

1 **Novel cholera toxin variant and ToxT regulon in environmental *Vibrio mimicus* strains:**  
2 **potential resources for the evolution of *Vibrio cholerae* hybrid strains**

3 **Running title:** New variant CTX $\Phi$  and ToxT regulon in *Vibrio mimicus* (51/54 characters)

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## 23 ABSTRACT

24 Atypical El Tor strains of *Vibrio cholerae* O1 harboring variant *ctxB* genes of cholera toxin  
25 (CT) are gradually becoming a major cause of recent cholera epidemics. *Vibrio mimicus*  
26 occasionally contains virulence factors associated with cholera, e.g., CT, encoded by *ctxAB*  
27 on CTX $\Phi$  genome; and TCP, the CTX $\Phi$ -specific receptor. This study carried out extensive  
28 molecular characterization of CTX $\Phi$  and ToxT regulon in *ctx*<sup>+ve</sup> strains of *V. mimicus*  
29 isolated from the Bengal coast. Southern hybridization, PCR, and DNA sequencing of  
30 virulence related-genes revealed the presence of an El Tor type CTX prophage (CTX<sup>ET</sup>)  
31 carrying a novel *ctxAB*, tandem copies of environmental type pre-CTX prophage (pre-  
32 CTX<sup>Env</sup>), and RS1 elements, which were organized in an array of RS1-CTX<sup>ET</sup>-RS1-pre-  
33 CTX<sup>Env</sup>-pre-CTX<sup>Env</sup>. Additionally, a novel variant of *tcpA* and *toxT* respectively, showing  
34 clonal lineage to a phylogenetic clade of *V. cholerae* non-O1/O139, was identified. The *V.*  
35 *mimicus* strains lacked the RTX and TLC elements, and *Vibrio* seventh pandemic islands of  
36 the El Tor strains, but contained five heptamer (TTTTGAT) repeats in *ctxAB* promoter region  
37 like some classical strains of *V. cholerae* O1. PFGE analysis showed all the *ctx*<sup>+ve</sup> *V. mimicus*  
38 strains were clonally related. However, their *in vitro* CT production and *in vivo* toxigenicity  
39 were variable, which could be explained by differential transcription of virulence genes along  
40 with ToxR regulon. Taken together, our findings strongly suggest that environmental *V.*  
41 *mimicus* strains act as potential reservoir of atypical virulence factors, including variant CT  
42 and ToxT regulon, and may contribute to the evolution of *V. cholerae* hybrid strains.

43 (248/250 words)

## 44 IMPORTANCE

45 Natural diversification of CTX $\Phi$  and *ctxAB* genes certainly influences disease severity and  
46 shifting patterns in major etiological agents of cholera, e.g., the overwhelming emergence of

47 hybrid El Tor variants, replacing the prototype El Tor strains of *V. cholerae*. This study  
48 showing the occurrence of CTX<sup>ET</sup> comprising a novel variant of *ctxAB* in *V. mimicus* points  
49 out a previously unnoticed evolutionary event, independent to that of the El Tor strains of *V.*  
50 *cholerae*. Identification and cluster analysis of the newly-discovered alleles of *tcpA* and *toxT*  
51 indicates their horizontal transfer from an uncommon clone of *V. cholerae*. The genomic  
52 content of ToxT regulon, and tandemly arranged multiple pre-CTX $\Phi$ <sup>Env</sup> and a CTX $\Phi$ <sup>ET</sup> in *V.*  
53 *mimicus* probably act as salient raw materials inducing natural recombination among the  
54 hallmark virulence genes of hybrid *V. cholerae* strains. This study will facilitate deeper  
55 understanding of the evolution of new variant CT and ToxT regulon, influencing cholera  
56 epidemiology.  
57 (150/150 words)

## 58 INTRODUCTION

59 *Vibrio mimicus* is genetically and ecologically very similar to *Vibrio cholerae*, the  
60 cholera bacterium and share similar environmental niche in freshwater and estuarine  
61 ecosystems, particularly in the tropical region like the Bengal delta. *V. mimicus* is known to  
62 be associated with sporadic cholera-like diarrhea cases. Despite a lot of efforts in hygiene  
63 promotion and therapeutic advances, cholera continues to pose as a major health problem  
64 worldwide, accounting for millions of episodes and thousands of deaths, with ca. 132,000  
65 cases in 2016 reported to the World Health Organization  
66 ([http://www.who.int/gho/epidemic\\_diseases/cholera/en/](http://www.who.int/gho/epidemic_diseases/cholera/en/)). The principal pathogenic factor  
67 instigating the disease is the cholera toxin (CT), encoded by the *ctxAB* operon, predominantly  
68 found in *V. cholerae* strains belonging to the O1 and O139 serogroups, and occasionally a  
69 few non-O1/non-O139 serogroups. Among the seven known cholera pandemics, the current  
70 seventh pandemic since 1961 is caused by the El Tor biotype of *V. cholerae* O1 while its  
71 classical biotype was associated with the sixth pandemic. In Bangladesh, the classical cholera  
72 re-emerged in 1983, later receded by the rise in El Tor cholera, and is believed to be extinct  
73 since 1993. However, since the last decade, hybrid El Tor strains producing classical-CT are  
74 the dominant cause of epidemic and endemic cholera replacing the prototype El Tor strains  
75 that produce El Tor CT (1). Occurrences of such type of variant El Tor strains have also  
76 reported to spread in many countries in Asia, Africa, and in Haiti (2, 3, 4). This indicates a  
77 cryptic existence of the variant or classical *ctxB*, and variant CTX $\Phi$  in environmental  
78 reservoirs, yet mostly unexplored. *In vitro* experiments have shown that CTX $\Phi$  can infect  
79 certain *V. mimicus* strains (5). In line with this, occurrence of *ctxAB* among *V. mimicus*  
80 strains, although isolated rarely, in Bangladesh, India, Japan and the United States, attests the  
81 hypothesis of inter-species genetic exchange (6, 7, 8, 9).

82           The *ctxAB* operon encoding the A and B subunits of CT is a part of the genome of  
83 CTX $\Phi$ , a filamentous bacteriophage. The precursor form of the CTX $\Phi$ , pre-CTX $\Phi$ , does not  
84 carry the *ctxAB* genes (8). Before this study, a total of 13 genotypes of *ctxB* have been  
85 distinguished based on single nucleotide polymorphisms (SNPs) at 10 loci of this toxigenic  
86 factor (Table 2). Notably, the *ctxB* genotypes 1 and 2 are typical for all classical strains and  
87 El Tor strains from Australia, respectively, while genotypes 3 and 7 are featured among the  
88 pandemic El Tor, and the Haitian variant strains. *V. cholerae* O1 El Tor strains are also  
89 characterized by the presence of TLC (Toxin linked cryptic) element and repeat in toxin  
90 (RTX) genes in the flanking region of CTX prophage, and two large genomic islands, termed  
91 as *Vibrio* Seventh Pandemic Islands (VSP-I and VSP-II) (10). Other known virulence factors  
92 of *V. cholerae*, particularly of the non-O1/non-O139 strains, include heat-stable enterotoxin  
93 (encoded by *stn*), type III secretion system (*vcsN2*), and cytotoxic cholix toxin (*chxA*) (11,  
94 12). Natural recombination events, compounded with the integration of phages contribute to  
95 evolution of genes, especially those related to virulence and ecological fitness (13). While  
96 persisting in the aquatic environment *V. cholerae* and *V. mimicus* interact with diverse  
97 phages, and a portion of their populations, harboring selective receptor, can integrate  
98 toxigenic phages into their genome.

99           The CTX $\Phi$  genome (~ 6.9 kb) contains core and RS2 regions. The core region  
100 includes genes involved in phage morphogenesis and CT production, including *ctxAB*, *zot*,  
101 and *orfU*. The RS2 region contains genes required for replication (*rstA*), integration (*rstB*)  
102 and regulation (*rstR*) of CTX $\Phi$  (14). Moreover, the upstream promoter of *ctxAB* possesses  
103 heptamer repeats, considered as evolutionary signature, while its downstream intergenic  
104 region contains site for CTX $\Phi$  integration, mediated by XerC and XerD recombinases (15).  
105 In El Tor strains, the prophage DNA is flanked by a genetic element known as RS1, which is  
106 a satellite phage (16). In comparison to RS2, the RS1 additionally contains *rstC* that encodes

107 an anti-repressor of *rstR* and promotes transmission of RS1 and CTX $\Phi$  (17). In *V. cholerae*  
108 strains, presence of both CTX prophage and RS1 element, as solitary and multiple copies  
109 with diverse arrays of genetic organization, have been documented (18). Based on nucleotide  
110 sequence polymorphism in its several genes, including *rstR* and *orfU* (gIII<sup>CTX</sup>), the CTX  
111 prophage can be differentiated into several types such as classical, El Tor, Calcutta and  
112 environmental (19). Among the El Tor variant or hybrid strains, two types of CTX  
113 prophages, one harboring classical *rstR* and classical *ctxB* (20) and the other containing El  
114 Tor *rstR* and classical *ctxB* (21) have been reported. Although extensive investigations have  
115 revealed nucleotide sequence polymorphism and diversity in the array of CTX prophages on  
116 *V. cholerae* genome (21) little is known for those of *V. mimicus* strains.

117 The transmission of CTX $\Phi$  into a *Vibrio* strain relies on the presence of a specific cell  
118 surface type IV pilus receptor, termed as toxin co-regulated pilus (TCP), which also plays a  
119 vital role aiding colonization of *V. cholerae* in human or animal intestine (22). The TCP is  
120 located on the *Vibrio* Pathogenicity Island (VPI), and produced by the action of a cluster of  
121 genes, termed as TCP island. The major structural subunit of TCP is encoded by *tcpA*. The  
122 expression of CT and TCP is activated by ToxT, present on the TCP island, and is under the  
123 control of the ToxR regulon, comprising *toxR*, *toxS*, *tcpP*, and *tcpH* (23). Based on the  
124 nucleotide sequence polymorphism in *tcpA*, the TCP can be differentiated into several types,  
125 e.g., El Tor, classical, Nandi, and Novais (24). CTX/pre-CTX prophages and genes of VPIs  
126 are found scattered throughout environmental isolates of *V. cholerae* (25). Despite the  
127 absence of the classical biotype strains along with the classical CTX phage particle (1), the  
128 increasing occurrence of hybrid El Tor strains of *V. cholerae* O1 harboring variant *ctxB* genes  
129 is intriguing and requires detail exploration for their environmental reservoirs. Being  
130 genetically the closest species of *V. cholerae*, there is high possibility for the environmental  
131 *V. mimicus* strains to act as potential reservoir of virulence genes associated with cholera and

132 diarrhea epidemics. However, our knowledge on the occurrence of genetic determinants of  
133 virulence, particularly cholera-like diarrhea, in environmental *V. mimicus* and their similarity  
134 to those of epidemic strains of *V. cholerae* is very limited.

135 In this study, several *ctx<sup>+</sup>* *V. mimicus* strains isolated from estuarine surface waters in  
136 Bangladesh were analyzed to ascertain whether they can act as reservoirs of the CTX $\Phi$   
137 carrying *ctxAB* variant present in *V. cholerae* strains associated with recent epidemics. The  
138 objectives were to investigate (i) the molecular diversity of genetic elements within CTX  
139 prophage and TCP islands, (ii) *in vitro* CT production, (iii) *in vivo* fluid accumulation using  
140 suckling mouse model, and (iv) differential expression of ToxT regulon in these  
141 environmental *V. mimicus* strains. Comparison of these phenotypic and genetic traits to those  
142 of toxigenic *V. cholerae* would aid in better understanding the evolution of new variant CT  
143 and ToxT regulon.

## 144 RESULTS

145 **Antimicrobial susceptibility.** Among the 11 antimicrobials tested, all *ctx<sup>+</sup>* *V.*  
146 *mimicus* strains examined in this study showed full resistance to ampicillin (10  $\mu$ g) and  
147 cephalothin (30  $\mu$ g), and intermediate resistance to erythromycin (15  $\mu$ g). However, two  
148 types of antimicrobial resistance pattern were observed based on the resistance to polymixin  
149 B (50  $\mu$ g) and gentamicin (10  $\mu$ g) (Table 1). Three out of six *V. mimicus* strains showed full  
150 resistance to polymixin B (50  $\mu$ g), while the other three strains showed intermediate  
151 resistance to gentamicin (10  $\mu$ g).

