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Genomic characterization of antibiotic resistance-encoding genes in clinical isolates of *Vibrio cholerae* non-O1/non-O139 strains from Kolkata, India; Generation of novel types of genomic islands containing plural antibiotic resistance genes

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Running title: Antibiotic resistance genes of Vibrio

ABSTRACTS

Non-O1/non-O139 nontoxigenic *Vibrio cholerae* associated with cholera-like diarrhea has been reported in Kolkata, India. However, the property involved in the pathogenicity of these strains has remained unclear. We examined the character of 25 non-O1/non-O139 nontoxigenic *V. cholerae* isolated during 8 years from 2007 to 2014 in Kolkata. Determination of serogroup showed that the serogroups O6, O10, O35, O36, O39, and O70 were represented by two strains in each serogroup, and the remaining isolates belonged to different serogroups. To clear the character of antibiotic resistance of these isolates, the antibiotic resistance test and the gene analysis were performed. According to antimicrobial drug susceptibility testing, 13 strains were classified as drug resistant. Among them, 10 strains were quinolone resistant and 6 of 13 strains were resistance against more than 3 antibiotics. To define the genetic background of the antibiotic character of these strains, we determined whole-genome sequences of these strains. From the analysis of these sequences, it becomes clear that all of quinolone resistance isolates have mutations in quinolone resistance-determining regions. Further search on the genome

sequence showed that 4 strains possess class 1 integrons in their genomes, and that three of four integrons are found to be located in their genomic islands. These genomic islands are novel type. This indicates that various integrons containing drug resistance genes are spreading among *V. cholerae* non-O1/non-O139 strains through the action of newly-generated genomic islands.

Key words antibiotic resistance, diarrhea, genome sequence, genomic island, integron, *Vibrio cholerae*,

Introduction

Cholera is a life-threatening acute diarrheal disease caused by toxigenic *Vibrio cholerae* in many developing countries (1). Of the 200 serogroups of *V. cholerae*, pandemics and epidemics of cholera have been caused by only *V. cholerae* O1 and O139. *V. cholerae* belonging to other serogroups are collectively designated as non-O1/ non-O139. Vibrios belonging to these multiserogroups do not have epidemic potential but have caused several diarrheal outbreaks worldwide (2, 3). It has been reported that 5 to 10% of vibriosis in Maryland in the USA has been associated with water-/foodborne *V. cholerae* non-O1/non-O139 strains (4, 5).

However, the number of laboratories which possess the ability to determine the serogroup of *V. cholerae* non-O1/non-O139 is limited (6). Thus, the relationship between the serogroup of *V. cholerae* non-O1/non-O139 and its pathogenicity has remained unclear.

V. cholerae non-O1/non-O139 strains cause cholera-like acute diarrhea, but their virulence and antimicrobial resistance (AMR) mechanism remains also unclear (7, 8). However, for the treatment of patients infected with *V. cholerae* non-O1/non-O139 strains, antibiotics have often been recommended (9). The role of aquatic ecosystems as reservoirs of antibiotic-resistant bacteria and antibiotic resistance genes has been established (10). The possibilities of acquiring AMR in *V. cholerae* are very high, as this bacterium is autochthonous to coastal waters.

The knowledges on the property involved in pathogenicity of *V. cholerae* non-O1/non-O139 strains obtained are not enough for understanding of the pathogenicity of these strains. In order to clear the property, we examined the groups and AMR pattern of 25 *V. cholerae* non-O1/non-O139 strains isolated in Kolkata. Subsequently, AMR-encoding genes of these AMR strains and the genetic

mechanisms to acquire the AMR-encoding genes were examined by analysing the whole-genome sequences of these strains. The examination showed that class 1 integrons encoded in genomic islands (GIs) have been incorporated into the chromosomes of some strains of *V. cholerae*. This indicates that multi-drug resistant *V. cholerae* non-O1/non-O139 strains possessing class 1 integrons will spread in our living environment, under the situation of usage of large amount of antibiotics.

MATERIALS AND METODS

Bacterial identification

V. cholerae non-O1/non-O139 were isolated from diarrheal patients in Kolkata by using standard bacteriological methods. Briefly, the stool samples were cultured in alkaline L-broth (pH 8.5) (Becton, Dickinson and Company, NJ, USA) and then on thiosulphate citrate bile-salt sucrose (TCBS) agar (Eiken Co., Tokyo, Japan) at 37 °C for 15 hours.

Sucrose-fermenting yellow colonies were examined for the oxidase reaction using *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (Sigma Aldrich, MD, USA) as substrate, and the identification of *V. cholerae* was confirmed by growth and characterization on a triple sugar iron agar slant. O1 or O139 serogroup determination was performed with slide agglutination test using commercially available antisera (Denkaseiken Co., Ltd., Tokyo). *V. cholerae* strains showing a negative reaction in the slide agglutination test were subjected to PCR targeting *ompW* for the species confirmation of *V. cholerae* non-O1/non-O139 (11). Confirmed *V. cholerae* non-O1/non-O139 were serogrouped using 206 O group-specific serum prepared by the National Institute of Infectious Diseases of Japan (6).

