

In silico Assessment of the Genotypic Distribution of Virulence and Antibiotic Resistance Genes in *Vibrio cholerae*

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ABSTRACT: *Vibrio cholerae* has long been reported as an important cause of death in developing countries. The study detected the virulence and antibiotic resistance gene of eight *V. cholerae* isolates through *in silico* tools. Cholera toxins, *ctxA* and *ctxB* were found in six isolates (75%). Seventy-five percent isolates were also found to be positive for zonula occludens toxin, *zot* which is known to increase the permeability by altering the tight junction of the small intestine. Accessory cholera enterotoxin *ace*, responsible for fluid accumulation, was detected in four *V. cholerae* strains. Seven isolates (87.5%) were positive for toxin-coregulated pilus, *tcp* which helps the bacteria to adhere to gut mucosa. Both *ompW* and *toxR* genes were found in 87.5% of the isolates. Twenty-five percent isolates harboured *strA*, *strB*, *sullI*, *dfrA1*, *floR* genes and SXT element demonstrating that they were multidrug-resistant (MDR) bacterium. One isolate was found to be positive for *tetA* gene while no erythromycin resistance gene, *ermA* and *ermB* was found. Virulence genes were found in all genotypes indicating that their distribution was not genotype-oriented while genotype 2 harboured no antibiotic resistance genes. This data helps to predict virulence genes associated with cholera and also demonstrates the presence of antibiotic resistance genes. Bacteria acquired the antibiotic resistance gene through natural process which cannot be stopped. So by analyzing the resistance pattern we can choose appropriate antibiotics. *In silico* study helps us to predict the antibiotic resistant genotypes and can easily identify antibiotic resistant strains which help us to treat cholera infections and reduce the morbidity and mortality rate of the infected individuals.

Key words: *Vibrio cholerae*, Virulence gene, Antibiotic resistance gene, SXT element, Genotype, *In silico*.

INTRODUCTION

Gram-negative *Vibrio cholerae* is responsible for causing water-borne diarrheal disease cholera in healthy communities especially in developing countries. More than 100000 people die annually due to cholera.¹ Rahmani *et al.* reported that O1 or O139 serogroup of toxigenic *V. cholerae* is mainly responsible for epidemic forms of cholera.² On the other hand, non-O1/non-O139 serogroups of *V. cholera* is associated with sporadic diarrhoeal disease and responsible for mild to severe morbidity.³⁻⁵ Ingestion of contaminated food and water caused this acute intestinal infection namely cholera.⁶ Estuarine,

and fresh water environments harboured *V. cholerae* isolates which were associated with endemic, epidemic and pandemic forms of cholera.⁷⁻⁹ *V. cholerae* produces several virulence factors such as cholera toxin encoding element (CTX), which encodes cholera toxin (CT)¹⁰ and the pathogenicity island toxin coregulated pilus (TCP). Acquisition of appropriate virulence genes converts nontoxigenic environmental strains into pathogenic *V. cholera* strain.¹¹ These genetic changes affect the presence and expression of virulence genes in *V. cholerae*. Several studies found the variability of virulence factors between environmental and clinical *V. cholerae* isolates.^{8,12,13} Antibiotic-resistant infections are already widespread across the globe. In 2013 the Centers for Disease Control and Prevention (CDC)

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declared that the human race is now in the “post-antibiotic era,” and the World Health Organization (WHO) in 2014 warned that the antibiotic resistance crisis is becoming dire. Acquisition of conjugative plasmid by *V. cholerae* developed multidrug-resistant bacterium as reported by several studies.¹⁴⁻¹⁶ Genetic element of *V. cholerae* have also been transferred by class 1 integron and SXT element which develops antibiotic resistant bacterium.¹⁷⁻²² *V. cholerae* O1 harboured antibiotic resistance gene continuously due to the presence of SXT constin.²³ Appropriate treatment is necessary to reduce the mortality rate of cholera patients and mortality rate may be increased from less than 1% to 50-60% with untreated cholera.^{16,24,25} But incorrectly prescribed antibiotics contribute to the development of resistant bacteria. Changes in antibiotic-induced gene expression can increase virulence, while increased horizontal gene transfer and mutagenesis promote antibiotic resistance and spread. Low levels of antibiotics have been shown to contribute to strain diversification in organisms. *In silico* modelling is a logical extension of controlled *in vitro* experimentation in which computer models are developed to model a pharmacologic or physiologic process. The computing power is used by the research scientist for computational model experiments which continually decrease overall cost. Unlike *in vivo* and *in vitro* experiments, *in silico* modelling is not bound to address ethical issues. *In silico* models, therefore, allow the researcher to include a virtually unlimited array of parameters, which render the results more applicable to the organism as a whole. This *in silico* study approach has the potential to predict effective antibiotics while reducing the need for expensive lab work and clinical trials.