152 **PFGE based screening for genomic relatedness.** PFGE of the undigested gDNA  
153 showed that the *ctx<sup>+</sup>* environmental *V. mimicus* strains possessed ca. 2.9 and 1.3 Mbp of  
154 large and small chromosomes, respectively, which were similar to *V. mimicus* type strain  
155 (ATCC33539<sup>T</sup>) but different from the classical (O395), El Tor (N16961) and non-O1/non-

156 O139 (VCE233) strains of *V. cholerae*. PFGE analysis of *NotI*- and *SfiI*-digested gDNA  
157 showed 0 to 3 band differences among the *ctx*<sup>+ve</sup> *V. mimicus* strains. According to Tenover  
158 *et al.* (1995), these strains were clonal in origin. However, comparison of the PFGE bands  
159 with two enzymes could reveal a total of five subtypes (Table 1). In case of *SfiI*, three  
160 patterns (designated as I, II and III, Fig. S1) could be assigned, but only two patterns  
161 (designated as a and b, Fig. S1) were observed in case of *NotI*. Taken together, four subtypes  
162 (patterns Ia, Ib, IIb and IIIb) were present in four out of six strains. The remaining two strains  
163 did not show any difference (pattern IIa) even after digestion with both the enzymes.

164 **Occurrence of the major virulence factors.** Among the hallmark genes associated  
165 with the toxigenic *V. cholerae* strains, several of them related to CT production were detected  
166 in the *V. mimicus* strains used in this study. Colony blot hybridization using <sup>32</sup>P-labelled  
167 probes for virulence related-genes showed the presence of *ctxA* and *zot* of CTXΦ, *rstC* of  
168 RS1 element, and *tcpA* of VPI. However, these strains did not harbor genes representing VSP  
169 I and II. They were also negative for TLC and RTX elements, which are commonly present in  
170 the flanking region of CTXΦ in *V. cholerae* El Tor and their hybrid strains. No other major  
171 toxigenic factors of *V. cholerae*, namely, *vcsN2*, *chxA*, and *stn* were detected in the *ctx*<sup>+ve</sup> *V.*  
172 *mimicus* strains.

173 **Characteristics of *ctxAB* and CTXΦ associated genetic elements.** Based on the  
174 results of MAMA-PCR, all the *V. mimicus* strains contained classical type of *ctxB*. Sequence  
175 analysis of the entire *ctxB* observed that the gene was identical in all six *V. mimicus* strains.  
176 Although showing signature changes at the 39th (tyrosine to histidine) and 68th (isoleucine to  
177 threonine) positions, similar to classical *ctxB* genotype 1, the *V. mimicus ctxB* had additional  
178 non-synonymous substitutions conferring subtle changes in the deduced amino acids at  
179 positions 46 (phenylalanine to leucine) and 67 (alanine to glutamic acid) (Table 2).  
180 Comparative analysis with other known *ctxAB* genotypes reported among *V. mimicus* and *V.*



181 *cholerae* revealed the existence of a new *ctxB*, designated as genotype 14. This genotype was  
182 almost similar to genotype 12, reported from a *V. mimicus* strain, but differed at amino acid  
183 position 67. In all other genotypes of *ctxB* the 67<sup>th</sup> position encoded alanine, but all the *ctxB*  
184 sequences of *V. mimicus* strains in this study detected glutamic acid at this position, thus  
185 unique for this novel genotype 14.

186 Sequencing analysis of *ctxA* of the environmental *V. mimicus* strains also observed  
187 alterations from the canonical gene and all available sequences in GenBank. This novel *ctxA*  
188 differed from that of the reference El Tor and classical strains with three amino acids at  
189 positions 46, 190 and 198, while the highest similarity was observed with *ctxA* of a *V.*  
190 *mimicus*, characterized with *ctxB* genotype 12 (Table 2). A unique change at amino acid  
191 position 198, with alteration of isoleucine to valine, of *ctxA* in the examined *V. mimicus*  
192 strains was noteworthy.

193 The presence of RS1 element was confirmed by the *rstC* gene-based PCR (Fig. S2),  
194 followed by sequencing analysis. The *rstC* gene in the environmental *V. mimicus* strains was  
195 identical to that of the reference El Tor strain. PCR-based genotyping of *rstR* showed the  
196 presence of two different alleles, one for El Tor (*rstR*<sup>ET</sup>) and the other for environmental  
197 (*rstR*<sup>Env</sup>), indicating the occurrence of multiple prophages, i.e., CTXΦ<sup>ET</sup> and CTXΦ<sup>ET</sup>.

198 **Genetic organization of CTXΦ associated elements.** Southern hybridization of  
199 chromosomal DNA digested with *BglI* and *BglIII*, which have single cutting site in CTXΦ at  
200 *rstR*<sup>ET</sup>/*rstR*<sup>Env</sup> and *zot*, respectively, showed identical RFLP patterns for all *V. mimicus*  
201 strains. Size-wise comparative analysis of the bands detected by hybridization, using different  
202 probes, of the enzyme digested gDNA revealed similar results, i.e., presence of two copies of  
203 pre-CTXΦ<sup>Env</sup> prophages and a single CTXΦ<sup>ET</sup> prophage, in all six strains of *ctx*<sup>+ve</sup> *V.*  
204 *mimicus*. Probing with *ctxA* and *rstR*<sup>Env</sup> of the *BglI*-digested gDNA generated a single  
205 positive band (ca. 10.5 Kb), and two positive bands (ca. 6.5 and 10.5 Kb), respectively. These

206 results indicated the presence of one copy of CTX $\Phi$  containing *ctxAB*, followed by a pre-  
207 CTX $\Phi^{\text{Env}}$  (lacking *ctxAB*) with an adjacent RS1, and pre-CTX $\Phi^{\text{Env}}$  without RS1 (Fig. 1, Fig.  
208 S3). Probing with *rstR^{\text{Env}}* and *rstR^{\text{ET}}* of the *Bgl*III-digested gDNA resulted one (ca. 18.8 kb),  
209 and three (ca. 3.5, 8.0 and 18.8 Kb) positive bands, justifying the adjacent locations of one  
210 RS1 followed by two pre-CTX $\Phi^{\text{Env}}$  prophages, along with the preceding occurrence of  
211 another RS1 and El Tor type full length CTX $\Phi$  containing *ctxAB*. Hybridization with *rstC*  
212 probe justified the presence of two copies of RS1 element, one before the CTX $\Phi^{\text{ET}}$  and the  
213 other preceding the adjacent CTX $\Phi^{\text{Env}}$ . However, there was no RS1 element between the two  
214 adjacent CTX $\Phi^{\text{Env}}$  prophages. Altogether, an array of RS1-CTX $\Phi^{\text{ET}}$ -RS1-pre-CTX $\Phi^{\text{Env}}$ -pre-  
215 CTX $\Phi^{\text{Env}}$  was deduced from the Southern hybridization analysis.

216 To verify the hybridization results, PCR arrays using the allele-specific forward and  
217 reverse primers, with multiple combinations, of *rstC*, *rstR*, *ctxAB* and *orfU* genes (Fig. 1)  
218 were conducted. All the six *ctx^{\text{+ve}}* *V. mimicus* strains yielded similar amplicons after PCR  
219 using primers specific for different regions of CTX element. Size-wise comparison of the  
220 PCR amplicons observed concordance with the published genetic organization of El Tor  
221 strains of *V. cholerae*. The results of PCR walking were in concordance with hybridization  
222 results, confirming the presence of the RS1, *rstR^{\text{ET}}* in RS2 of the CTX $\Phi$  carrying *ctxAB*, and  
223 *rstR^{\text{Env}}* allele in RS2 of pre-CTX $\Phi$  element(s), which lacked the *ctxAB* operon (Fig. S2, Fig.  
224 S3). The flanking sequences of CTX $\Phi^{\text{ET}}$  harbored both the RS1 and RS2 elements, similar to  
225 the reference *V. cholerae* O1 El Tor strain N16961. Sequencing analysis of several core and  
226 intergenic regions of CTX element (Fig.1), confirmed the tandem presence of three copies of  
227 the CTX element, including one intact CTX $\Phi^{\text{ET}}$  and two pre-CTX $\Phi^{\text{Env}}$  prophages lacking  
228 *ctxAB*.

229 **Genomic signatures in *ctxAB* promoter and intergenic sequences.** The sequential  
230 integration of pre-CTX $\Phi^{\text{Env}}$  (lacking *ctxAB*) and CTX $\Phi^{\text{ET}}$  in *V. mimicus* strains isolated from

231 the estuarine environment prompted sequencing analysis of intergenic regions, particularly  
232 *ctxAB* promoter and the prophage flanking regions to reveal genomic signatures associated  
233 with their lysogenic transformation. Forward and reverse primers of the adjacent genes, i.e.,  
234 *zot*, *ctxA*, *ctxB* and *rstR*<sup>ET</sup> for El Tor CTX prophage, and *zot* and *rstR*<sup>Env</sup> for environmental  
235 pre-CTX prophage amplified the desired parts of *ctxAB* promoter and intergenic regions.  
236 Sequencing analysis of the El Tor CTX prophage showed that the promoter at the 5'-  
237 upstream of *ctxAB* contained 5 heptamer (TTTTGAT) repeat spanning between -90 and -57  
238 bp, which is a characteristic of the classical type *ctxAB* promoter, while the RNA polymerase  
239 binding sites at -35 bp (TTTACT) and -10 bp (CAATTA) were conserved (Fig. 1). The 3'-  
240 end of *ctxAB* was characterized by attR sequences coupled with the XerC and XerD binding  
241 sites for CTX $\Phi$  integration, which started 106 bp downstream similar to the reference El Tor  
242 strain N16961 of *V. cholerae*. On the other hand, the 3'-end of pre-CTX<sup>Env</sup> prophage was  
243 characterized by a 13 bp gap between *zot* (the last gene of phage core region) and attR  
244 sequences, followed by XerC and XerD binding sites (Fig. 1).

245 **Sequence diversity in *orfU* of CTX prophage core region.** Because the *orfU* gene is  
246 instrumental in studying the diversity in the core region of CTX prophage, this gene was  
247 PCR-amplified from *V. mimicus* strains and subjected to sequencing, followed by  
248 phylogenetic analysis. Interestingly, identical sequence homology among all the study strains  
249 and also within CTX $\Phi$ <sup>ET</sup> and pre-CTX $\Phi$ <sup>Env</sup> prophages was observed. Comparative analysis  
250 with the reference El Tor and classical strains of *V. cholerae* and a recently studied *V.*  
251 *mimicus*, of the deduced amino acid sequences of *orfU*, indicated that the environmental *V.*  
252 *mimicus* strains of this study did not completely match any of them rather they possessed nine  
253 unique changes with a total of 31 polymorphic sites observed within these strains. The  
254 highest similarity was observed with the reference El Tor strain N16961 of *V. cholerae* O1,  
255 which differed by 11 amino acids in *OrfU*. In comparison to *OrfU* of *V. mimicus* strains of

256 this study, both the reference classical O395 strain of *V. cholerae* O1 and another *V. mimicus*  
257 strain differed, although not identical, by 27 amino acid substitutions. Thus, sequencing  
258 analysis indicated the presence of a variant OrfU (Fig. S4) in the genome of environmental *V.*  
259 *mimicus* strains. Phylogenetic analysis using partially available nucleotide sequences (702 of  
260 1083 bp) of *orfU* in GenBank database showed this gene in the study strains did not cluster  
261 with the classical *V. cholerae* strains like the previously reported strains of *V. mimicus* (7).  
262 However, the *orfU* variant in environmental *V. mimicus* strains grouped into a cluster  
263 comprising of the El Tor, El Tor variant O1, O139, and several non-O1/non-O139 strains of  
264 *V. cholerae* (Fig. 2).