Antimicrobial susceptibility test

The susceptibility of *V. cholerae* strains to antibiotics was examined by the disc diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2014; 2015) (12, 13) using commercially available discs containing ampicillin (10 µg), meropenem (10 µg), azithromycin (15 µg),

chloramphenicol (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), erythromycin (15 µg), furazolidone (100 µg), gentamicin (10 µg), neomycin (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), ofloxacin (5 µg), sulfamethoxazole-trimethoprim (23.75 µg/1.25 µg), streptomycin (10 µg), and tetracycline (30 µg) (Becton, Dickinson and Company, MD, USA). *Escherichia coli* (ATCC25922) was used as the quality control strain for the assay.

Extraction of genomic DNA, next-generation sequencing and alignment analysis

The procedures and analysis of genomic DNA was performed using previously described method (14). Briefly, genomic DNA was extracted from overnight broth cultures of *V. cholerae* strains using a DNeasy Blood & Tissue Kit (Qiagen Sciences, Maryland, USA) according to the manufacturer's instructions. We prepared Illumina libraries using a Nextera XT DNA Library Preparation Kit (Illumina) and sequenced paired-end Illumina short reads for each library on HiSeq 2500 (Illumina) or MiSeq (Illumina) sequencers. The reads were assembled using the de novo genome assembly program of CLC Genomics Workbench, and a

multicontig draft genome was prepared for each sample. Nucleotide sequence data were submitted to the DDBJ Sequenced Read Archive (DRA), and each accession number is listed in Table S1.

Sequence analysis for identification of antimicrobial resistance genes was performed using BLASTn against contig data. Identification of integron was performed using the Integron Database INTEGRALL (http:// integrall.bio.ua.pt/) (15). A comparative alignment was performed with reference to sequences of PGI1 (*Proteus mirabilis* genomic island 1, GenBank number, KJ411925), AGI1 (*Acinetobacter baumannii* genomic island 1, GenBank number, KP054476), and GI*Vch*Hai8 (*Vibrio cholerae* HE-45 vcoHE45.contig.17, GenBank number, ALED01000018.1).

Ethics statement

This study was approved by the Institutional Ethics Committee (IEC) of the National Institute of Cholera and Enteric Diseases in Kolkata, India (registration number: A-1/2015-IEC). Written consent was obtained from each adult patient or parent/guardian of the child patient enrolled in this study.

RESULTS

Serogroups of non-O1/non-O139 *V. cholerae* and their susceptibility to antibiotics

We analyzed 25 non-O1/non-O139 strains of *V. cholerae* isolated from unrelated patients in this study. Nineteen serogroups were detected among these 25 strains (Table 1). Serogroups O6, O10, O35, O36, O39, and O70 were identified from 2 patients each, whereas the other serogroups (O8, O11, O12, O27, O34, O37, O44, O51, O54, O67, O128, O149 and O178) were identified from a single case of diarrhea. Seven of the 25 strains showed antimicrobial resistance to <4 antibiotics examined, and 6 strains (IDH-01006, IDH-01215, IDH-02653, IDH-02822, IDH-04886, and IDH-05249) showed resistance to >4 antibiotics (Table 1).

Determination of the genome sequence and analysis of genes related to antibiotic resistance

We determined the genomic DNA sequences of these strains by next-generation sequencing methods. Genome sequence accession numbers of *V. cholerae* strains analyzed in this study are shown in supplementary Table 1 (Table S1).

Quinolone resistance gene

As shown in Table 1, resistance to quinolones was prominent among strains examined. Quinolones target DNA gyrase (GyrA and GyrB) and topoisomerase IV (ParC and ParE) of bacteria. The binding region of the quinolone antibiotic is very important for determining the susceptibility of the bacteria, which is designated the quinolone resistance-determining region (QRDR). Mutation(s) in this region has been reported to induce a change in the bacteria from a susceptible to a resistant phenotype (16).

We found 10 quinolone-resistant strains in this experiment and examined the mutation of the gene in the QRDR of these strains. The mutation was detected

at two codons, codons at position 83 (codon; AGT) of GyrA and position 85 (TCG) of ParC. The former codon was changed to ATT which was codon for isoleucine. This mutation (GyrA S83I) was detected in all strains examined (Table 2). As all 10 *V. cholerae* strains were resistant to nalidixic acid, the mutation at one site (83rd) from serine to isoleucine might convert the phenotype from susceptible to resistant.

Another mutation in the codon for serine at position 85 (codon; TCG) of *parC* to encode leucine (codon; TTG) was detected in 8 strains (Table 2). Among these isolates, five strains (IDH-01006, IDH-01215, IDH-02653, IDH-04886 and BCH-03594) showed resistance to norfloxacin and ofloxacin (Tables 1 and 2). This suggests that the structure of amino acid residue at position 85 of ParC is related with the resistance activity of the bacteria against norfloxacin and ofloxacin.