The aim of the study was to identify the disease associated virulence genes in 8 *V. cholerae* strains and also detect the antibiotic resistance genes and SXT element for successful treatment of *V. cholerae* infections.

METHODS AND MATERIALS

Strains used in the study: Strains used in the study are summarized in Table 1.

Table 1. Name of the isolates.

Sl. No.	Isolate
1.	NC_002505 <i>Vibrio cholerae</i>
2.	NC_016944 <i>Vibrio cholerae</i> IEC224
3.	NC_017270 <i>Vibrio cholerae</i> LMA3984-4
4.	NC_012578 <i>Vibrio cholerae</i> M66-2
5.	NC_012668 <i>Vibrio cholerae</i> MJ-1236
6.	NC_016445 <i>Vibrio cholerae</i> O1 str. 2010EL-1786
7.	NC_012582 <i>Vibrio cholerae</i> O395
8.	NC_009456 <i>Vibrio cholerae</i> O395 chromosome 1

PCR amplification: All primers used are listed in Table 2 and Table 3. An online software, available at <http://insilico.ehu.es/PCR/> was used to perform *in silico* PCR amplification.^{33,34}

Table 2. Primers for detection of virulence genes.

Gene	Primer sequence (5'-3')	Amplicon size (bp)
<i>toxR</i>	CCTTCGATCCCCTAAGCAATAC AGGGTTAGCAACGATGCGTAAG	779 ²⁶
<i>ctxA</i>	CGGGCAGATTCTAGACCTCCTG CGATGATCTTGGAGCATTCCCAC	564 ²⁶
<i>ctxB</i>	TGAATTATGATTAATAAATTTG TTTATATCTTAATTTGCCATACTAA	391 ²⁶
<i>zot</i>	TCGCTTAACGATGGCGCGTTTT AACCCCGTTTCACTTCTACCCA	947 ²⁶
<i>ompW</i>	CACCAAGAAGGTGACTTTATTGTG GAACTTATAACCACCCGCG	588 ²⁷
<i>ace</i>	GCTTATGATGGACACCCCTTTA GTTAACGCTCGCAGGGCAAA	283 ²⁸
<i>tcpA</i>	ATTCTGGTGATCTCATGATAAGG TTAATTCACCACAAATATCTGCC	295 ²⁷

PFGE digestion: Pulse-field gel electrophoresis (PFGE) digestion and construction of the dendrogram was done *in silico* using the website <http://insilico.ehu.es/digest/>.^{33,34} The enzyme used for the digestion was *SgrDI* and lambda ladder was used to compare the bands.

RESULTS AND DISCUSSION

Pulsed-field gel electrophoresis (PFGE) analysis was performed to determine the genetic diversity of the isolates. Restriction enzyme *SgrDI* was used that recognized the CGTCGA_{CG} restriction sequence. Bands were separated according to their molecular weight in 1.2% agarose gel and lambda ladder was used to compare the band size. Results were scored into binary data and interpreted by SPSS software (IBM, USA) to construct a dendrogram (Figure 1). Isolates were grouped into 3 genotypes. Genotype 1 contained 50% of the isolates. Genotype 2 and 3 harboured 12.5% and 37.5% of the isolates, respectively (Figure 2).

