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Research paper

Genetic diversity of environmental *Vibrio cholerae* O1 strains isolated in Northern Vietnam

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ABSTRACT

Cholera epidemics have been recorded periodically in Vietnam during the seventh cholera pandemic. Since cholera is a water-borne disease, systematic monitoring of environmental waters for *Vibrio cholerae* presence is important for predicting and preventing cholera epidemics. We conducted monitoring, isolation, and genetic characterization of *V. cholerae* strains in Nam Dinh province of Northern Vietnam from Jul 2013 to Feb 2015. In this study, four *V. cholerae* O1 strains were detected and isolated from 110 analyzed water samples (3.6%); however, none of them carried the cholera toxin gene, *ctxA*, in their genomes. Whole genome sequencing and phylogenetic analysis revealed that the four O1 isolates were separated into two independent clusters, and one of them diverged from a common ancestor with pandemic strains. The analysis of pathogenicity islands (CTX prophage, VPI-I, VPI-II, VSP-I, and VSP-II) indicated that one strain (VNND_2014Jun_6SS) harbored an unknown prophage-like sequence with high homology to vibriophage KSF-1 phi and VCY phi, identified from Bangladesh and the USA, respectively, while the other three strains carried *tcpA* gene with a distinct sequence demonstrating a separate clonal lineage. These results suggest that the aquatic environment can harbor highly divergent *V. cholera* strains and serve as a reservoir for multiple *V. cholerae* virulence-associated genes which may be exchanged *via* mobile genetic elements. Therefore, continuous monitoring and genetic characterization of *V. cholerae* strains in the environment should contribute to the early detection of the sources of infection and prevention of cholera outbreaks as well as to understanding the natural ecology and evolution of *V. cholerae*.

1. Introduction

Cholera is a severe and sometimes lethal human diarrheal disease caused by a gram-negative bacterium *Vibrio cholerae*. Repeated cholera pandemic episodes have been recorded in the past 200 years (Abbott et al., 2007), and the disease still remains a significant health issue in many parts of the world. WHO reported the estimation of 1.4 to 4.3 million cholera cases, and 28,000 to 142,000 disease-related deaths every year (WHO, 2015). Among the nearly 200 recognized serogroups of *V. cholerae*, only two, O1 and O139, are associated with epidemics and global pandemics of cholera. However, many *V. cholerae* virulence genes or their homologs are located in mobile genetic elements and may be acquired by strains with low or no pathogenicity. For example, the gain of virulence-related genes has been detected in *Vibrio* spp. isolated from the aquatic environment (Faruque et al., 1998; Karaolis et al., 1999; Xie et al., 2005; Gennari et al., 2012) which, thus, could present a potential gene cradle where bacteria can exchange genetic elements, leading to the emergence of pathogenic *Vibrio* strains (Alam et al., 2006).

Two virulence factors, cholera toxin (CTX) and toxin-coregulated pilus (TCP), play a pivotal role in the pathogenicity of *V. cholerae* (Klose, 2001). CTX is responsible for severe watery diarrhea associated with cholera. The toxin is encoded by *ctxAB* located on the lysogenic bacteriophage CTX phi and comprises the *ctxA* and *ctxB* genes and accessory genes such as *zot* and *ace* (Ramamurthy and Bhattacharya, 2010). TCP is a type IV bundle-forming pilus which mediates the interaction among *V. cholerae* cells and promotes their colonization of the intestinal epithelium; it also serves as a receptor for the CTX-encoding phage CTX phi (Ramamurthy and Bhattacharya, 2010). TCP is formed by

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polymerization of the major pilin subunit TcpA encoded by *tcpA* gene which is located within the Vibrio pathogenicity island (VPI). The gene demonstrates sequence variability and is widely distributed among *V. cholerae* non-O1/non-O139 serogroups (Boyd and Waldor, 2002; Kumar et al., 2011).