265 **PCR, sequencing, and phylogenetic analysis of *tcpA* and *toxT*.** PCR using a  
266 forward primer from the beginning part of the 5'-terminal conserved region (*tcpA-F*) and a  
267 reverse primer for El Tor or classical type of *tcpA* did not yield any amplicon. However,  
268 using *tcpA-F* and a reverse primer from *tcpQ* (*tcpQ-R*) yielded a 2.1-kb product for all the  
269 *ctx*<sup>+ve</sup> *V. mimicus* strains. DNA sequencing showed that all of the strains had identical *tcpA*  
270 and BLAST search analysis revealed the occurrence of a new *tcpA* allele, designated as  
271 *tcpA*<sup>Env-Vm</sup>. Phylogenetic analysis observed that this gene had sequence homology between  
272 69.3 and 96.4 % when compared to other reported *tcpA* sequences, and could be categorized  
273 into a novel cluster clearly separated from other major *tcpA* clusters, including the classical,  
274 El Tor, Nandi, and Novais types. The novel *tcpA*<sup>Env-Vm</sup> in *V. mimicus* strains showed the  
275 closest similarity with a couple of *V. cholerae* non-O1/non-O139 strains isolated from India  
276 and USA, and also with a *V. mimicus* strain (Acc. no. ACYV01000002) isolated from USA  
277 (Fig. 3). Most of the diversities observed among the *tcpA* alleles were in the carboxy-terminal  
278 half, but the amino-terminal region was almost conserved among the compared sequences.  
279 Comparative sequence analysis with the reference classical and El Tor strains of *V. cholerae*  
280 O1, showed that the *tcpA*<sup>Env-Vm</sup> had 74% homology at the DNA level to that of the El Tor

281 (N16961) and classical (O395) *tcpA*, with 40 and 43 substitutions, respectively, among 224  
282 deduced amino acids of the *tcpA* gene (Fig. 3, Fig. S6). The Nandi and Novais types TcpA  
283 differed by 15 and 45 amino acid residues in comparison to that of the environmental *V.*  
284 *mimicus* of this study. Phylogenetic analysis also observed high sequence homology of one *V.*  
285 *mimicus* strain isolated from Brazil and another strain from China to the canonical TcpA of  
286 classical and El Tor O1 *V. cholerae*, respectively. However, these classical and El tor types  
287 TcpA of *V. mimicus* had 40 and 42 differences in amino acids, respectively, when compared  
288 to the TcpA<sup>Env\_Vm</sup>.

289         Similar to *tcpA* amplification, PCR using the conventional primers for *toxT* did not  
290 produce any amplicon for the environmental *V. mimicus* strains of this study. However,  
291 application of newly designed primers (Table S5), considering variations in classical, El Tor,  
292 and environmental types of *toxT*, successfully yielded specific amplicon of this gene in the  
293 study strains. Sequencing results showed identical sequence homology of *toxT* in all the  
294 environmental *V. mimicus* strains. Comparative analysis identified the presence of a new  
295 allele, with several unique substitutions, and 76.9-78.0 % homology among the deduced  
296 amino acid residues in comparison to the canonical *toxT* of the classical and El Tor *V.*  
297 *cholerae* strains (Fig. S7). Higher diversity was observed in the amino-terminal half of ToxT  
298 sequences when comparing those of the environmental *V. mimicus* and *V. cholerae* O1  
299 strains. Phylogenetic analysis clearly differentiated *toxT* genes into two major clusters, one  
300 including the usual *toxT* commonly found in epidemic *V. cholerae* O1 strains and the other  
301 comprising the variant *toxT* identified in this study and several *V. cholerae* non-O1/non-O139  
302 strains from India (Fig. 3). However, the variant ToxT in environmental *V. mimicus* strains  
303 was novel in terms of the acquired differences in 11 amino acid residues in comparison to  
304 that of the non-O1/non-O139 *V. cholerae* in the same phylogenetic cluster, and 59-60 amino  
305 acid residues with the canonical ToxT found in classical and El Tor *V. cholerae* O1.

306           **Competitive survival of *V. mimicus* in microcosm.** In competition with the  
307 predominant estuarine vibrios, i.e., *V. cholerae* and *V. parahaemolyticus*, the inoculated *V.*  
308 *mimicus* strain could be cultured on TTGA agar up to 14, 45 and 55 days at 0.1, 3.5 and 11.5  
309 ppt water salinities, respectively, in microcosm environment. The survival rate of *ctx*<sup>+ve</sup> *V.*  
310 *mimicus* strain was comparable to a strain of epidemic *V. cholerae* O1. In contrast to a rapid  
311 decrease in culturable counts with time observed for *V. parahaemolyticus* at lower salinity  
312 (<5 ppt), the inoculated *V. mimicus* strain showed better potential to persist as culturable form  
313 at all the tested water salinities, representing their environmental habitats (Fig. S8).

314           **CT production capacity and virulence potential.** All the environmental *V. mimicus*  
315 strains showed identical pattern for the major virulence related genes, including those of the  
316 predicted amino acid sequences in CTX prophages and TCP island, which indicated their  
317 probable functional capability to produce CT and virulence related proteins. Therefore, bead-  
318 ELISA was carried out following established conditions for both the El Tor and classical  
319 strains of *V. cholerae* O1 to check the functional CT production capacity and its variation, if  
320 any, among the environmental *V. mimicus* strains. Results showed that the CT production  
321 capacity varied among the environmental *V. mimicus* strains, and was better under the *in vitro*  
322 conditions favorable for the classical (LB, pH 6.6, 30 °C) strains than El Tor conditions (AKI,  
323 pH 7.4, 37 °C) for *V. cholerae* strains. Out of the six *V. mimicus* strains, one strain (Vm7)  
324 showed high CT production capacity (110 and 30 ng mL<sup>-1</sup> in LB and AKI, respectively)  
325 while the toxin production was very low (0.1-0.5 ng mL<sup>-1</sup>) in others (Table 1).

326           SMA-based experiments produced results in congruence with CT production capacity  
327 for the *V. mimicus* strains (Table 1). Both live cells (10<sup>6</sup> to 10<sup>7</sup> CFU) and culture filtrates of  
328 the *V. mimicus* strain Vm7 producing high CT induced fluid accumulation and diarrhea in all  
329 the experimental mice, hence concluded to be enterotoxigenic. SMA score, representing fluid  
330 accumulation ratio, ranged between 0.083 and 0.090 (0.087 ± 003) for the high CT producing

331 Vm7 strain. However, none of the experimental mice produced diarrhea when a low CT-  
332 producing *V. mimicus* strain Vm2 was administered at normal dose ( $10^7$  CFU) and even at  
333 higher dose ( $> 5 \times 10^9$  CFU). The fluid accumulation ratio by this strain with attenuated CT  
334 production ranged between 0.065 and 0.070 ( $0.0068 \pm 002$ ), which was similar to that of the  
335 negative control ( $0.062 \pm 002$ ) (Table S9).

336 **Transcriptional analysis of genes associated with CT production.** According to the  
337 results of bead-ELISA, the optimum culture condition for CT production, i.e., classical type  
338 condition using LB medium, was selected for transcriptional analysis of *ctxAB* and its known  
339 regulatory genes by qRT-PCR in the high (Vm7) and low (Vm2) CT producing  
340 environmental *V. mimicus* strains used in SMA *in vivo* experiments. As expected, a  
341 significantly lower transcription of *ctxA* in the low-CT producing strain in comparison to the  
342 high CT producer was observed. Similarly, significantly low-level transcription of *tcpA* and  
343 *toxT*, which are known to directly interact with CT production, was also observed. While  
344 checking the transcription of other genes in the ToxR regulon influencing CT production, the  
345 high and low transcription of *ctxA* was observed to be correlated with the mRNA  
346 transcription of *toxR*, *toxS*, and *tcpP* (Fig. 4). In the low CT-producing strain, the  
347 transcription of *ctxA*, *tcpA* and *toxT* genes was significantly lower by about 15- to 25-fold ( $P$   
348  $< 0.005$ ), in comparison to those of the high CT producing strains. Similarly, *toxR* expression  
349 was also significant lower, about 4-fold ( $P < 0.01$ ), while both *toxS* and *tcpP* showed about 2-  
350 fold ( $P < 0.05$ ) lower transcription. In the low CT producing strain, *tcpH* transcription was  
351 about 1.4-fold lower but not significant in comparison to the high CT producer. On the other  
352 hand, an opposite trend was observed for *hns* transcription; the high CT producing strain  
353 showed about 1.3-fold lower *hns* transcription, which was not significant, than the low CT  
354 producer.

## 355 DISCUSSION

356 Understanding the adaptive evolutionary mechanism of the CTX $\Phi$  and *ctxAB* genes  
357 encoding the cholera toxin (CT) is highly important because of its direct relation to severe  
358 diarrhea such as cholera, which is causing health hazards throughout the world. In this aspect,  
359 through acquisition of toxigenic *ctxAB*, *V. mimicus* might play a salient role for its  
360 maintenance and propagation in the natural environment. Due to the lack of systematic  
361 surveillance of environmental and clinical samples, our knowledge of the occurrence and  
362 diversity of virulence genes associated with CT production in *V. mimicus* is very limited. In  
363 this study, the genetic traits and virulence potential of several estuarine strains of *ctx*<sup>+ve</sup> *V.*  
364 *mimicus* were analyzed for better understanding of the role of this bacterium in the evolution  
365 of CTX $\Phi$ , *ctxAB*, and related pathogenic factors.

366 **Novel *ctxAB* allele in CTX<sup>ET</sup> prophage in environmental *V. mimicus* strains.**

367 Comparing the core and flanking regions of CTX prophage in *V. mimicus* with those of *V.*  
368 *cholerae* strains shows that some unique changes in amino acid residues, which were  
369 previously unidentified, with respect to the reference homologous genes have occurred in  
370 these *V. mimicus* strains. In the reference El Tor strain N16961, the phage integration site,  
371 characterized by the *attR* sequence followed by XerC and XerD, starts at 106 bp downstream  
372 of *ctxAB* intergenic region (27). Similar phage integration site starting at 106 bp downstream  
373 of *ctxAB* of CTX $\Phi$ <sup>ET</sup>, which is different from pre-CTX $\Phi$ <sup>Env</sup> integration site, i.e., starting at 13  
374 bp downstream of *zot*, has been observed in this study. Remarkably, comparison of amino  
375 acid residues with the known *ctxB* sequences has identified the presence of a novel *ctxB*  
376 variant (Table 2) in the *V. mimicus* strains. Phylogenetically, this newly discovered *ctxB*  
377 genotype 14 is distantly related to the El Tor genotype 3, but more closely related to the  
378 classical genotype 1 and Haitian genotype 7 (Fig. 2). However, sequencing results and  
379 comparison of *orfU* of CTX prophage in *V. mimicus* strains could identify its close homology  
380 with that of the El Tor type *V. cholerae* O1. Nonetheless, several unique changes in the



381 amino acid residues within the first two (D1 and D2) of three domains of *orfU* (28) indicates  
382 the ongoing evolution of CTX prophage in environmental *V. mimicus* in parallel to those of  
383 the epidemic *V. cholerae* O1. According to Wang *et al.* (15), these polymorphic residues  
384 most likely interact with TolA and the ‘adsorption’ domains, associated with phage  
385 penetration.

386 Genome walking through hybridization and PCR demonstrated that the *ctx*<sup>+ve</sup> *V.*  
387 *mimicus* strains actually contain one mature El Tor type prophage (CTXΦ<sup>ET</sup>) with *ctxAB*, and  
388 two environmental type pre-CTX prophages (pre-CTXΦ<sup>Env</sup>) without *ctxAB*. Existence of pre-  
389 CTXΦ in some epidemic strains of *V. cholerae* O1 and O139 has been known (25).  
390 Integration of at least two types of CTXΦ (El Tor and Environmental) within the genome of  
391 *V. mimicus* is an interesting novel observation. All of the *V. mimicus* strains in this study  
392 were observed to produce replicative forms of both CTXΦ<sup>ET</sup> and pre-CTXΦ<sup>Env</sup> when induced  
393 by mitomycinC in the culture filtrates, which was detected by PCRs after DNase and RNase  
394 treatment (data not shown). On the other hand, like the El Tor strains of *V. cholerae*, the  
395 environmental *V. mimicus* strains also harbored *rstC*, i.e., the RS1 element, which has been  
396 recently observed to promote diversity by the loss of CTX prophage and lysogenic immunity  
397 to make room for new CTX prophage to be integrated (29). Presence of *rstC* has been shown  
398 to increase *rstA* transcription and CTXΦ production (17), which may influence *ctxAB*  
399 transcription and diversification. It is assumed that the El Tor strains possess greater  
400 ecological fitness than the classical strains. In comparison to the canonical El Tor type  
401 strains, the hybrid El Tor strains with classical type *ctxB* genotype is considered as more  
402 virulent than the El Tor CT producer (30). The acquisition of hybrid CTXΦ<sup>ET</sup> by the *V.*  
403 *mimicus* strains might have equipped them with greater evolutionary fitness, since these  
404 pathogenic strains can utilize the chance of becoming selectively enriched in the intestine of  
405 humans and animals.