However, three strains, IDH-01793, IDH-02822 and IDH-06582, were sensitive against norfloxacin and ofloxacin, though the mutation of S85L in ParC has generated in these three strains (Tables 1 and 2). On the contrary, the amino acid residue at position 85 of IDH-05249 was not mutated, but the strain was resistant against norfloxacin and ofloxacin (Tables 1 and 2). These indicate that the

mutation (ParC S85L) is not sufficient to induce the resistance of *V. cholerae* non-O1/non-O139 against norfloxacin and ofloxacin. Other activities of the bacteria, such as influx activity and efflux activity of these quinolones, might be deeply involved in the resistance of the bacteria against these quinolones.

Other antibiotic resistance genes

In the whole-genome analysis, the presence of other antimicrobial resistance genes in *V. cholerae* strains was identified by BLASTn analysis. As shown in Table 1, several antimicrobial resistance genes, such as *dfrA* (encoding dihydrofolate reductase for trimethoprim resistance), *sul1* (encoding dihydropteroate synthase for sulfonamide resistance), *floR* (resistance gene for chloramphenicol), *tet* (tetracycline resistance-encoding gene), *bla* $_{\text{CARB-9}}$ (encoding β-lactamase for β-lactam antibiotic resistance), and the *mer* operon (a mercury resistance operon), were detected. There is some correlation between the possession of these antibiotic resistance genes and resistance to antibiotics in *V. cholerae* strains (Table 1).

Sequence-based analysis of transferable AMR genes

Class 1 integron of isolates

To analyze how *V. cholerae* O1 strains acquire AMR genes, we searched for integrase genes in the genome sequences (Table 1). These genes includes *intI1*, *intI2* and *intSXT*. These three integrase genes are components of class 1 integron, class 2 integron and SXT element, respectively. These integrase genes are involved in the horizontal transfer of AMR genes (17, 18). *IntI1* was detected in 4 strains (IDH-01215, IDH-02822, IDH-03944, and IDH-06641), and one strain, IDH-06641, harbored *intSXT*. None of the strains had *intI2*.

Based on this information, the regions encoding integrons of each strain were referred to based on genome sequence (Table S1). Our results showed that the class 1 integrons of IDH-01215 and IDH-03944 included *tni* module genes. Therefore, these class 1 integrons were regarded as members of a In16 family (Fig 1) (19). Additionally, as class 1 integrons of IDH-02822 contained IS*6100*, class 1 integrons of IDH-02822 might be related with In4 family (Fig 1) (19). As the class 1 integron of IDH-06641 contains the gene cassette, *dfrA*15-*qacE*Δ1-*sul*1, the

integron was regarded as the member of In192. As the integron of IDH-03944 also contains the cassette, *dfrA*15-*qacE*Δ1-*sul*1, the integron of IDH-03944, might be a member of In192 (15).

The IS*26*-*mphA*-*mrx*- *mphR*-IS*6100* unit, which was found in the class 1 integron of IDH-02822, has been shown to be a mobile genetic element (MGE) carrying a macrolide-resistance operon (Fig 1) (20). *MphA*, *mrx* and *mphR* encode genes for a phosphotransferase, a factor involved in the expression of *mphA* and a negative regulator of transcription of *mphA*, respectively, and the bacteria expressing these genes usually show macrolide-resistance activity. Here, the strain IDH-02822 was resistant to EM (erythromycin), which is a macrolide (Table 1). Although the other 2 strains, IDH-01215 and IDH-03944, possessed class 1 integrons, they did not contain MGE carrying the macrolide-resistance operon. Therefore, they were sensitive to erythromycin (Table 1). *Int*SXT of IDH-06641 did not contain AMR genes. However, it was evident that the SXT element of IDH-06641 contained a *mer* operon encoding the mercury resistance gene (data not shown).

GIs of isolates possessing class 1 integrons

Although integrons play an important role in the horizontal transfer of AMR genes from host bacteria to recipient bacteria, especially in the insertion of the gene into a replication gene of the recipient cell, the integron itself does not possess the ability to achieve horizontal gene transfer from host bacteria to recipient bacteria (21). To accomplish gene transfer, GIs are often involved in the transfer of integrons (17). We identified the existence of GIs in 4 strains possessing class 1 integrons (IDH-01215, IDH-02822, IDH-03944, and IDH-06641). As two GIs were detected in IDH-02822, they were designated GI of IDH-02822-1 and GI of IDH-02822-2 (Fig 2). These GIs detected in IDH-01215, IDH-02822 and IDH-03944 are located in the VC0003 region of each genome. However, no specific chromosomal sequence was not found in either end of GI in IDH-06641.

Framework of GIs

GIs of IDH-01215, IDH02822-1 and IDH-03944 were located at the 3' end of *trmE* in the VC0003 region of each genome (Fig 2). The site at which IDH-02822-2 located was not ben identified. As GIs are usually found at the 3' end of *trmE* (18),

it is likely that IDH-02822-2 is placed adjacent to the 3' end of IDH-02822-1 and that these two GIs (IDH-02822-1 and IDH-02822-2) are arranged in tandem.

GIs are composed of two regions, a multidrug resistance region (MDR) and a core region. To clarify the relation between GIs of *V. cholerae* and other bacterial GIs, we examined the similarity of the core region of the GIs of IDH-01215, IDH-02822-1, IDH-02822-2 and IDH-03944. The arrangement of the GIs of IDH-01215 and IDH-02822-1 resembled that of AGI1, which is a member of the SGI1 (*Salmonella* genomic island 1) family. The SGI1 family has been found in several bacteria, including *Salmonella* species and *Acinetobacter baumannii* (22, 23).