One of the adaptation strategies employed by various bacterial species to survive in the changing environment is genetic variation by positional mutations or horizontal transfer of foreign genes. Several mobile genetic elements harboring virulence-associated genes have been identified in *V. cholera* genome, including VPI-I, VPI-II, and Vibrio seventh pandemic islands (VSP-I, VSP-II) (Faruque et al., 2003; Jermyn and Boyd, 2005; Murphy and Boyd, 2002; Gennari et al., 2012). VPI-I is a 41-kb region integrated into the transfer-messenger (tm) RNA (*ssrA*) loci which includes TCP-related genes (Murphy and Boyd, 2002). VPI-II is a 57.3-kb island consisting of 52 open reading frames (ORFs) (VC1758 to VC1809 in the N16961 strain), which is integrated into the tRNA-Ser-II gene locus (Jermyn and Boyd, 2005; Gennari et al., 2012). VSP-I is a 16-kb region spanning 11 ORFs (VC0175 to VC0185) and present mainly in the *V. cholerae* serogroup O1 biotype El Tor and serogroup O139 isolates (Dziejman et al., 2002; Jermyn and Boyd, 2005). VSP-II is a 27-kb region encoding homologs of a type IV pilus; it contains 26 ORFs and is positioned at the tRNA-methionine locus (O'Shea et al., 2002; Murphy and Boyd, 2002).

Genome sequence analysis is a widely used approach for comprehensive evaluation of phylogenetic relations by identifying single nucleotide polymorphisms (SNPs), which was instrumental in revealing the phylodynamics of current *V. cholerae* pandemics (Mutreja et al., 2011; Reimer et al., 2011; Dutilh et al., 2014). In addition, whole-genome data can be applied for the characterization of endemic strains and estimation of *V. cholerae* transmission routes by degerming how given strains from distinct areas were genetically related each other (Shah et al., 2014; Abd El Ghany et al., 2014; Okada et al., 2014; Pang et al., 2016; Siriphap et al., 2017.).

Repeated cholera epidemics have been recorded in Vietnam until 2010 (Duc Anh et al., 2014; Nguyen et al., 2016), and endemic episodes have also been reported in the neighboring Southeast Asian countries (WHO, 2016). Although improved sanitation can reduce the risk of cholera outbreaks, systematic and continuous monitoring of environmental waters for *V. cholerae* presence is important for the prevention of epidemics (Alam et al., 2006; Kahler et al., 2015, Mala et al., 2017, Das et al., 2016). In this study, we tried to detect *V. cholerae* strains carrying toxin-related genes, and/or O1/O139 antigen-encoding genes from the environment in Vietnam, and tried to make a phylogenetic tree analyses seeking for any possibilities whether those strains were originated from a common ancestor with any of the 7th pandemic strains.

2. Materials and methods

2.1. Sample collection and screening

Nam Dinh province located in Northern Vietnam was chosen as an area of sample collection. The area faces the sea, is rich in tidal creeks, and not a few numbers of cholera cases were reported in an outbreak occurred in 2007–2008 season (unpublished data). A 500 ml each of surface water was collected at 10 sites in the area (Fig. S1) at approximately 2-month interval in the period between July 2013 and February 2015, and 110 samples were collected in total, and analyzed. Each sample was cultured with Alkaline Peptone Water (Nissui, Tokyo, Japan) at 37 °C overnight, then total DNA was extracted from the cultures by boiling, and used for templates for PCR targeting *toxR*, *ctxA*, *O1-rfb*, and *O139-rfb* genes (Table S1) (Nguyen et al., 2012).

2.2. Isolation of V. cholerae from environmental water samples

Each culture was streaked onto thiosulfate citrate bile salt (TCBS; Nissui, Tokyo, Japan) agar and incubated overnight at 37 °C. Eight *V. cholerae*-like colonies on each plate were chosen and sub-cultured on nutrient agar (NA) plates. Each strain was examined by API 20 kit (bioMerieu, Marcy l'Etoile, France), by serotyping with anti-O1 or anti-O139 antisera (Denka Seiken, Niigata, Japan), and further by PCR for the presence of *toxR*, *ctxA*, *O1-rfb*, and *O139-rfb* genes (Table S1), for the identification of *V. cholerae*.