406           **Diverse TCP and ToxT alleles in *V. mimicus* strains.** Not only the CTX elements  
407 but also the TCP genes in *V. cholerae* can be mobilized by a generalized transduction (31).  
408 The sequence of the *tcpA* locus in the TCP element is known to be more divergent compared  
409 to other loci in the VPI (32). Similarly, by phylogenetic analysis we have observed a high  
410 diversity in *tcpA* sequences, compared to not only among the O1/O139 and non-O1/non-  
411 O139 strains of *V. cholerae* but also among the previously reported *ctx*<sup>+ve</sup> *V. mimicus* strains.  
412 The observed homology of *tcpA* of *V. mimicus* and *V. cholerae*, showing as less as ca. 70% at  
413 nucleotide level, is in congruence with other studies analyzing strains belonging to different  
414 serotypes and biotypes of *V. cholerae* (33, 34). The environmental *ctx*<sup>+ve</sup> *V. mimicus* strains  
415 contain a novel type of *tcpA* claimed as *tcpA*<sup>Env-Vm</sup>, which showed higher sequence homology  
416 to that of *V. cholerae* serogroups O56 and O115. Thus, *tcpA* of the environmental *V. mimicus*  
417 strains of this study might have acquired this gene from *V. cholerae* strains belonging to the  
418 O56 and O115 serogroups, and/or vice versa. This observation is not coherent with the  
419 previously reported *V. mimicus tcpA* genotypes, which were affiliated to the phylotgenic  
420 clades containing the canonical classical and El Tor strains of *V. cholerae* O1. Most likely,  
421 this diversity is a reflection of diversifying selection to CTX $\Phi$  susceptibility during  
422 adaptation to the aquatic environment or host intestine. Sequencing results also indicate that  
423 *V. mimicus* strains contain a novel allele of *toxT*, affiliating with the atypical *toxT* of certain  
424 non-O1/non-O139 but not the canonical *toxT* of epidemic classical and El Tor O1 strains of  
425 *V. cholerae* (24).

426           Comparative analysis of *tcpA* sequences has clearly identified a high substitution rate  
427 in the carboxy-terminal half, encoding the exposed part of the TCP pilus on cell surface.  
428 Among the many differences between the present *tcpA*<sup>Env-Vm</sup> allele and the *tcpA*<sup>Cla</sup> allele  
429 (classical) is the c.187V>K substitution, which is shown to be correlated with increase in  
430 pilus-mediated autoagglutination in the context of *tcpA*<sup>cla</sup> (35). In comparison, the amino-

431 terminal region, encoding the basal part of the mature pilus structure, was observed to be  
432 more conserved among the *V. mimicus* and other strains compared. In case of *toxT*, the  
433 diversity in amino acid residuals in comparison to the reference strain was higher in the  
434 amino-terminal half, which is in accordance with a previous study (24). The relatively  
435 conserved carboxy-terminal half is known to determine the specificity of ToxT protein  
436 binding to DNA regulatory sites (24). Apart from acting as a virulence factor, TCP may also  
437 aid in the environmental persistence, e.g., biofilm formation on aquatic particles, and  
438 organisms, particularly, chitinous zooplankton (36). The occurrence of a new variant *tcpA*,  
439 with possible alterations in cell surface epitopes, among the toxigenic *V. mimicus* strains of  
440 the present study might be due to an adaptive evolutionary response to the changes in  
441 environmental niche.

442 **Variation in CT production, virulence potential, and its regulatory framework in**  
443 ***V. mimicus*.** Results of bead-ELISA showed that CT production level in *V. mimicus* strains is  
444 preferentially induced under the *in vitro* growth conditions favorable for the classical *V.*  
445 *cholerae* O1 strains. Most of the *V. mimicus* strains did not cause fluid accumulation or  
446 diarrhea in experimental mice, in concordance with the very low CT production ( $<0.5 \text{ ng mL}^{-1}$ ).  
447 Yet these environmental *V. mimicus* strains can be considered as potentially toxigenic  
448 because of their acquisition of genes related to pathogenic factors, including CT and TCP.  
449 This is reflected in at least one strain of this study producing considerable amount ( $>100 \text{ ng}$   
450  $\text{mL}^{-1}$ ) of CT to induce fluid accumulation in suckling mice. We also cannot rule out the  
451 possibility that the current assay condition may not be suitable for inducing the CT  
452 production especially for low CT producing strains and that need to be further investigated  
453 using different culture conditions and growth media like M9-minimal medium. In case of *V.*  
454 *cholerae*, strains producing at least  $\sim 20 \text{ ng mL}^{-1}$  concentration of CT are known to cause fluid  
455 accumulation or diarrhea (37). Absence of any other potential virulence factors, e.g., TTSS,

456 ST, ChxA and RTX indicates that the observed enterotoxicity in mice intestine is due to CT  
457 produced by the environmental *V. mimicus* strains. Interestingly, attenuation of some  
458 bacterial virulence factors has been attributed to the effect of repeated subculture *in vitro*,  
459 e.g., reduction of heat-labile enterotoxin (LT) in *E. coli*, and CT production in *V. cholerae*.  
460 (38; N. Chowdhury *et al.*, unpublished). On the other hand, the higher CT production in one  
461 *V. mimicus* strain of this study may be due to its pre-exposure to the intestine of mammals,  
462 fish or any potential aquatic animal (39).

463 The studied *V. mimicus* strains showed resistance to polymixin B, ampicilin,  
464 cephalotin and reduced susceptibility to gentamicin. Resistance to polymixin B has been  
465 shown to be typical for the El Tor strains, while most of the O1 strains of both classical and  
466 El tor biotypes are usually resistant to ampicilin. When compared to the recent clinical  
467 strains from patients with cholera, the observed resistance to a few antimicrobials in the  
468 environmental *V. mimicus* strains is in congruence with the results obtained for *V. cholerae*  
469 O1 strains isolated from natural surface water (40). The widespread use of antimicrobials  
470 might have provided an additional selective pressure for the sporadic emergence of the multi-  
471 drug resistant *V. mimicus* strains.

472 Despite the presence of CTX $\Phi$  and TCP element the variation in CT production is  
473 likely influenced by other genetic or physiological factors in *V. cholerae*. The expression of  
474 CT and TCP is activated by ToxT, which is regulated by the TcpP-TcpH-ToxR-ToxS  
475 complex of the ToxR regulon (22). In case of the high CT-producing *V. mimicus* strain,  
476 higher transcription of *ctxA* in conjunction with that of *tcpA* and *toxT* corroborates with the  
477 known ToxT-mediated genetic regulation influencing CT production in *V. cholerae*.  
478 Moreover, *ctxA* transcription was correlated with significant induction in the transcription of  
479 the upstream-regulatory genes *toxR/toxS*. In addition to ToxR regulon, the histone-like  
480 nucleoid structuring protein (H-NS) encoded by *hns*, a global prokaryotic gene regulator, has

481 been shown to repress the transcription of several virulence genes including *toxT*, *ctxAB* and  
482 *tcpA* in *V. cholerae* (41). However, the variation in CT production in *V. mimicus* strains is not  
483 influenced by the H-NS since its transcription did not show considerable change in parallel to  
484 that of *ctxAB*. Therefore, the gene transcription results indicate that the ToxR/ToxS, in  
485 conjunction with ToxT, controls the CT production in *V. mimicus* strains. The ToxR regulon  
486 is thought to be controlled by environmental stimuli, such as temperature, pH and osmolarity  
487 (26). Hence, understanding the precise genetic and physiological mechanisms behind the very  
488 low or high level of CT production in environmental *V. mimicus* strains requires more  
489 extensive research, which is beyond the scope of this study.

490 **Probable role of *V. mimicus* in the evolution of CTX $\Phi$ .** The presence of the  
491 recombinase XerC and XerD binding sequences at both ends of the pre-CTX $\Phi$ <sup>Env</sup> and  
492 CTX $\Phi$ <sup>ET</sup> prophages support the phage-mediated integration events of these external genetic  
493 elements. The lack of CTX $\Phi$  element in some *tcpA*-positive O1 and non-O1/non-O139  
494 strains supports the hypothesis that *tcpA* is acquired first and then integration of *ctxAB* genes  
495 happens during the evolution of pathogenic *V. cholerae* from their non-pathogenic  
496 progenitors (42). On the other hand, special forms of the CTX $\Phi$  family, designated as pre-  
497 CTX $\Phi$ , do not carry *ctxAB* but contain other genes considered to be CTX $\Phi$  precursors (6).  
498 The step-wise occurrence of RS1-CTX<sup>ET</sup>-RS1-CTX<sup>Env</sup>-CTX<sup>Env</sup> indicates an evolutionary  
499 signature of CTX $\Phi$  insertion events in *V. mimicus*. The observed prevalence of novel types of  
500 *ctxAB*, *tcpA*, *toxT*, and *orfU*, indicates that CTX prophage on *V. mimicus* genome might have  
501 evolved independently of the 7<sup>th</sup> pandemic El Tor clones, probably through independent  
502 integration of pre-CTX $\Phi$ <sup>Env</sup>, in duplicate, and then a primeval CTX $\Phi$ <sup>ET</sup>. The absence of RTX  
503 and TLC elements, which are usually located on the flanks of the CTX element in *V. cholerae*  
504 El Tor strains, also support this assumption for the environmental *ctx*<sup>+ve</sup> *V. mimicus* strains.  
505 Similarly, these *V. mimicus* strains were devoid of the VSP I and VSP II genes cluster of

506 pandemic El Tor strains of *V. cholerae*. Comparative whole genome sequence analysis also  
507 indicates horizontal transfer of virulence-related genes from an uncommon clone of *V.*  
508 *cholerae*, rather than the seventh pandemic strains, may have generated the pathogenic *V.*  
509 *mimicus* strain carrying *ctx* genes (9, 43). This is further supported by the observations of this  
510 study showing existence of five heptamer (TTTTGAT) repeats in the promoter region of  
511 *ctxAB* in *V. mimicus*, a characteristic genomic signature of the classical O1 strain isolated  
512 during 1960s, while the El Tor O1 strains, isolated since 1970s onward, contained four  
513 heptamer repeats (27). Therefore, the evolution of different types of CTX $\Phi$  not only involves  
514 their integration into the epidemic strains of *V. cholerae* O1 but also environmental *tcp*<sup>+ve</sup> *V.*  
515 *mimicus*. Based on the results of this study and previous reports of others, a hypothetical  
516 evolutionary map for the genomic drift associated with pathogenic traits in *V. mimicus* and *V.*  
517 *cholerae* has been depicted in Fig. 5.

518 Isolation of clonally related *V. mimicus* strains with almost identical PFGE pulsotypes  
519 during different months of the year indicates their unique ancestral origin and high adaptation  
520 capacity in the estuarine environment. The microcosm results also support this notion as it  
521 shows prolong persistence of the *ctx*<sup>+ve</sup> *V. mimicus* strains at least in culturable form, similar  
522 to *V. cholerae*, which are usually observed to co-occur in estuaries. This kind of *V. mimicus*  
523 strains, therefore, probably serves a cryptic but important natural reservoir of the CTX $\Phi$ ,  
524 TCP and related virulence genes. In the aquatic environment, lytic phage mediated transfer of  
525 virulence gene from classical *V. cholerae* to *V. mimicus* strain may have also happened  
526 through natural transformation in aquatic microhabitats including chitinous surface and  
527 biofilm (44). The probable influence of environmental *V. mimicus* on the ongoing population  
528 shift of typical El Tor strains to hybrid El Tor strains carrying classical and variant type *ctxB*  
529 cannot be overruled. However, clinical and environmental surveys until now have mainly  
530 focused on the detection of *ctx*<sup>+ve</sup> *V. cholerae* strains. Thus, the cryptic existence of pre-

531 CTX $\Phi$  in both the *V. cholerae* and *V. mimicus* populations, which might provide significant  
532 evolutionary signatures regarding evolution of CTX $\Phi$  and TCP element, has been so far  
533 neglected.