The arrangement of backbone genes of the GI of IDH-01215 is almost similar to that of AGI1, with identity of more than 99%, and the MDR region is inserted into the A025 site (Fig 2). However, the backbone of the GI of IDH-02822-1 comprised the regions from PGI1 in addition to the region from AGI1 (Fig 2). The MDR region of IDH-02822-1 was inserted adjacent to the position of A027 site, which is a different position from that in the GI of

IDH-01215. Interestingly, IDH-02822 possessed another GI, IDH-02822-2, which shared 100% homology with GI*Vch*Hai8 of the *V. cholerae* strain HE-45 isolated in Haiti (21). The GI of IDH-03944 was also basically composed of the components of GI*Vch*Hai8 (Fig 2). Both the upstream and downstream terminal ends of the GI of IDH-03944 were composed of the gene regions corresponding with those of GI*Vch*Hai8.

MDR region

MDR regions of GIs are variable among vibrios. In the evolution of MDR, multiple actions of gene transfer, such as by integrons, ISs and transposons, selfishly function to induce the incorporation of AMR genes into this region. This phenomenon leads to the formation of various MDR phenotypes. Therefore, it is often observed that the compositions of AMR genes in two GIs that possess a similar core region are quite different (24). MDR regions in the GIs of IDH-01215, IDH-02822-1 and IDH-03944 contained several AMR genes, namely, a *dfrA*1, *sul*1 and *mer* operon; *dfrA*1, *sul*1 and *mphA* operon; and *dfrA*15, *sul*1 and *mer* operon, respectively. This finding shows that the MDR regions of these GIs are composed

of their corresponding class 1 integrons (Fig 1). These GIs detected in three strains are novel types of GI.

Phylogenetic analysis of integrase

To check the correlation of GIs examined in this study, phylogenetic analysis of integrase genes of these GIs was carried out. The positions of *int*IDH-02822-1 and *int*IDH-01215 in the phylogenetic tree were close to that of *int*AGI1 (Fig 3). Similarly, the positions of *int*IDH-02822-2 and *int*IDH-03944 were close to that of *int*GI*Vch*Hai8. These positional relations are coincident with the result from the homology search performed for these integrons (Fig 2).

PGI1 and SGI1 are encoded in *Proteus* and *Salmonella*, respectively and are regarded as the source of other bacterial integron 1 sequences. *Int*PGI1 was placed at a position distant from those of *int*IDH-02822-2 and *int*IDH-03944. The two integrases encoded in PGI1 and SGI1 were positioned very close in the phylogenetic tree (Fig 3). This result indicates that mutual transfer of integron 1 between *Proteus* and *Salmonella* generated during the initial stage of integron 1

evolution, followed by its transfer into *V. cholerae* non-O1/non-O139 strains, with some changes in the nucleotide sequence.

DISCUSSION

The hospitalized patients infected with *V. cholerae* non-O1/non-O139 strains presented acute diarrhea resembling cholera caused by *V. cholerae* O1. Very limited studies have been carried out to investigate the virulence and AMR of *V. cholerae* strains other than O1 and O139 serogroups. The Indian subcontinent is said to be the homeland of cholera and the source of several variants of *V. cholerae* O1 (25-27). Therefore, much attention has to be paid to clinical strains of *V. cholerae* showing antibiotic resistance. With this background, we examined the AMR pattern in *V. cholerae* non-O1/non-O139 isolates and analyzed the genes responsible for the AMR.

In the analysis of quinolone resistance gene, we noted the mutation in GyrA at position 83 (S83I) and ParC at position 85 (S85L). The former mutation caused the resistance of every bacteria examined to NA. It can be concluded that serine

residue at position 83 of GyrA is an important residue for NA to express the activity.

However, the effect of mutation of ParC S85L on the resistance of bacteria was not uniform and the mutation was not always required to express the resistance against norfloxacin and ofloxacin as observed in IDH-05249 (Table 2).

We examined the sensitivity of bacteria by disc diffusion method and regarded the bacteria expressing resistance to antibiotics at the level of more than intermediate as resistant bacteria to the antibiotics. The bacteria expressing resistance at low level was regarded as sensitive bacteria in this assay, though they exhibited weak resistance. To conclude the resistance of these strains against norfloxacin and ofloxacin, further assays, such as determination of minimal inhibitory concentration, is necessary. Based on the data obtained, we should discuss the function of amino acid residue at position 85 of ParC.

We think other possibilities. The mutations to change the resistance of the bacteria to norfloxacin and ofloxacin may happen in some strains. To find out such

mutations, more careful searching of gene sequence is necessary. To carry out these searching, the search using other tools such as ResFinder is necessary.

In this study, 4 *V. cholerae* non-O1/non-O139 strains (IDH-01215, IDH-02822, IDH-03944 and IDH-06641) had resistance-encoding genes in class 1 integrons. This integron seems to play an important role in the acquisition of AMR in *V. cholerae* non-O1/non-O139 strains as well as in other bacteria (28-31).