2.3. Whole-genome sequencing and construction of phylogenetic trees

Whole genome sequencing was performed using the next-generation sequencing platform. Genomic DNA was extracted from each strain identified as *V. cholerae* O1 using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA), and 1 ng DNA was used to prepare a library for the paired-end sequencing (Nextera XT DNA Kit; Promega, Fitchburg, WI, USA). The constructed libraries were sequenced on Illumina MiSeq using Reagent Kit v.3 (Illumina, San Diego, CA, USA), and 300-bp paired-end reads were assembled by *de novo* with the Velvet software (Zerbino and Birney, 2008), and several prophage regions of the strains were analyzed by phylogenetic trees made by kSNP3 v2 software (Gardner and Hall, 2013).

3. Results and discussion

The majority of the 110 water samples (95%) were tested positive for *toxR*, a transcriptional activator for toxin-related genes including *ctxAB*. However, only five samples (4.6%) were positive for *O1-rfb* gene and none were positive for *ctxA* or *O139-rfb* (Table 1). A limited number of the samples analyzed in this study would not allow to make a statistically confirmed implication, however, it is intriguing that *O1-rfb* gene were detected in environment only in July and September 2013, and in June 2014, one of the hot and wet seasons in Northern Vietnam. What we have estimated in the study was *V. cholerae* strains were relatively abundant in the environment in the study area in Nam Dinh province throughout a year, however, *V. cholerae* strains carrying O1 antigen were few and circulated in the environment preferably in hot and wet season. *V. cholera* strains were isolated from four out of the five *O1-rfb*-positive water samples. Of those, two strains were isolated from samples collected in July 2013; one from a sample collected in September 2013; and one from a sample collected in June 2014, and designated as VNND_2013Jul_3SS, VNND_2013Jul_5SS, VNND_2013Sep_5SS, and VNND_2014Jun_6SS, respectively. Serological analyses identified each of the four isolates as *V. cholera* serogroup O1 and serotype Ogawa.

Phylogenetic analysis indicated that the three *V. cholerae* O1 isolates (VNND_2013Jul_3SS, VNND_2013Jul_5SS, and VNND_2013Sep_5SS) out of four formed a tight cluster which placed a proximity to clusters to which *V. cholerae* O1 pandemic classical or the 7th pandemic strains belonged (Fig. 1, Table S2), indicating that those three strains might have evolved from a close ancestor from which pandemic *V. cholerae* had been originated. The remaining one strain, VNND_2014Jun_6SS,

Table 1
Summary of PCR screening.

Sampling site[a]	No. of positive samples for each target gene			
	O1-rfb	*O139-rfb*	*ctxA*	*toxR*
#1	0	0	0	11
#2	0	0	0	10
#3	1	0	0	11
#4	1	0	0	10
#5	2	0	0	11
#6	1	0	0	11
#7	0	0	0	11
#8	0	0	0	10
#9	0	0	0	11
#10	0	0	0	11
Total	5/110	0/110	0/110	107/110

[a] Sampling sites were described in Fig. S1.

Fig. 1. Phylogenetic relationship of O1 isolates from environmental waters in Vietnam. The SNP-based phylogenetic tree was constructed including 135 reported *V. cholerae* draft or complete sequences (Table S1) using the kSNP v3.0 software. The names of strain shown in bold letters indicate the environmental strains isolated in this study. O1 classical and seventh pandemic clusters are shown by triangles, respectively. The branches in heavy lines were O1 isolates.