Detection of virulence genes is important for treating *V. cholerae* infections. Several studies documented that cholera toxin, *ctx* play role in diarrheal pathogenesis by inducing dehydration.^{35,36} A number of virulence factors are involved in cholera pathogenesis which mediate the bacterium to colonize the epithelium layer of the small intestine and disrupt the ion balance by producing enterotoxins.²⁶ Blackstone *et al.* stated that epidemic cholera caused life threatening cholera gravis. *V. cholera* harbouring the cholera toxin, *ctxAB* is involved directly in the pathogenesis of diarrhea.³⁷ Previous study detected 85% *ctxA* gene by multiplex PCR technique³⁸ while another study found *ctxB* gene in 90.8% of the isolates.³⁹ The present study found that 75% isolates (n=6) had both *ctxA* and *ctxB* genes and they gave 564 bp and 391 bp gene product, respectively. Two out of the 8 isolates harboured neither *ctxA* nor *ctxB* gene. Alishahi *et al.* study

found that 11 isolates had no *ctxA* gene and concluded that the presence of *ctxA* is not important for diarrheal pathogenesis.³⁸ Sheikh *et al.* stated that environmental *V. cholerae* does not harbour cholera toxin.⁴⁰ Fasano *et al.* documented that zonula occludens toxin altered the tight junction of small intestine which increased permeability in the intestinal mucosa.⁴¹ Six isolates (75%) were found to be positive for *zot* gene. These six isolates were also seen to harbor *ctxA* and *ctxB* gene. Trucksis *et al.* stated that accessory cholera enterotoxin, *ace* is responsible for fluid accumulation

Table 3. Primers for detection of antibiotic resistance genes.

Gene	Primer sequence (5'-3')	Amplicon size (bp)
<i>strA</i>	GAGAGCGTGACCGCCTCATT TCTGCTTCATCTGGCGCTGC	862 ²⁹
<i>strB</i>	CGTTGCTCCTTCTCCATC TGCCTTCTGCCCTTCTCC	430 ²³
SXT	ATGGCGTTATCAGTTAGCTGGC GCGAAGATCATGCATAGACC	1035 ³⁰
<i>sulII</i>	TCAAGGCAGATGGCATTCC ACGACGAGTTTGGCAGATG	157 ²³
<i>dfrA1</i>	TGATGTTTACTTTCCTGAAATCCC ATCCGTTGCTGCCACTTG	121 ²³
<i>floR</i>	TTATCTCCCTGTCGTTCCAGCG CCTATGAGCACACGGGGAGC	627 ²¹
<i>ermA</i>	TAACATCAGTACGGATATTG AGTCTACACTTGGCTTAGG	139 ³¹
<i>ermB</i>	CCGAACACTAGGGTTGCTC ATCTGGAACATCTGTGGTATG	200 ³¹
<i>tetA</i>	GTAATTCTGAGCACTGTCCG CTGCCTGGACAACATTGCTT	957 ³²

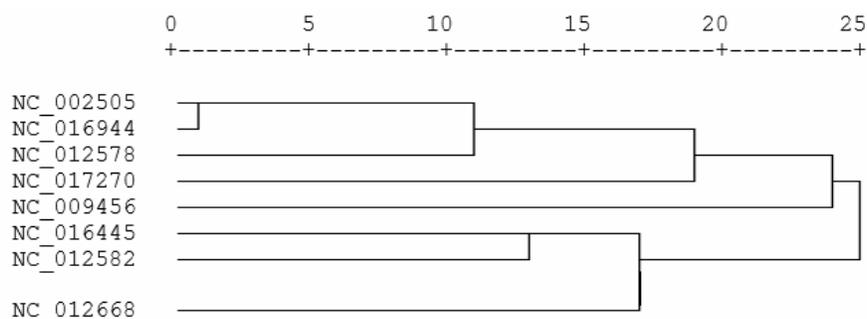


Figure 1. Phylogenetic diversity of *Vibrio cholerae* identified by PFGE.