As class 1 integrons cannot transfer on their own, other MGEs, such as GIs and/or conjugative plasmids, are necessary for their transfer to other bacteria (17, 21). We detected 3 types of GIs, including class 1 integrons inserted at *trmE* that differed from other reports (21). Though the presence of class 1 integrons has been reported in other *V. cholerae* non-O1/non-O139 isolates (28-30), our integrons are quite different from those integrons. Thus, our GIs are novel types of GI. It indicates that new type of GI has constantly emerged in *V. cholerae* non-O1/non-O139 strains and *V. cholerae* non-O1/non-O139 strains containing these new GIs have already spread to a certain extent.

Apart from *Salmonella enterica*, the *int* gene encoding a site-specific recombinase family in SGI1 has been reported in many species, including *P. mirabilis* and *A. baumannii* (23, 32-34). Moreover, the SGI1 family may contain the NDM β-lactamase gene (35, 36), and hence, the SGI1 family has the potential to grow up the gene cluster containing several AMR-encoding genes in several bacterial species.

Our results indicate that *V. cholerae* can also acquire the SGI1 family-encoding AMR genes from other species by horizontal gene transfer. We have shown that some strains of *V. cholerae* can contain two GIs. This result suggests that *V. cholerae* non-O1/non-O139 strains have the potential to generate strains that are highly resistant to several antimicrobials.

The acquisition of AMR genes in *V. cholerae* O1 which is mediated by the SXT element and IncA/C conjugative plasmid has been reported (37). Further studies showed that other genetic factors are involved in the acquisition SXT element (38). In this study, we have showed that GIs containing class 1 integrons in the downstream region of *trmE* are also important factors for antimicrobial

resistance in *V. cholerae* non-O1/non-O139 strains. The site where GIs locate is different from the site where the SXT element is inserted (39). *V. cholerae* O1 containing both these GIs and the IncA/C plasmid has not been reported, yet. However, as shown in this study, a certain number of non-O1/non-O139 *V. cholerae* have already acquired GIs. It may happen that non-O1/non-O139 *V. cholerae* strains acquired both GIs and the IncA/C plasmid appear and that the number of these strains increases under antibiotic stress in near future. It is important to monitor the appearance of these types of *V. cholerae* non-O1/non-O139 and prevent the generation of *V. cholerae* O1/O139 with extensive drug resistance.

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DISCLOSURE

The authors have no conflict of interest to declare.

REFERNCES

1. Harris J. B., LaRocque R. C., Qadri F, Ryan E. T., Calderwood S. B. (2012) Cholera. *Lancet* **379**: 2466-2476.

2. Trubiano J. A., Lee J. Y., Valcanis M., Gregory J., Sutton B. A., Holmes N. E. (2014) Non-O1, non-O139 *Vibrio cholerae* bacteraemia in an Australian population. *Intern Med J* **44**: 508-511.

3. Schirmeister F., Dieckmann R., Bechlars S., Bier N., Faruque S. M., Strauch E. (2014) Genetic and phenotypic analysis of *Vibrio cholerae* non-O1, non-O139 isolated from German and Austrian patients. *Eur J Clin Microbiol Infect Dis* **33**: 767-778.

4. Jones E. H., Feldman K. A., Palmer A., Butler E., Blythe D., Mitchell C.S. (2013) Vibrio infections and surveillance in Maryland, 2002-2008. *Public Health Rep* **128**: 537-545.

5. Newton A., Kendall M., Vugia D.J., Henao O.L., Mahon B.E. (2012) Increasing rates of vibriosis in the United States, 1996-2010: Review of surveillance data from 2 systems. *Clin Infect Dis* **54**: S391-S395.

6.Yamai S., Okitsu T., Shimada T., Katsube Y. (1997) Distribution of serogroups of *Vibrio cholerae* non-O1 non-O139 with specific reference to their ability to produce cholera toxin, and addition of novel serogroups. *Kansenshogaku Zasshi* **71**: 1037-1045.

7. Dutta D., Chowdhury G., Pazhani G.P., Guin S., Dutta S., Ghosh S., Rajendran K., Nandy R. K., Mukhopadhyay A. K., Bhattacharya M. K., Mitra U., Takeda Y., Nair G.B., Ramamurthy T. (2013) *Vibrio cholerae* non-O1, non-O139 serogroups and cholera-like diarrhea, Kolkata, India. *Emerg Infect Dis* **19**: 464-467.

8. Ceccarelli D., Chen A., Hasan N.A., Rashed S.M., Huq A., Colwell R. R. (2015) Non-O1/non-O139 *Vibrio cholerae* carrying multiple virulence factors and *V. cholerae* O1 in the Chesapeake Bay, Maryland. *Appl Environ Microbiol* **81**: 1909-1918.

9. Daniels N. A., Shafaie A. (2000) A review of pathogenic vibrio infections for clinicians. *Infect Med* **17**: 665-685.

10. Biyela P. T., Lin J. Bezuidenhout C.C. (2004) The role of aquatic ecosystems as reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. *Water Sci Technol* **50:** 45-50.