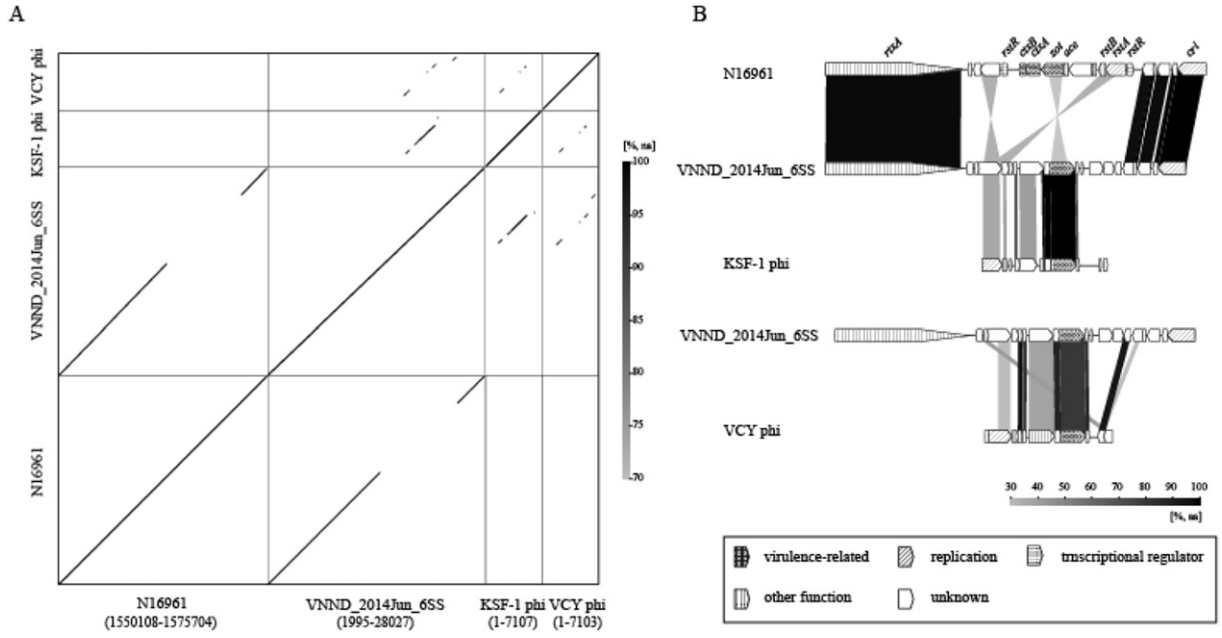

Fig. 2. Sequence comparison of several prophage and prophage-like regions integrated on chromosome in *V. cholerae* strains. VNND_2014Jun_6SS, newly identified prophage-like region in this study, was compared with KSF-1 phi, VCY phi, and CTX phi of *V. cholerae* O1 biovar El Tor str. N16961 at nucleotide level (A) and amino acid level (B). Dot-plot matrices and genetic organization were represented using GenomeMatcher (Ohtsubo et al., 2008). Orthologous genes (encoding same function) are indicated in matching design. Compared regions were shown in parentheses.