534 **Conclusion.** It can be inferred that certain clonally related environmental *V. mimicus*  
535 strains can act as reservoir of variant *ctxB*, designated as genotype 14, which is  
536 phylogenetically more close to the currently predominant genotypes 1 and 7 associated with  
537 cholera outbreaks worldwide. This study provides molecular insight into the virulence  
538 potential of *ctx*<sup>+ve</sup> *V. mimicus* strains, which could potentially serve as reservoirs of not only  
539 novel or variant type of *ctxAB*, but also *tcpA*, *toxT*, and *orfU* in the estuarine environment.  
540 The genomic content of tandemly arranged multiple pre-CTX $\Phi$ <sup>Env</sup> and a CTX $\Phi$ <sup>ET</sup> with novel  
541 classical type *ctxAB* probably act as salient raw materials for the natural recombination  
542 events driving the evolution of virulence genes related to CT production. Though CT  
543 production in some of this kind of environmental *V. mimicus* strains can be naturally  
544 attenuated, they may be potentially toxigenic in favourable conditions and can instigate  
545 cholera-like diarrhea. The variation in CT production capacity in *V. mimicus* is shown here to  
546 be controlled by the ToxR regulon, which is influenced by the physicochemical changes in  
547 the environment. The cryptic existence of the virulence genes related to CT production in *V.*  
548 *mimicus* genome points out an unnoticed event in the evolutionary pathway of CTX $\Phi$   
549 ecology and cholera epidemiology. Systematic environmental surveillance of non-epidemic  
550 strains, including *V. mimicus* and *V. cholerae*, and their detail molecular genetic analysis  
551 would allow us better understanding the evolution of new variant *ctx* element, and CTX $\Phi$ , as  
552 well as the genes that regulate them.

## 553 MATERIALS AND METHODS

554           **Bacterial strains and their antimicrobial susceptibility.** Six *ctx*<sup>+ve</sup> *V. mimicus*  
555 strains (Table 1) were obtained from the culture collection of Environmental Microbiology  
556 Laboratory of ICDDR,B. These strains were isolated during post-monsoon and early-winter  
557 months in 2000 from the Karnaphuli River estuary, Bangladesh. The *ctx*<sup>+ve</sup> strains were  
558 screened from 1600 presumptive *V. mimicus* colonies, grown on thiosulfate citrate bile salts  
559 sucrose (TCBS) agar after enrichment of environmental samples in alkaline peptone water  
560 (pH 8.0) (APW). All strains were grown in Luria–Bertani (LB) broth, and their identity was  
561 verified according to standard protocol (45). Strains stored as glycerol stock at -80°C were  
562 grown in APW and subsequently on TCBS agar (Difco), Gelatin Agar (Difco), and LB at  
563 37°C whenever needed. Several reference strains of *V. cholerae*, i.e., N16961 and O395,  
564 representing the El Tor and classical biotypes, respectively, VCE233 and AS522, non-  
565 O1/non-O139 strains containing environmental and Calcutta type CTX prophages, SG6, a  
566 Type III Secretion System (TTSS)-positive non-O1/non-O139, GP156, a *stn*-positive O1 El  
567 Tor and C9, a *chxA*-positive non-O1/non-O139, and *V. mimicus* ATCC 33653<sup>T</sup> were used as  
568 controls. Each of the *ctx*<sup>+ve</sup> *V. mimicus* strains were examined for resistance to some  
569 commonly used antibiotics (Table 1) by disc diffusion method according to the Clinical and  
570 Laboratory Standards Institute (<http://www.clsi.org>) using Mueller-Hinton agar (Difco  
571 Laboratories, MI, USA) and commercially available discs (Oxoid, Hampshire, England).

572           **Pulsed-field gel electrophoresis (PFGE).** PFGE was performed according to the  
573 Pulse Net USA protocol (<http://www.cdc.gov/pulsenet/protocols.htm>) with slight  
574 modifications. Briefly, freshly grown of *V. mimicus* strains were embedded into 1% Seakem  
575 Gold agarose followed by lysis of the cells with 0.5 mg mL<sup>-1</sup> Proteinase K (P8044-5G,  
576 Sigma) and 1% Sarcosine (Sigma) at 54°C for 1 h. Agarose blocks containing genomic DNA  
577 were digested with *NotI* and *SfiI* (30 and 40 U, respectively; Takara Bio Inc, Otsu, Japan)  
578 using appropriate buffer at 37°C for 3 h. DNA fragments were electrophoresed in 1% pulsed-



579 field certified agarose gel (BioRad) using a CHEF MAPPER (Bio-Rad). Gels were stained  
580 for 30 min, de-stained twice for 15 min each and images were captured using a Gel-Doc 2000  
581 (Bio-Rad). Lambda ladder (Bio-Rad) was used as a molecular mass standard. The PFGE  
582 fingerprints were analyzed by Fingerprinting II software (Bio-Rad).

583 **Colony blot hybridization of virulence related genes.** DNA probes for colony blot  
584 hybridization included the major toxigenic factors, e.g., cholera toxin (*ctxA*), zonula  
585 occuldens toxin (*zot*, part of CTX phage), RS1 element (*rstC*) and *Vibrio* Seventh Pandemic  
586 island (VSP I and II, marker of present pandemic O1 El Tor biotype), TLC element, and  
587 other known virulence genes of *V. cholerae*, namely, *vcsN2*, *chxA*, *stn*, and *rtxA*, encoding  
588 Type III secretion system, cytotoxic cholix toxin, heat stable enterotoxin, and repeat in toxin,  
589 respectively. DNA templates of reference *V. cholerae* strains were subjected to PCR targeting  
590 the above mentioned virulence related genes. Standard PCR reaction mixture was prepared,  
591 applying primers for the toxigenic genes as mentioned in Table S5. PCR amplified genes  
592 were labeled by random priming with [ $\alpha$ -<sup>32</sup>P]-dCTP (370 MBq mmol<sup>-1</sup>) using Multi-Prime  
593 DNA Labeling System (GE Healthcare, Buckinghamshire, UK). Environmental *V. mimicus*  
594 strains were grown on nitrocellulose membrane, overlaid on LB agar at 37°C for 4-6 h, and  
595 subjected to colony blot hybridization following the procedure described by Yamasaki *et al.*  
596 (46). Radioactivity in the hybridized membrane was detected using BAS FLA-3000 system  
597 (Fuji film, Tokyo, Japan).

598 **PCR based typing of virulence genes and CTX phage element.** Template DNA  
599 was prepared by standard boiling method and stored at -30°C until use. The mismatch  
600 amplification mutation assay (MAMA)-PCR (47) was employed to detect *ctxB* genotype in *V.*  
601 *mimicus* strains to define their potential of classical or El Tor type CT production. The  
602 presence of the RS1 element was determined by the *rstC* gene-based PCR (17). Genotypes of  
603 *rstR*, namely, classical, El Tor, Calcutta and environmental, were also determined by PCR

604 using a newly designed primer set for the environmental type, and previously established  
605 protocols for others (19). The genotypes of *tcpA*, belonging to the VPI, were also checked by  
606 PCR using previously established methods (48). Details of the primers and PCR conditions  
607 for screening these genes are mentioned in Table S5.

608 **Southern hybridization and PCR arrays to understand genetic organization of**  
609 **CTXΦ.** Southern hybridization of *BglI* and *BglII* digested gDNA of *V. mimicus* strains were  
610 carried out with probes for selected virulence related genes, including *ctxAB*, *rstR<sup>ET</sup>*, *rstR<sup>Env</sup>*,  
611 and *rstC*. Briefly, 5-μg aliquots of total gDNA were digested with the restriction enzymes  
612 using appropriate buffer and electrophoresed in 0.8% pulsed-field certified agarose gel  
613 (BioRad) using a CHEF MAPPER (BioRad). Once separated, the gDNA fragments were  
614 subjected to Southern transfer and blotted onto nylon membranes (Hybond-N<sup>+</sup>; Amersham).  
615 The genomic blots were hybridized with the gene probes, labeled by random priming with [ $\alpha$ -  
616 <sup>32</sup>P]-dCTP (370 MBq mmol<sup>-1</sup>), and autoradiographed as described previously (12). In order to  
617 verify the genetic organization of CTXΦ and associated elements, a series of PCR arrays  
618 were performed using the forward and reverse allelic primers, with multiple combinations, of  
619 genes *rstC*, *rstR<sup>ET</sup>*, *rstR<sup>Env</sup>*, *ctxAB* and *orfU* as shown in Fig. 1 using respective primers (Table  
620 S5).

621 **Nucleotide sequencing and phylogenetic analysis of virulence genes diversity.** *V.*  
622 *mimicus* strains were subjected to nucleotide sequencing analysis for several target virulence  
623 genes of CTXΦ, including *ctxAB*, *orfU*, *rstR*, and associated flanking regions comprising *zot*,  
624 intergenic regions (ig-1 and ig-2), and of TCP element, namely, *tcpA* and *toxT*. Briefly, PCRs  
625 using primers (Table S5) targeting these virulence genes and flanking regions were conducted  
626 following standard protocols. The amplified products were purified using QIAquick  
627 Purification Kit (QIAGEN GmbH, Hilden, Germany), then cycle sequencing was carried out  
628 using BigDye Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's

629 instruction (Applied Biosystems). Afterwards, a further purification was done using  
630 CleanSEQ (Agencourt Bioscience), and nucleotide sequences were determined by an ABI  
631 PRISM 3100 Avant Genetic Analyzer (Applied Biosystems). The obtained gene sequences  
632 were assembled and aligned by DNA Lasergene software (DNASTAR, WI, USA).  
633 Homology search was performed using the BLAST program  
634 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the nucleotide and deduced amino acid  
635 sequences were compared with published genes. Phylogenetic tree was constructed using  
636 ClustalW algorithm to understand the genetic lineage and sequence diversity within the study  
637 strains and other representative sequences of target gene of *V. mimicus* and *V. cholerae*  
638 published in GenBank.

639 **Microcosm experiments.** Microcosm experiments were conducted to understand the  
640 competitive survival of *ctx*<sup>+ve</sup> *V. mimicus* with predominant vibrios in water. Surface water  
641 samples, collected at the three isolation sites of *ctx*<sup>+ve</sup> *V. mimicus* strains in the Karnaphuli  
642 estuary, with different salinities, i.e., 11.5, 3.5 and 0.1 ppt, and pH between approx. 7.6 and  
643 8.0, were filter sterilized. Three microcosm sets, representing the isolation sites environment,  
644 were prepared in triplicate, each with 250 mL of sterile estuarine water in glass conical flasks  
645 (500 mL). One representative strain of each of *ctx*<sup>+ve</sup> *V. mimicus*, *V. cholerae* O1, and *V.*  
646 *parahaemolyticus*, isolated from the same estuary, was added at  $\sim 10^5$  CFU mL<sup>-1</sup> to each  
647 microcosm and incubated at 25 °C. At regular intervals 100  $\mu$ L sample was plated on  
648 Tauracholate Tellurite Gelatin Agar (TTGA, pH 7.5) and culturable vibrios were enumerated  
649 in triplicate following standard procedures. Colonies of the three species were differentiated  
650 according to their different size and morphology, biochemical test for sucrose utilization, and  
651 serology with specific antiserum for *V. cholerae* O1. Median ( $n = 3$ ) counts of the culturable  
652 populations of each *Vibrio* species were compared.

653           **Measuring CT production by bead enzyme-linked immunosorbent assay (bead-**  
654 **ELISA).** The *ctx*-positive *V. mimicus* strains were grown in AKI-medium (pH 7.4) and Luria  
655 broth (L-broth, pH 6.6) (Difco, KS, USA) for 12 h at 37 and 30 °C, respectively, to compare  
656 their CT production in conditions favorable for the El Tor and classical strains of *V. cholerae*  
657 (31, 49). Subsequently, the OD<sub>600</sub> nm of the bacterial cultures were adjusted to 1.0, followed  
658 by 100-fold dilution in respective media and incubation at stationary and shaking conditions,  
659 for 4 h each, at 180 rpm (49). The cell free supernatant (CFS) of each culture was prepared by  
660 centrifugation at 12,000 xg for 10 min followed by filtration through 0.22 µm filter (IWAKI,  
661 Tokyo, Japan). The CFS from each culture was diluted 10, 100 and 500 times with phosphate  
662 buffered saline (10 mM NaCl, pH 7.0) and the produced CT was measured by bead-ELISA.  
663 Purified CT was obtained following methodology described by Uesaka *et al.* (50) and used as  
664 controls for known concentration. Preparation of polyclonal rabbit antisera against CT,  
665 conjugation of Fab' of antitoxin IgG with horseradish peroxidase, and estimation of CT  
666 secreted by each strain bead-ELISA were done according to Oku *et al.* (51). All experiments  
667 were done in triplicate.