11. Nandi B, Nandy R.K., Mukhopadhyay S, Nair G.B., Shimada T, Ghose A.C. (2000) Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW**.** *J Clin Microbiol* **38**:4145-4151.

12. CLSI (Clinical and Laboratory Standards Institute). Performance standards for antimicrobial susceptibility testing; Twenty-fourth informational supplement. Wayne, PA: Clinical and Laboratory Standards Institute; 2014.

13. CLSI (Clinical and Laboratory Standards Institute). Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious

bacteria. 3rd ed., CLSI document M45. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.

14. Imamura D., Morita M., Sekizuka T., Mizuno T., Takemura T., Yamashiro T., Chowdhury G., Pazhani, G. P., Mukhopadhyay A. K., Ramamurthy T., Miyoshi S., Kuroda M, Shinoda S., Ohnishi M. (2017) Comprehensive genome analysis of VSP-II and SNPs reveals heterogenic variation in contemporary strains of *Vibrio cholerae* O1 isolated from cholera patients in Kolkata, India. *PLoS Negl Trop Dis.* **11**: e0005386. doi: 10.1371

15. Moura A, Soares M, Pereira C, Leitao N, Henriques I, Correia A. (2009) INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. Bioinformatics **25**: 1096–1098.

16. Baranwal S., Dey K., Ramamurthy T., Nair G.B., Kundu M. (2002) Role of active efflux in association with target gene mutations in fluoroquinolone resistance in clinical isolates of *Vibrio cholerae*. *Antimicrob Agents Chemother* **46**: 2676-2678.

17. Deng Y., Bao X., Ji L., Chen L., Liu J., Miao J., Chen D., Bian H., Li Y., Yu G. (2015) Resistance integrons: Class 1, 2 and 3 integrons. *Ann Clin Microbiol Antimicrob* **20**: 14-45.

18. Mala W., Faksri K., Samerpitak K., Yordpratum U., Kaewkes W., Tattawasart U., Chomvari C. (2017) Antimicrobial resistance and genetic diversity of the SXT element in *Vibrio cholerae* from clinical and environmental water samples in Northeastern Thailand. *Infect Genet Evol* **52**: 89-95.

19. Partridge S. R., Brown H.J., Hall R.M. (2002) Characterization and movement of the class 1 integron known as Tn2521 and Tn1405. *Antimicrob Agents Chemother* **46**: 1288-1294.

20. Noguchi N., Emura A., Matsuyama H., O'Hara K., Sasatsu M., Kono M. (1995) Nucleotide sequence and characterization of erythromycin resistance determinant that encodes macrolide 2'-phosphotransferase I in *Escherichia coli*. *Antimicrob Agents Chemother* **39**: 2359-2363.

21. Carraro N., Rivard N., Ceccarelli D., Colwell R.R., Burrus V. (2016) IncA/C conjugative plasmids mobilize a new family of multidrug resistance Islands in clinical *Vibrio cholerae* non-O1/Non-O139 isolates from Haiti. *mBio*. **7**: e00509-00516.

22. Doublet B., Boyd D., Mulvey M.R., Cloeckaert A. (2005) The salmonella genomic Island 1 is an integrative mobilizable element. *Mol Microbiol* **55**: 1911-1924.

23. Hamidian M., Holt K.E, Hall R.M. (2015) Genomic resistance island AGI1 carrying a complex class 1 integron in a multiply antibiotic-resistant ST25 *Acinetobacter baumannii* isolate. *J Antimicrob Chemother* **70**: 2519-2523.

24. Adams M.D., Goglin K., Molyneaux N., Hujer K.M., Lavender H., Jamison J.J., MacDonald I. J., Martin K. M., Russo T., Campagnari A. A., Hujer A. M., Bonomo R. A., Gill S.R. (2008) Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *J Bacteriol.* **190**: 8053-8064.

25. Sack D. A, Sack R. B, Nair G.B., Siddique A. K. (2004) Cholera. *Lancet.* **363**: 223-233.

26. Banerjee R., Das B., Nair G.B., Basak S. (2014) Dynamics in genome evolution of *Vibrio cholerae. Infect Genet Evol* **23**: 32-41.

27. Kim E.J., Lee C.H., Nair G.B., Kim D.W. (2015) Whole-genome sequence comparisons reveal the evolution of *Vibrio cholerae* O1. *Trends Microbiol* **23**: 479-489.

28. Shi L., Fujihara K., Sato T., Ito H., Garg P., Chakrabarty R., Ramamurthy T., Nair G.B., Takeda Y, Yamasaki S. (2006) Distribution and characterization of integrons in various serogroups of *Vibrio cholerae* strains isolated from diarrhoeal patients between 1992 and 2000 in Kolkata, India. *J Med Microbiol* **55**: 575-583.

29. Dalsgaard A., Forslund A., Serichantalergs O., Sandvang D. (2000) Distribution and content of class 1 integrons in different *Vibrio cholerae* O-serotype strains isolated in Thailand. *Antimicrob Agents Chemother* 44: 1315-1321.