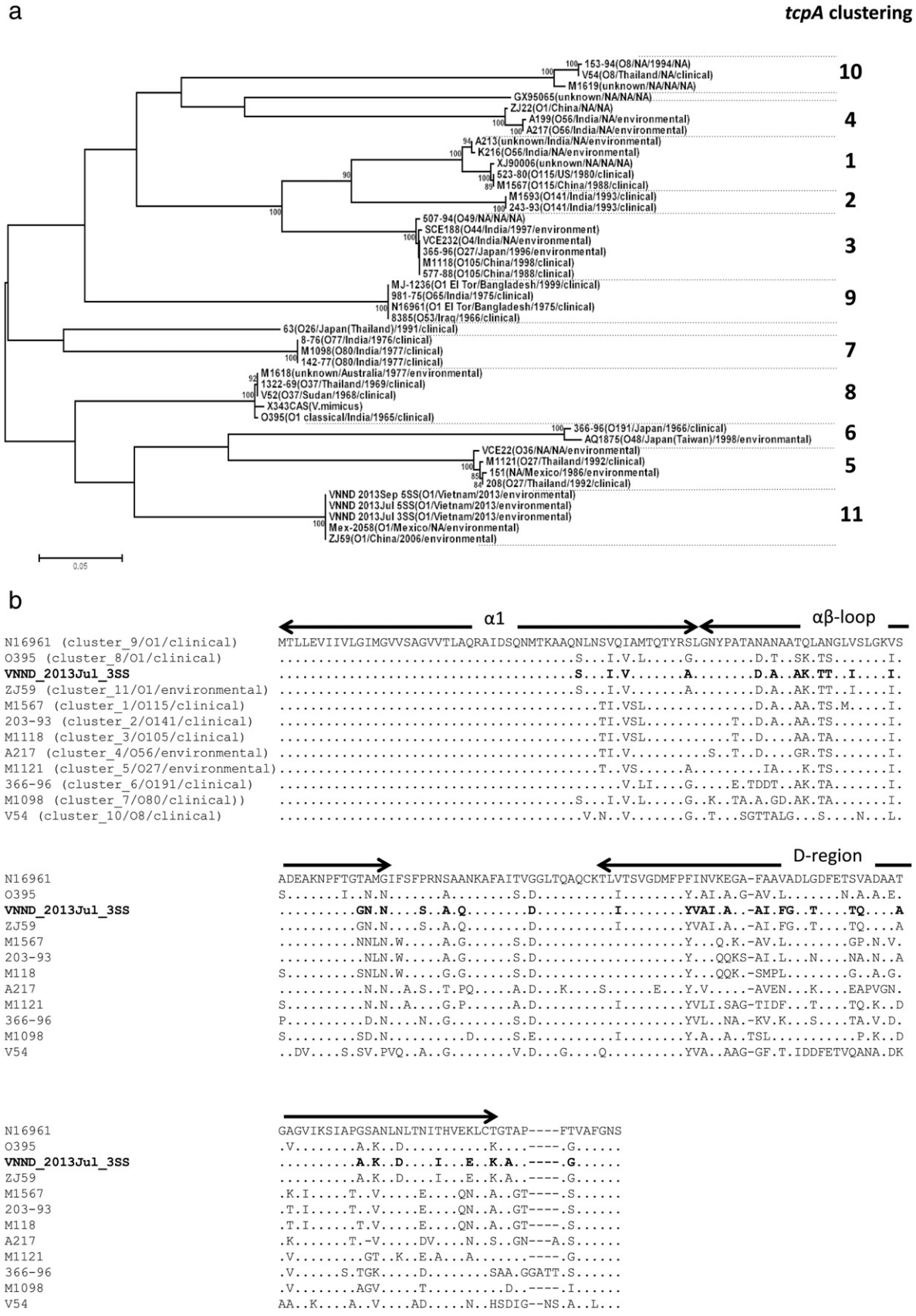

Fig. 3. Phylogenetic tree of *tcpA* and deduced amino acid sequence alignment of TcpA. (a) Phylogenetic tree was constructed by maximum likelihood methods *tcpA* genotypes were extracted from Kumar et al. (2011). The bootstrap values higher than 80 are indicated at the nodes. The meta information of each isolates were shown in the parenthesis as order of O-antigen type/country/year/source. (b) Deduced amino acid sequence alignment of TcpA was performed using ClustalW2 multiple sequence alignment program. VNND_2013Jul_3SS isolated in this study was compared with other representative strains in each clusters (Thompson et al., 1994).

segregated into a different cluster to which several non-O1/O139 (NAG) strains belonged (data not shown), and was separated from the ones to which *V. cholerae* pandemic strains converged. The closest relatives of the VNND_2014Jun_6SS strain were VC311 (non-O1/O139, Australia, 1986) and LMA3984-4 (O1, Brazil, 2007), both of which originated from geographically distinct areas from Vietnam.

Our analysis revealed that none of the *V. cholerae* isolates carried well known pathogenic islands including CTX prophage, VPI-II, VSP-I, or VSP-II. However, the VNND_2014Jun_6SS strain was identified to harbor an unknown phage-like sequence in the CTX integration site, corresponding to the one between VC1451 and VC1465 of strain N16961, a referral strain of *V. cholerae* biotype El Tor (Fig. 2). The phage-like sequence contained 14 ORFs; of those, ORF_8 and ORF_9 showed high homology to the ORF III–ORF IX region (approximately 3619 bp) of KSF-1 phi (Faruque et al., 2003), while ORF_5 and ORF_14 were highly homologous to ORF 9 (189 bp) and ORF 11 (354 bp) of VCY phi, respectively (Faruque et al., 2005; Xue et al., 2012). KSF-1 phi is reported to serve as a helper phage for packaging the CTX phage genome, while VCY phi circulates among the environmental *V. cholerae* isolates from the USA and can exist both in a plasmid-like and host genome-integrative forms. Thus, the phage-like sequence found at the CTX integration site of the VNND_2014Jun_6SS strain was estimated to represent a mosaic structure of KSF-1 phi, VCY phi, and unknown exogenous DNA.