668           **Detection of pathogenic potential *in vivo*.** Two strains of *ctx*<sup>+ve</sup> *V. mimicus* showing  
669 similar PFGE pulsotype but producing high and low CT, as detected by ELISA, were selected  
670 for evaluating enterotoxigenic potential *in vivo*. Suckling mice assay (SMA), using three-day-  
671 old Swiss albino suckling mice, was performed according to standard procedures (52).  
672 Briefly, an aliquot (0.1 mL) of freshly grown bacterial culture in LB medium, and also its  
673 filtrate (using 0.2 µm filter), was mixed with Evans Blue (0.01%, w/v) and intragastrically  
674 inoculated into each suckling mouse. Approximately 10<sup>7</sup> CFU was inoculated as normal dose,  
675 however, higher and lower dose for low and high CT producer, respectively, were also  
676 administered. After 6 h of incubation, their intestines were removed, pooled and weighed.  
677 Fluid accumulation score in SMA was expressed as the ratio of weight of the intestine to the

678 remaining body weight and a ratio of  $\geq 0.08$  was considered as positive. Culture filtrates of  
679 the reference strains of *ctx*<sup>+ve</sup> *V. cholerae* O1 (O395), and *ctx*<sup>-ve</sup> *V. mimicus* (ATCC 33653<sup>T</sup>)  
680 were used as positive and negative controls, respectively. Pathogenic potential of each strain  
681 was verified using five and three mice for live cells and culture filtrates, respectively.

682 **RNA isolation and qRT-PCR assay.** The *ctx*<sup>+ve</sup> *V. mimicus* strains, expressing high  
683 and low CT, were freshly grown up to the mid-logarithmic phase ( $\sim 10^8$  CFU mL<sup>-1</sup>) in LB  
684 medium following the classical condition of CT production (49). Total RNA was extracted  
685 and purified using Trizol reagent (Gibco-BRL, NY) according to the manufacturer's  
686 instructions. The qRT-PCR assay was carried out with the primers and probes for genes,  
687 namely *ctxA*, *tcpA*, *toxT*, *toxR*, *toxS*, *tcpP*, *tcpH* and *hns*, which are known to regulate CT  
688 production and a housekeeping *recA* gene as an internal control (Table S5) following the  
689 TaqMan probe method. Each probe was labeled with FAM and TAMRA as 5'-reporter, and  
690 3'-quencher dyes, respectively. Reverse transcription for cDNA synthesis from RNA template  
691 (1  $\mu$ g) was carried out using the quick RNA-cDNA kit (Applied Biosystems Inc., CA)  
692 according to the manufacturer's instruction. Real-time PCR was carried out using the  
693 amplified cDNA and TaqMan Gene Expression master mix containing each set of primer and  
694 probe (Applied Biosystems Inc.). PCR conditions were 50 °C for 2 min, 95 °C for 10 min and  
695 40 cycles, each having 95 °C for 15 sec and 60 °C for 1 min, in an ABI PRISM 7000  
696 sequence detection system (Applied Biosystems Inc.). The relative transcription in  
697 comparison with the internal control was analyzed according to Hagihara *et al.* (53).

698 **Statistical analysis.** Statistica (ver. 10.0, StatSoft, Oklahoma, USA) was used to  
699 explore the differences between the mean values applying Student's two-sample *t*-test. A *p*-  
700 value of <0.05 was considered as significant.

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710 data. ZHM and MSI performed field studies. SPA, MA and AH helped design the study and  
711 participated in laboratory experiments. SBN, NC and SY wrote the draft of manuscript. All  
712 authors read and approved the final manuscript.

713 **Conflict of interest.** The authors declare that there is no conflict of interest.

714

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897 **Table 1.** Antimicrobial susceptibility, cholera toxin production, PFGE pattern and  
 898 enterotoxigenicity in *ctx*<sup>+ve</sup> *V. mimicus* strains

Strain ID <sup>1</sup>	Date of Isolation	Antimicrobial resistance <sup>2</sup>						CT production <sup>3</sup> (ng mL <sup>-1</sup> )		PFGE pattern		Suckling mice assay <sup>4</sup> (n = 5)	
		PB 50	CF 30	EM 15	ABPC 10	GM 10	Others <sup>#</sup>	LB	AKI	S/II	NotI	FA ratio	Diarrhea
Vm1	17-Jul-00	R	R	I	R	S	S	0.3	0.1	I	a	nd	nd
Vm2	05-Aug-00	R	R	I	R	S	S	0.4	0.2	II	a	0.068	0 / 5
Vm5	22-Aug-00	R	R	I	R	S	S	0.2	0.1	I	b	nd	nd
Vm6	11-Sep-00	S	R	I	R	I	S	0.2	0.1	III	b	nd	nd
Vm7	03-Oct-00	S	R	I	R	I	S	110	30	II	b	0.087	5 / 5
Vm8	27-Oct-00	S	R	I	R	I	S	0.5	0.4	II	a	nd	nd
V.c. 0395	1948	S	I	S	R	S	S/R	270	150			0.098	5 / 5
V.c. N16961	1975	R	S	S	I	S	S/R	1.6	2.5			nd	nd

899

900 <sup>1</sup>V.c. O395 and N16961 representing *V. cholerae* reference strains of classical and El Tor  
 901 biotypes, respectively.

902 <sup>2</sup>PB, CF, EM, ABPC, and GM indicate polymixin B, streptomycin, cephalothin,  
 903 erythromycin, ampicilin, and gentamicin, respectively. Units (µg) of antimicrobials used are  
 904 mentioned in parenthesis. S, R and I designate susceptibility, resistant and intermediate  
 905 pattern.

906 <sup>#</sup>other antibiotics, e.g., furazolidon, trimethoprim/sulfamethoxazole, nalidixic acid,  
 907 ciprofloxacin and tetracycline were also used at standard doses, i.e., 100, 1.25/23.75, 10, 5,  
 908 and 30 µg, respectively.

909 <sup>3</sup>CT production was measured by bead-ELISA after cells were cultured (4 h static + 4 h  
 910 shaking, 120 rev min<sup>-1</sup>) in Luria Broth and AKI medium representing inducible conditions for  
 911 classical and El Tor types, respectively. Mean values are given based on three experiments  
 912 for each strain.

913 <sup>4</sup>Fluid accumulation ratio in suckling mice assay >0.08 indicates enterotoxigenic; mean  
 914 values (n = 5) are given; ‘nd’, not done.

915

916 **Table 2.** Comparative diversity in *ctxAB* gene among *V. mimicus* and *V. cholerae*

Strains <sup>1</sup>	Isolation		<i>ctxA</i> (aa positions)					<i>ctxB</i> (aa positions) <sup>2</sup>								Designated Genotype	Reference		
	Country	Year	46	190	198	226	255	20	24	28	34	36	<b>39</b>	46	55			67	<b>68</b>
VC O1, CL, O395 (CP000627)	India	1948	S	R	I	V	K	H	Q	D	H	T	H	F	K	A	T	1	[1]
VC O1, Australia ET	Australia		-	-	-	-	-	H	Q	D	H	T	H	L	K	A	T	2	[39]
VC O1, ET, N16961 (NC_002505)	Bangladesh	1975	S	R	I	V	K	H	Q	D	H	T	Y	F	K	A	I	3	[39]
VC O139 (FJ821557)	Bangladesh	1998	-	-	-	-	-	H	Q	D	H	T	Y	F	K	A	T	4	[39]
VC O139 (FJ821556)	Bangladesh	2005	-	-	-	-	-	H	Q	A	H	T	H	F	K	A	T	5	[39]
VC O139 (FJ821581)	Bangladesh	2007	-	-	-	-	-	H	Q	D	P	T	Y	F	K	A	T	6	[39]
VC O1 (EU496273, L19089)	India, Haiti	2007, 2010	-	-	-	-	-	N	Q	D	H	T	H	F	K	A	T	7	[39]
VC O27 (AF390572)	Japan	1996	S	R	I	V	E	H	H	A	H	T	H	F	K	A	T	8	[39]
VC O37 (D30052)	Sudan	1968	N	R	I	V	K	H	Q	D	H	T	H	L	N	A	T	9	[39]
VC O1 (EU932878)	Zambia	1996						H	Q	D	P	T	Y	F	K	A	I	10	[54]
VC O1 (EU932881)	Zambia	2003						H	Q	D	P	T	H	F	K	A	T	11	[54]
VM (ACYV01000039)	USA	1990	N	I	I	I	K	H	Q	D	H	T	H	L	K	A	T	12	[19]
VC O1 (SH65928)	China	1965	-	-	-	-	-	H	Q	D	H	A	Y	L	N	A	T	13	[55]
<b>VM (This study)</b>	Bangladesh	2000	<b>N</b>	<b>I</b>	<b>V</b> *	V	K	H	Q	D	H	T	H	L	K	<b>E</b> *	T	<b>14</b>	This study

917

918 <sup>1</sup>VC, VM, CL and ET represent *V. cholerae*, *V. mimicus*, classical and El Tor, respectively.  
 919 Known serogroups of *V. cholerae* strains are shown, accession number of the gene sequences  
 920 are given in parenthesis.

921 <sup>2</sup>The deduced amino acid (aa) positions are indicated by vertical numbering; bolded 39 and  
 922 68 positions bear the amino acid markers, differentiating classical and El Tor type *ctxB* gene.

923 \*Unique change in deduced amino acid of *ctxAB* in *V. mimicus* strains of this study.

## 924 FIGURE LEGENDS

925 **Fig. 1.** Organization of CTX $\Phi^{\text{El Tor}}$ , RS1 and pre-CTX $\Phi^{\text{Env}}$  in *V. mimicus* strains. (A) Filled  
926 bars indicate PCR arrays used to check probable locations of genes and sizes of PCR  
927 products are given on top. Hashed bars indicate the genetic regions (names mentioned on top)  
928 used as probes for Southern hybridization after restriction digestion with *BglI* or *BglII*  
929 enzymes; arrows indicate RS1, RS2 or core prophage where dotted regions were analyzed by  
930 sequencing. Lines (filled and dotted) in the bottom show the distances between specific  
931 genetic locations determined by Southern hybridization analysis of the *BglI*- or *BglII*-digested  
932 genomic DNA using specific probes. (B) Region between *zot* and *rstR* in pre-CTX $\Phi^{\text{Env}}$  and  
933 CTX $\Phi^{\text{El Tor}}$  in *V. mimicus*. The *ctxAB* promoter of CTX $\Phi^{\text{El Tor}}$  contains 5 heptamer  
934 (TTTTGAT) repeats, shown by filled black arrows, which is characteristic of classical type  
935 *ctxAB*. In the reference El Tor strain, N16961, the attR sequence is also located 106 bp  
936 downstream of *ctxAB*, followed by XerC and XerD.