30. Thungapathra M., Amita S.K.K., Chaudhuri S.R., Garg P., Ramamurthy T., Nair G. B., Ghosh A. (2002) Occurrence of antibiotic resistance gene cassettes aac(6')-Ib, dfrA5, dfrA12, and ereA2 in class I integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. *Antimicrob Agents Chemother* **46**: 2948-2955.

31. Dalsgaard A., Forslund A., Petersen A., Brown D.J., Dias F., Monteiro S., Molbak K., Aaby P., Rodrigues A., Sandström A. (2000) Class 1 integron-borne, multiple-antibiotic resistance encoded by a 150-kilobase conjugative plasmid in epidemic *Vibrio cholerae* O1 strains isolated in Guinea-Bissau. *J Clin Microbiol* **38**: 3774-3779.

32. Siebor E., Neuwirth C. (2014) Proteus genomic island 1 (PGI1), a new resistance genomic island from two *Proteus mirabilis* French clinical isolates. *J Antimicrob Chemother* **69**: 3216-3220.

33. Havlickova H., Hradecka H., Bernardyova I,. Rychlik I. (2009) Distribution of integrons and SGI1 among antibiotic-resistant *Salmonella enterica* isolates of animal origin. *Vet Microbiol* **133**: 193-198.

34.Sung J. Y., Kim S., Kwon G., Koo S.H. (2017) Molecular characterization of salmonella genomic Island 1 in *Proteus mirabilis* isolates from Chungcheong Province, Korea. *J Microbiol Biotechnol* **27**: 2052-2059.

35. Girlich D., Dortet L., Poirel L., Nordmann P. (2015) Integration of the blaNDM-1 carbapenemase gene into proteus genomic island 1 (PGI1-PmPEL) in a *Proteus mirabilis* clinical isolate. *J Antimicrob Chemother* **70**: 98-102.

36. Schultz E., Haenni M., Mereghetti L., Siebor E., Neuwirth C., Madec J.Y., Cloeckaert A, Doublet B. (2015) Survey of multidrug resistance integrative mobilizable elements SGI1 and PGI1 in *Proteus mirabilis* in humans and dogs in France, 2010-13. *J Antimicrob Chemother* **70**: 2543-2546.

37. Wang R., Li J., Kan B. (2016) Sequences of a co-existing SXT element, a chromosomal integron (CI) and an IncA/C plasmid and their roles in multidrug resistance in a *Vibrio cholerae* O1 El tor strain. *Int J Antimicrob Agents* **48**: 305-309.

38. Kitaoka M., Miyata S.T., Unterweger D., Pukatzki S. (2011) Antibiotic resistance mechanisms of *Vibrio cholerae*. *J Med Microbiol* **60**: 397-407.

39. Hochhut B., Waldor M.K. (1999) Site-specific integration of the conjugal *Vibrio cholerae* SXT element into prfC. *Mol Microbiol* **32**: 99-110.

Figures

Fig 1. Components Constituting the Class 1 Integron of the Strains IDH-01215, IDH-02822, IDH-03944 and IDH-06641.

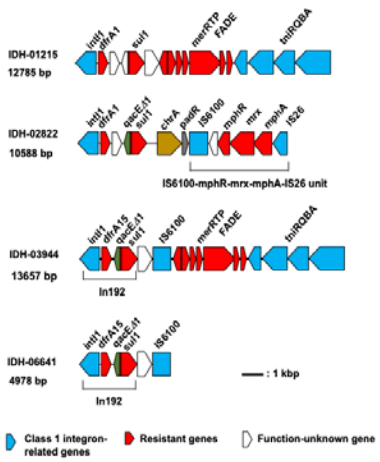


Fig. 2. **Components of the GIs detected in isolates and comparison with related GIs.** GIs in strains were detected by homology research as described in the text. The arrangements of PGI1, AGI1 and GI*Vch*Hai8 were designated from GenBank accession numbers KJ411925, KP054476 and ALED01000018.1, respectively.

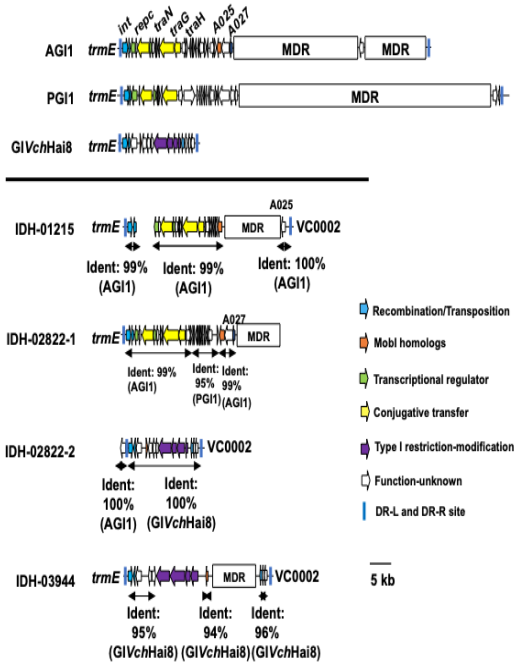
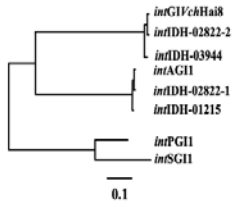


Fig. 3. **Molecular phylogenetic analysis of the integrase genes encoded in genomic islands inserted at *trmE*.** Integrase genes encoded in each genomic island inserted at *trmE* were analyzed to draw phylogenetic trees. The analysis was performed by the Clustal W (https://www.genome.jp/tools-bin/clustalw) algorithm using default parameters.