Three isolates (VNND_2013Jul_3SS, VNND_2013Jul_5SS, and VNND_2013Sep_5SS) harbored a pathogenic island VPI-I which contained *tcpA* gene encoding TcpA, pilus protein of TCP, a colonization factor of *V. cholerae*. *TcpA* alleles of the three isolates were identical to each other, and shared sequence homology at approximately 72.7% and 80.2% with that of strain N16961 and O395, a referral strain of biotype classical, respectively. The phylogenetic tree analysis revealed that *tcpA* carried by these three environmental isolates were clustered into a unique group together with those of strains isolated in Mexico (Mex-2058, Alam et al., 2014), or in China (ZJ59, accession No. EU622531), which was different from any other 10 genotypes reported previously (Kumar et al., 2011), hence, could be categorized as a new genotype of *tcpA*, designated as cluster_11 (Fig. 3a). The cluster_11 *tcpA* allele were carried by *V. cholerae* strains isolated in Latin America (Mex-2058), East Asia (ZJ59), and Southeast Asia (this study), indicating that *tcpA* allele was distributed relatively a wide area.

The deduced amino acid sequence alignment of TcpA of a strain representing each of 11 *tcpA* cluster revealed that the cluster_11 TcpA (VNND_2013Jul_3SS, ZJ59) showed a relatively higher homology to that of the cluster_8 to which most of classical strains converged, rather than the cluster_9 to which representative El Tor strains belonged (Fig. 3b), which was consistent with DNA sequence analysis. Amino acid substitutions found in the cluster_11 TcpA, same as the ones in other clusters, were located principally in αβ-loop and D-region of TcpA (Fig. 3b), which were portions composing a surface structure of the TCP filament, then are exposed directly to surrounding environment (Lim et al., 2010). Corresponding regions of the αβ-loop and D-region of other type IV pilins were reported also highly variable (Lim et al., 2010). TCP has been reported to serve as a receptor for CTX phage to infect *Vibrio* bacteria, then integrate into bacterial genome. The fact needed to be reminded is that all the three environmental *V. cholerae* O1 strains harboring the cluster_11 *tcpA* allele did not carry CTX phage region in their genome. It is important to determine whether TcpA protein is expressed out of the cluster_11 *tcpA*, and has a potential to assemble as a functional form to serve as a receptor, or not.

Several waves of cholera transmission originated probably from the Bengal area have been reported in Vietnam before 2010, however, no cholera cases were observed afterwards. In this study, *V. cholerae* strains carrying pathogenic gene clusters of VPI-II, VSP-I, or VSP-II were not isolated from environment in Nam Dinh province, Northern Vietnam, indicating that pathogenic strains were relatively rare in population, which may explain a temporal extinction of cholera in Northern Vietnam. Our data supported an abundant inhabitation of non-pathogenic *V. cholerae* in aquatic environment. Phylogenetic tree analysis indicated that three *V. cholerae* O1 non-pathogenic strains formed a cluster proximity to those for pandemic classical or El Tor strains (Fig. 1). Taken together, it is important to pay attention that abundant population of environmental *V. cholerae* O1 has a potential to serve as a reservoir for the emergence of pathogenic *V. cholerae* strains by transferring pathogenic gene clusters among *V. cholerae* strains. Therefore, monitoring of virulent genes among environmental *V. cholerae* strains is important for the sake of detecting possible cholera epidemics to occur in the area at relatively early timing.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2017.06.017.

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