive, distributed onto a 96-tube PCR plate, and amplified using 50 cycles. Beads with amplified DNA were recovered from the emulsion and purified. Beads were packed into a picotiter plate along with the reaction mixture and packing beads, and the plates were loaded into the GS Junior instrument. Sequencing was performed using 200 nucleotide cycles.

Illumina MiSeq sequencing was performed using library DNA prepared from 50 ng of DNA and the Nextera DNA library kit, according to the manufacturer's protocol. Multiple strains were sequenced simultaneously using the Illumina Multiplex kit. Following amplification and purification of library DNA, libraries were diluted to 6.0 pM, and equal volumes were combined prior to addition to the MiSeq sequencing cartridge. Paired-end, 150-bp reads were generated using the MiSeq and standard protocols (31).

Sequence analysis. The raw sequencing reads, from both the Roche GS Junior (SFF file) and Illumina MiSeq (FASTA file), were assembled into contigs using GS *de novo* Assembler software (version 2.7; Roche). Assembled contigs were analyzed using NCBI BLAST to confirm *V. cholerae* species identification. Annotation of assembled contigs was done using the RAST Annotation Server (8) and the annotation service of the Institute for Genome Sciences (Baltimore, MD). All completed genome projects for *Vibrio cholerae* in the NCBI database were utilized as reference strains for mapping of reads from the Philippines strains, using GS Reference Mapper software (version 2.7; Roche).

Variant analysis. Detection of single-nucleotide polymorphisms (SNPs) and structural variants was performed using an in-house pipeline consisting of mapping shotgun sequencing reads from the Philippines isolates to *V. cholerae* El Tor reference strain N16961 and Roche gsMapper (version 2.7). The BAM file output was sorted and indexed, and reads were aligned using mpileup (SAMTools) to generate variant call files (VCF). VCF were converted to Annovar format files, and the location and type of variant were determined using Annovar and the annotated *V. cholerae* N16961 genome from NCBI. Comparison of the completed *V. cholerae* genomes with *V. cholerae* N16961 was done by *in silico* read generation using MetaSim, and simulated reads were mapped and processed as described above. In-house scripts were compiled and compared to Annovar output to generate a list of common variants.

Genetic analysis of CTXΦ and flanking regions. The orientation of CTXΦ and its flanking regions was performed using primers and conditions described previously (3, 12, 32). Sequencing of PCR products was accomplished after purification of the DNA fragments followed by Sanger sequencing (First Base, Singapore).

Comparative genomics and phylogeny. Genome-to-genome comparison was performed using methods described previously (8). Genomic islands (GIs) were defined as a continuous array of five or more coding sequences (CDSs) discontinuously distributed among genomes of test strains. Identified GIs were annotated using a BLASTP search of member CDSs against the GenBank NR database. Regions orthologous to *V. cholerae* N16961 were identified by comparisons based on similarity (95%), and the resultant 1,051 orthologs were used to generate a phylogenetic tree. The set of orthologous regions for each CDS of a reference genome was identified according to nucleotide similarity and aligned using CLUSTALW2. The resultant multiple alignments were concatenated to form genome scale alignments, which were then used to generate the neighbor-joining phylogenetic trees (33).

Nucleotide sequence accession number. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under accession no. AWWD00000000, AWWE00000000, and AWWF000000 (BioProject PRJNA218554).

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