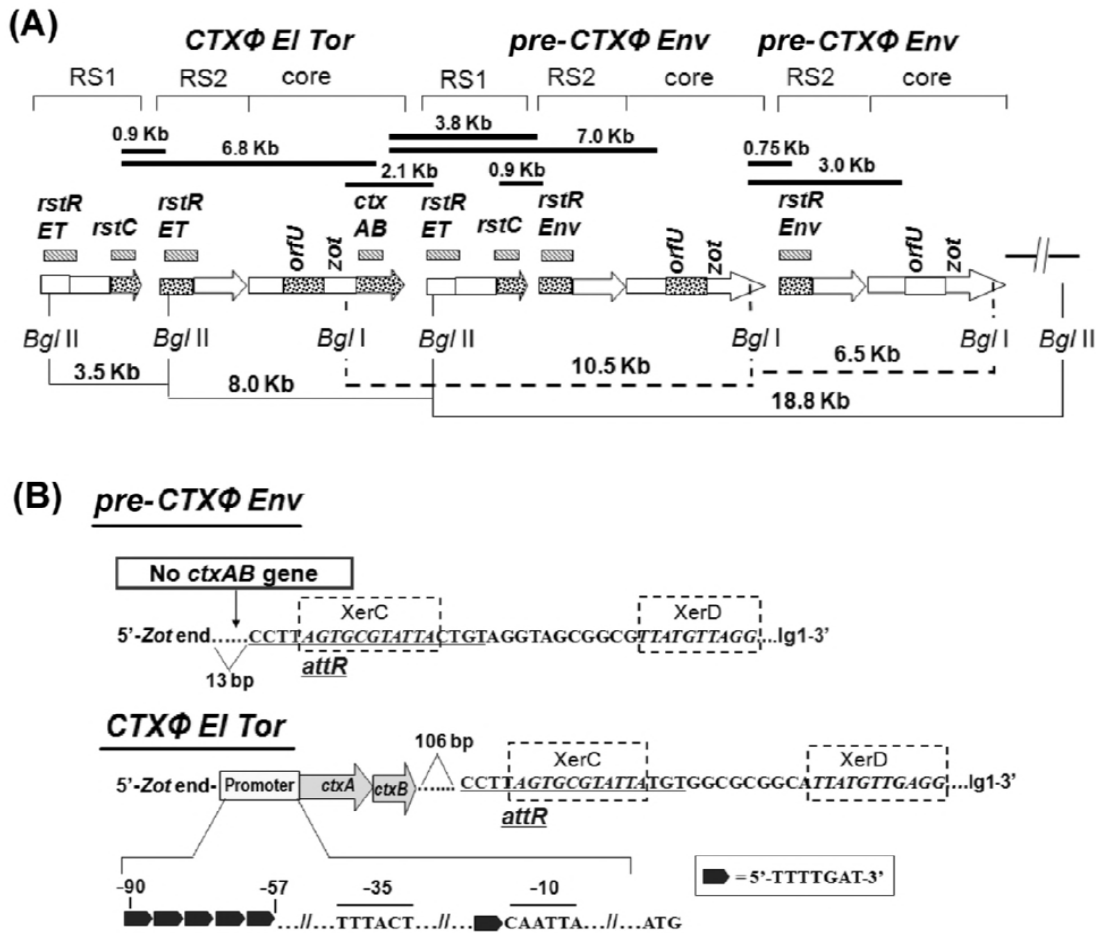
937 **Fig. 2.** Genetic relatedness among *ctxB* and *orfU* genes of *V. mimicus* and *V. cholerae* strains.  
938 (A) The novel *ctxB* genotype 14 of *V. mimicus* showed closeness with both the genotypes 1  
939 and 7, representing classical and Haitian strains, respectively. (B) The *orfU* of *V. mimicus* of  
940 this study showed close affiliation to the strains grouped into the El Tor clade.

941 **Fig. 3.** Genetic relatedness of *tcpA* and *toxT* genes among different serogroup strains of *V.*  
942 *cholerae* and *V. mimicus*. (A) The novel *tcpA* of *V. mimicus* of this study did not cluster in  
943 classical, El Tor, and other types of strains but formed a separate clade showing closeness to  
944 serogroups O56 and O115 strains of *V. cholerae*. (B) The novel *toxT* of *V. mimicus* of this  
945 study did not group into the major cluster comprising the *toxT* of *V. cholerae* O1 classical, El  
946 Tor, O139, non-O1/non-O139 and other *V. mimicus* strains but grouped into a separate cluster  
947 with atypical *toxT* reported in a few non-O1/non-O139 strains.



948 **Fig. 4.** Variation in mRNA transcription of virulence and its regulated genes between a high  
949 and low CT-producing strains of *Vibrio mimicus*. Transcriptional levels of various virulence-  
950 related genes were analyzed by qRT-PCR. The relative transcriptional level of each gene was  
951 normalized with the housekeeping *recA* gene. The mRNA transcription level of each gene in  
952 a low CT-producing strain was compared with that of a high CT-producing strain. The  
953 transcriptional level of each virulence related gene of the high CT-producing strain was  
954 arbitrarily considered as 1 (Relative Arbitrary Unit). Statistically significant differences were  
955 calculated using the two-sample *t*-test. A P-value of <0.05 was considered as significant (\*\*  
956 = P <0.005; \*\* = P <0.01; \* = P <0.05).

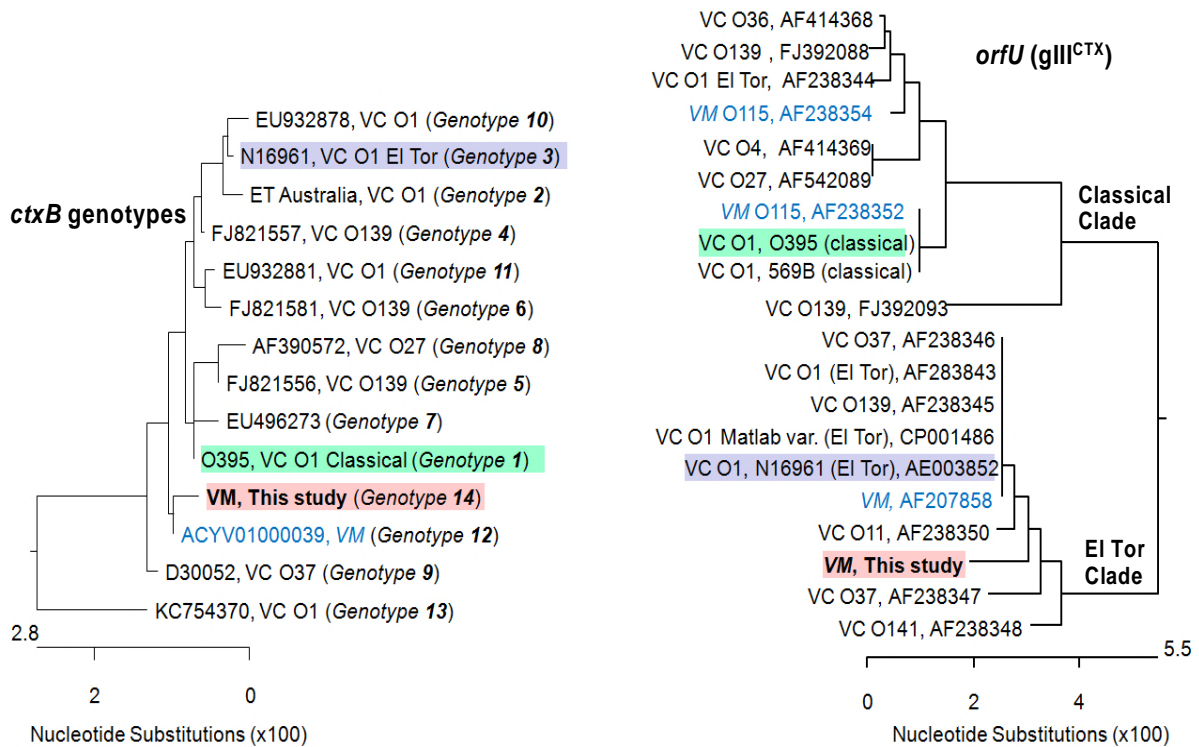
957 **Fig. 5.** A hypothetical scenario of the evolution of CTX and variant virulence genes in *V.*  
958 *cholerae* and *V. mimicus*. Environmental *V. mimicus* may play a salient role by acting as an  
959 important reservoir of variant genes aiding the evolution. Line connectors with arrows  
960 indicate probable routes of origin of *V. mimicus* and *V. cholerae* strains containing *ctxB*  
961 variants. Bacteria, CTX $\Phi$ , and *ctxB* gene are shown in different shapes, with solid line  
962 border. VM, VC, ET, Cla, VSP, and TLC indicate *V. mimicus*, *V. cholerae*, El Tor, Classical,  
963 *Vibrio* Seventh Pandemic island, and Toxin Linked Cryptic element, respectively. In the  
964 bottom, the light blue oval, with dotted border, indicates an interactive environmental pool  
965 facilitating generation of new clones of atypical *V. cholerae* O1 El Tor and *V. mimicus* strains  
966 possessing variant *ctxB* gene.



968 **Fig. 1.** Organization of  $CTX\Phi^{EI Tor}$ , RS1 and  $pre-CTX\Phi^{Env}$  in *V. mimicus* strains. (A) Filled  
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 973 sequencing. Lines (filled and dotted) in the bottom show the distances between specific  
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 975 genomic DNA using specific probes. (B) Region between *zot* and *rstR* in  $pre-CTX\Phi^{Env}$  and  
 976  $CTX\Phi^{EI Tor}$  in *V. mimicus*. The *ctxAB* promoter of  $CTX\Phi^{EI Tor}$  contains 5 heptamer  
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 978 *ctxAB*. In the reference El Tor strain, N16961, the *attR* sequence is also located 106 bp  
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981



982 (A) Phylogenetic diversity among *ctxB*

(B) Phylogenetic relatedness among *orfU*

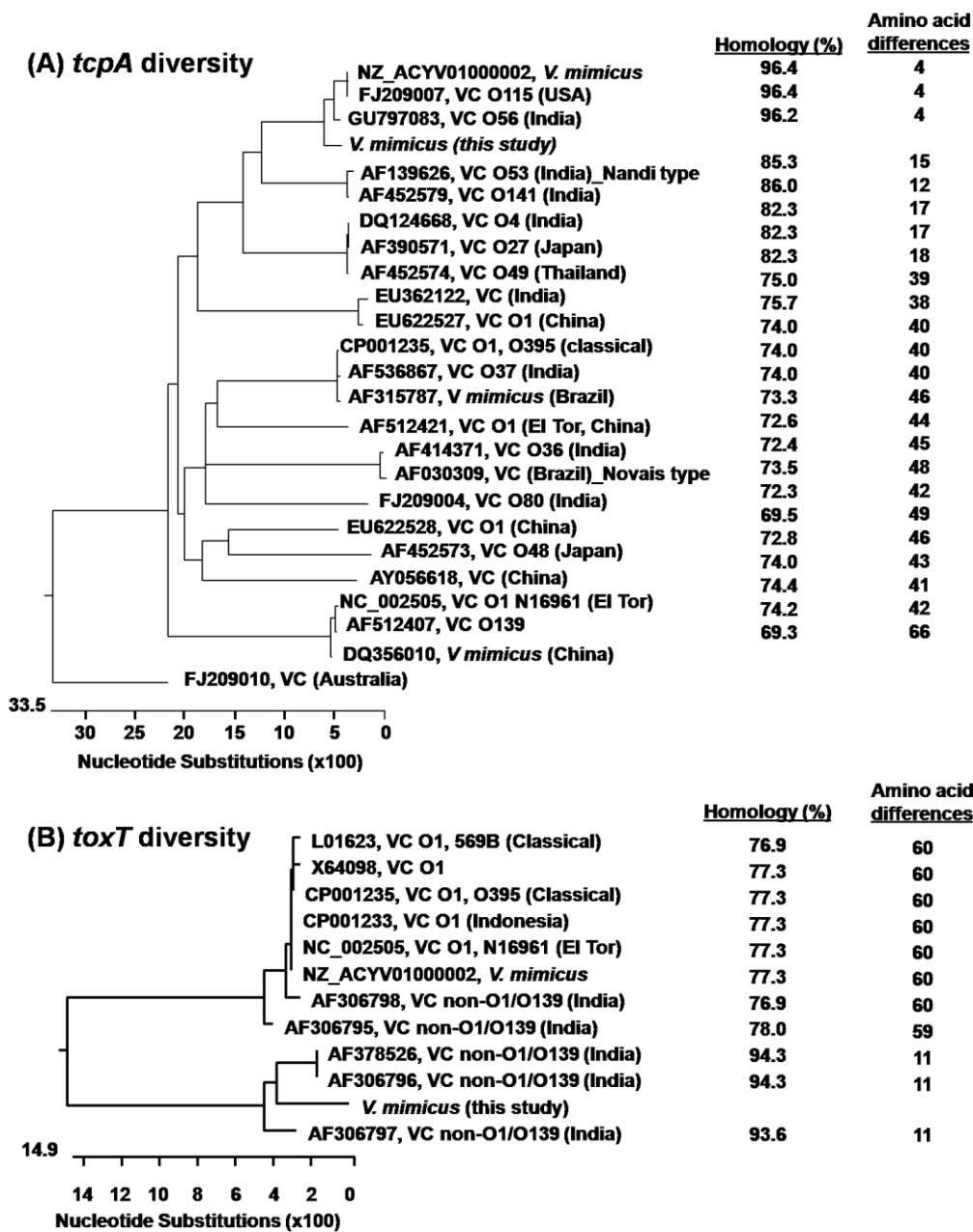
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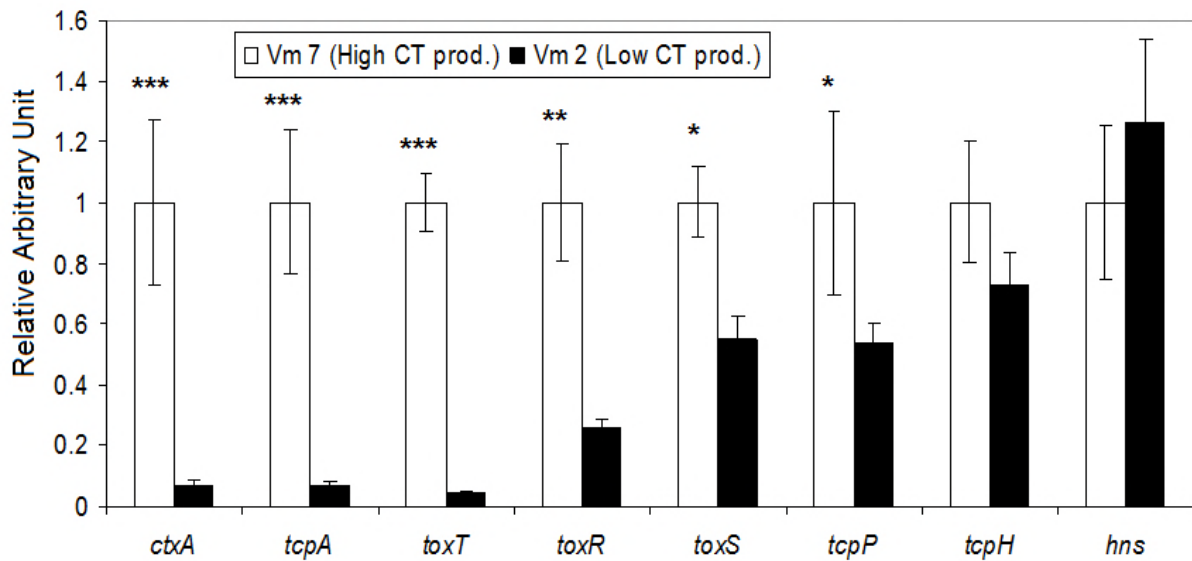