List of Abbreviations:

AM, ampicillin; AMR, antimicrobial resistance; CIP, ciprofloxacin; CLSI, clinical and laboratory standards institute; EM, erythromycin; GIs, genomic islands; MDR, multidrug resistance region; MGE, mobile genetic element;

NA, nalidixic acid; NDM, new delhi metallo-β-lactamase; NOR, norfloxacin; OFX, ofloxacin; QRDR, quinolone resistance-determining region; SM, streptomycin; SXT, sulfamethoxazole with trimethoprim; TCBS, thiosulphate citrate bile-salt sucrose; TE, tetracycline.

Table 1. Detection of O-antigen, antimicrobial resistance, antimicrobial resistance-encoding gene and integrase gene of non-O1/non-O139 *V. cholerae*

Strain	**Year**	**O-antigen**	**Resistance profile**[†]	**Antimicrobial resistance-encoding gene** [‡]	**Integrase gene**
IDH-00012	2007	O149	SXT	*dfrA6(31)*	n. d.
IDH-00024	2007	O11	None	n. d.	n. d.
IDH-00749	2007	O39	None	n. d.	n. d.
IDH-00812	2008	O70	None	n. d.	n. d.
IDH-00828	2008	O67	None	n. d.	n. d.
IDH-00904	2008	O70	None	n. d.	n. d.
IDH-01006	2008	O6	CIP, NA, NOR, OFX	*m-gyrA*, *m-parC*[e]	n. d.

IDH-01215	2008	O37	CIP, NA, NOR, OFX	*dfrA1*, *sul1*, *merRTPFADE*, *m-gyrA*, *m-parC*	*intI1*
IDH-01460	2009	O8	None	n. d.	n. d.
IDH-01580	2009	O39	None	n. d.	n. d.
IDH-01620	2009	O27	None	n. d.	n. d.
IDH-01793	2009	O34	NA	*m-gyrA*, *m-parC*	n. d.
IDH-02372	2009	O6	None	n. d.	n. d.
IDH-02653	2009	O51	CIP, NA, NOR, OFX, TE	*sul2*, *tetA(D)*, *m-gyrA*, *m-parC*	n. d.
IDH-02822	2010	O35	EM, NA, SM, SXT, TE	*dfrA1*, *sul1*, *sul2*, *floR*, *mphA*, *mrx*, *mphR*, *m-gyrA*, *m-parC*	*intI1*
IDH-03148	2010	O128	None	n. d.	n. d.
IDH-03257	2010	O178	None	n. d.	n. d.
IDH-03297	2010	O35	None	n. d.	n. d.
IDH-03944	2011	O44	NA, SM, SXT	*dfrA15*, *sul1*, *floR*, *merRTPFADE*, *m-gyrA*	*intI1*

IDH-04713	2012	O54	SM	*dfrA6(31)*	n. d.
IDH-04886	2012	O12	CIP, NA, NOR, OFX, SXT	*dfrA6(31)*, *m-gyrA*, *m-parC*	n. d.
BCH-03594	2013	O36	NA, NOR, OFX	*m-gyrA*, *m-parC*	n. d.
IDH-05249	2013	O36	CIP, NA, NOR, OFX	*m-gyrA*	n. d.
IDH-06582	2014	O10	NA, SXT	*dfrA6(31)*, *m-gyrA*, *m-parC*	n. d.
IDH-06641	2014	O10	AM, SXT	*dfrA15*, *sul1*, $bla_{\text{CARB-9}}$, *mer* operon	*intI1*, *int*SXT

[†]Antibiotics to which strains were resistant with activity of more than intermediate action. None: no antibiotics to which the strain examined is resistant.

[‡]n.d.: not detected. *m-gyrA* and *m-parC* are mutant *gyrA* and mutant *parC* genes, respectively, described in Table 2.

Table 2. Mutations in the QRDR of quinolone-resistant *V. cholerae* strains

Mutation in the QRDR	**Strain**	**Resistance profile**
GyrA S83I	IDH-03944	NA
GyrA S83I	IDH-05249	NA, NOR, OFX, CIP
GyrA S83I and ParC S85L	IDH-01006	NA, NOR, OFX, CIP
GyrA S83I and ParC S85L	IDH-01215	NA, NOR, OFX, CIP
GyrA S83I and ParC S85L	IDH-01793	NA
GyrA S83I and ParC S85L	IDH-02653	NA, NOR, OFX, CIP
GyrA S83I and ParC S85L	IDH-02822	NA
GyrA S83I and ParC S85L	IDH-04886	NA, NOR, OFX, CIP
GyrA S83I and ParC S85L	BCH-03594	NA, NOR, OFX
GyrA S83I and ParC S85L	IDH-06582	NA