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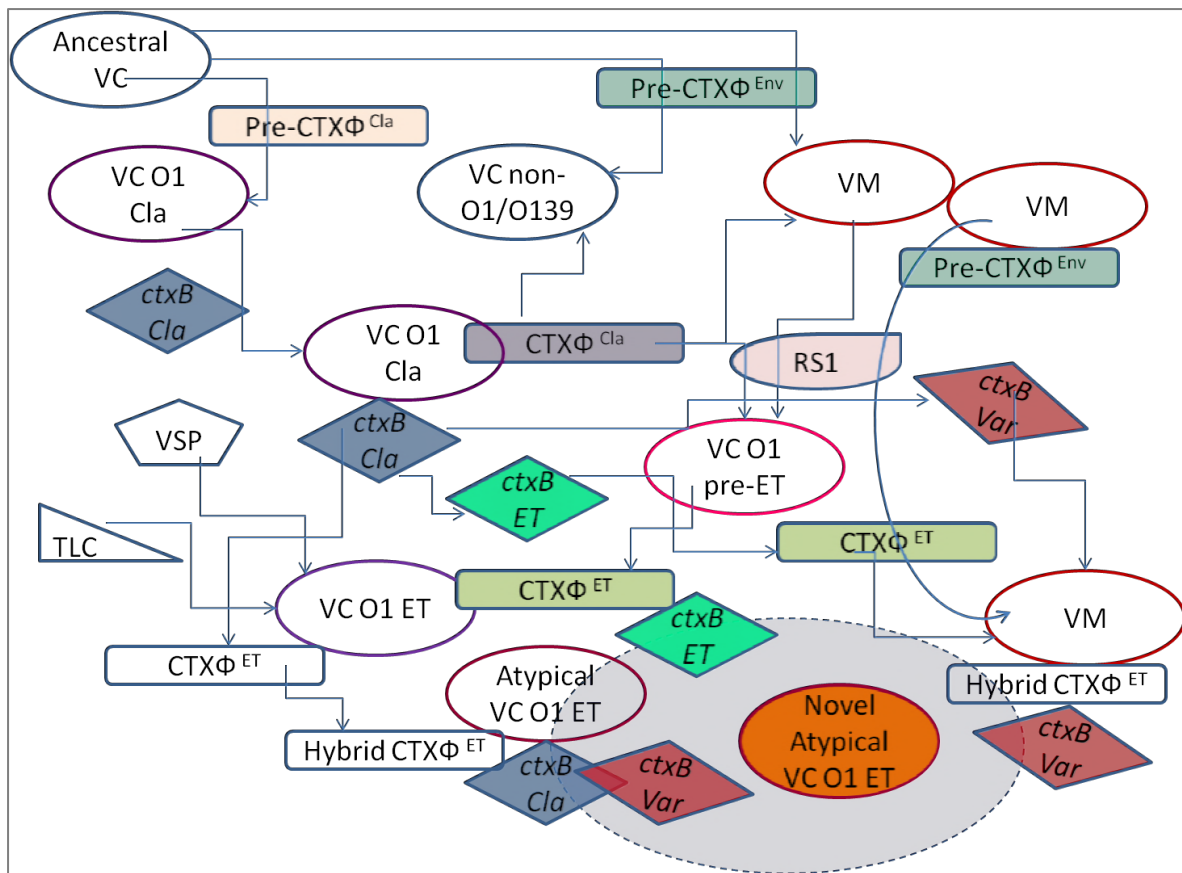


1000

1001 **Fig. 4.** Variation in mRNA transcription of virulence and its regulated genes between a high  
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1005 a low CT-producing strain, Vm 2, was compared with that of a high CT-producing strain, Vm  
1006 7. The transcriptional level of each virulence related gene of the high CT-producing strain  
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1014 **Fig. 5.** A hypothetical scenario of the evolution of CTX and variant virulence genes in *V.*

1015 *cholerae* and *V. mimicus*. Environmental *V. mimicus* may play a salient role by acting as an

1016 important reservoir of variant genes aiding the evolution. Line connectors with arrows

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1018 variants. Bacteria, CTXΦ, and *ctxB* genes are shown in different shapes, with solid line

1019 border. VM, VC, ET, Cla, VSP, and TLC indicates *V. mimicus*, *V. cholerae*, El Tor, Classical,

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1022 facilitating generation of new clones of atypical *V. cholerae* O1 El Tor and *V. mimicus* strains

1023 possessing variant *ctxB* genes.

1024

1025 **SUPPLEMENTARY INFORMATION**

1026 **Fig. S1.** PFGE analysis of environmental  $ctx^{+ve}$  and reference (ATCC) strains of *V. mimicus*  
1027 (VM), and  $ctx^{+ve}$  non-O1/non-O139 (VCE 233), O1 El Tor (VC N16961) and O1 classical  
1028 (VC O395) strains of *V. cholerae*. Left gel image, PFGE profiles of undigested gDNA  
1029 showing similar size of the two chromosomes of  $ctx^{+ve}$  VM and ATCC VM strains. The  
1030 middle and right gel images, PFGE patterns of *NotI*- and *SfiI*-digested gDNA of  $ctx^{+ve}$  VM  
1031 and the reference strains. The  $ctx^{+ve}$  VM strains were clonal but differing in 1-2 bands,  
1032 indicated by arrows. *NotI*- and *SfiI*-digested gDNA of  $ctx^{+ve}$  VM strains generated two (a and  
1033 b) and three (I, II and III) PFGE profiles. Taken together, four PFGE profiles (Ia, Ib, IIa, IIb,  
1034 and IIIb) could be distinguished among the six  $ctx^{+ve}$  VM strains. MW, molecular weight,  
1035 representing the lambda ladder (Bio-Rad).

1036 **Fig. S2.** PCR detection of the *rstC* (RS1),  $rstR^{El\ Tor}$ ,  $rstR^{Calc}$ ,  $rstR^{Cla}$  and  $rstR^{Env}$  genes, and the  
1037 presence or absence of *ctxAB* in the El Tor type CTX $\Phi$  and environmental type CTX $\Phi$  in *V.*  
1038 *mimicus* (Vm) strains. *V. cholerae* (Vc) strains belonging to O1 El Tor (N16961), O1classical  
1039 (O395), and non-O1/O139 (AS522 and VCE233) were used as controls. Environmental *V.*  
1040 *mimicus* strains were positive for *rstC* (RS1 element),  $rstR^{El\ Tor}$ , and  $rstR^{Env}$  genes but did not  
1041 contain  $rstR^{Calc}$  and  $rstR^{Cla}$  genes. Similar to a *V. cholerae* non-O1/O139 strain, VCE233, all  
1042 of the environmental *V. mimicus* strains contained *ctxAB* in the El Tor type CTX $\Phi$  but did not  
1043 possess any *ctxAB* in the environmental type CTX $\Phi$ .

1044 **Fig. S3.** Probable genetic organization of El tor and environmental types of CTX $\Phi$ , and RS1  
1045 element, deduced by comparison of the restriction map of the marker genes, i.e., *ctx*,  $rstR^{El}$   
1046  $Tor$ ,  $rstR^{Env}$ , and *rstC*, respectively, in *V. mimicus* strains. Top panel: autoradiographed images  
1047 of gDNA, of *V. mimicus* strains, digested by restriction enzymes (*BglI* or *BglII*) and detected  
1048 by  $^{32}P$ -labelled PCR products of the marker genes. Bottom panel: a schematic diagram with

1049 location of RS1, RS2 and Core of the CTX prophages, with lines (filled and dotted) showing  
1050 the distance between the restriction sites, and bars mimicking the results of Southern  
1051 hybridization using different probes. Taken together, the results indicated an array of RS1 -  
1052 CTX $\Phi^{\text{El Tor (ET)}}$  (with *ctxAB*) - RS1 - CTX $\Phi^{\text{Env}}$  (without *ctxAB*) - CTX $\Phi^{\text{Env}}$  (without *ctxAB*).

1053 **Fig. S4.** Comparative variations in deduced amino acids of *orfU* gene sequences in selected  
1054 *V. mimicus* and *V. cholerae* strains. Sequences were aligned by ClustalW algorithm. Amino  
1055 acid positions are shown as a heading scale. Strain details are shown on the right border at  
1056 each row. In comparison to *V. mimicus* strain in this study, only mismatched amino acids of  
1057 *orfU* genes in other selected strains are shown while their identical amino acids are indicated  
1058 by dots.

1059 **Table S5.** Primers and probes used in this study.

1060 **Fig. S6.** Genetic diversity of amino acid residues in the novel variant *tcpA* in *V. mimicus*  
1061 strains of this study in comparison to that of the selected reference strains of *V. cholerae*.  
1062 Sequences were aligned by ClustalW algorithm. Amino acid positions are shown as a heading  
1063 scale. Strain details are shown on the right border at each row. In comparison to *V. mimicus*  
1064 strain in this study, only mismatched amino acids of *tcpA* genes of other selected strains are  
1065 shown while their identical amino acids are indicated by dots.

1066 **Fig. S7.** Variation in amino acid residues in the novel variant *toxT* in *V. mimicus* strains of  
1067 this study in comparison to other *toxT* genes in selected reference strains of *V. cholerae*.  
1068 Sequences were aligned by ClustalW algorithm. Amino acid positions are shown as a heading  
1069 scale. Strain details are shown on the right border at each row. In comparison to *V. mimicus*  
1070 strain in this study, only mismatched amino acids of *toxT* genes in other selected strains are  
1071 shown while their identical amino acids are indicated by dots.



1072 **Fig. S8.** Competitive survival of *ctx*<sup>+ve</sup> *V. mimicus* (Vm2), *V. cholerae* O1 and *V.*  
1073 *paraheamolyticus* strains co-cultured in microcosm water having different salinities and pH.  
1074 Filter sterilized water of three estuarine sites where *V. mimicus* strains were isolated was used  
1075 in microcosm. Water samples of sites 1, 2 and 3 had salinity of 11.5, 3.5 and 0.1 ppt,  
1076 respectively, and pH of 8.0, 7.7, and 7.6, respectively.

1077 **Table S9:** Suckling mice assay showing enterotoxigenic potential of the *ctx*<sup>+ve</sup> *V. mimicus*  
1078 strains.