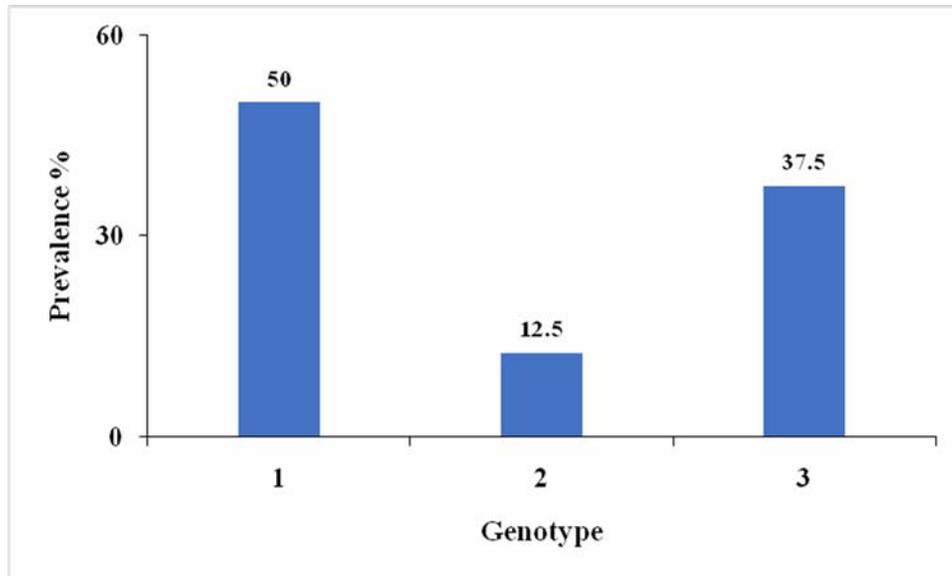
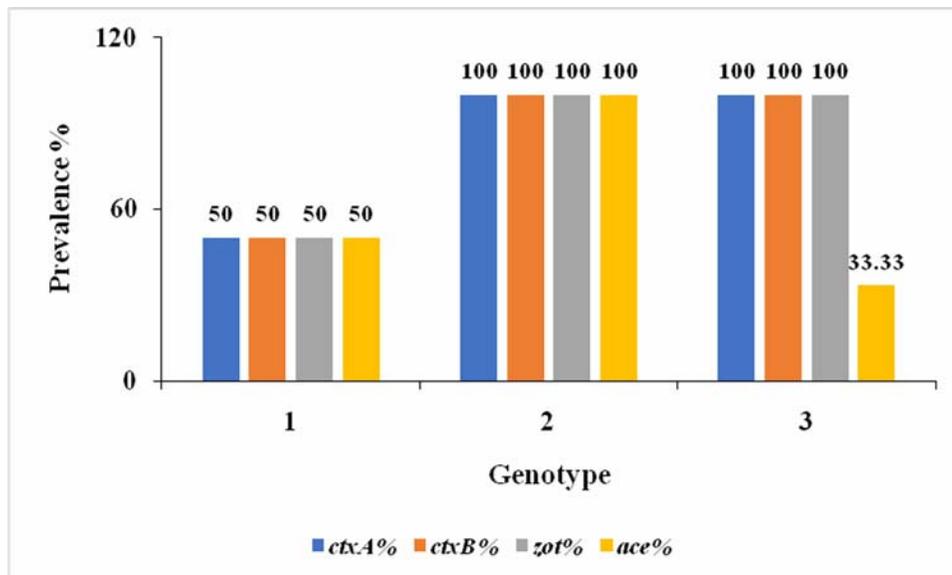


Figure 2. Prevalence of genotypes.

Figure 3. Genotypic distribution of *ctxA*, *ctxB*, *zot* and *ace* genes.

in rabbit ileal loops.⁴² Our study found four *ace* positive *V. cholerae* strains. Each genotype carried all four genes as mentioned above. Genotype 2 was composed of only NC_009456 *Vibrio cholerae* O395 chromosome 1 isolate and this strain harboured all four virulence genes (100%) (Figure 3). Fifty percent isolates present in genotype 1 expressed *ctxA*, *ctxB*, *zot* and *ace* genes. All the isolates present in genotype 3 carried *ctxA*, *ctxB* and *zot* genes but *ace*

gene was present in about 33.33% of the isolates in genotype 3.

Sharma and Chaturvedi reported that toxin-coregulated pilus, *tcp* is regulated with cholera toxin (CT) and also stated that continuously cholera producing *Vibrio* carried the *tcp* gene.⁴³ Another study found that the pilus *tcp* was involved in adherence of human gut mucosa.⁴⁴ Previous study found that *ctx* and *tcp* genes are associated with

clinical strains of *V. cholerae* O1 and O139 serogroups.⁴⁵ Strains of non-O1 and non-O139 *V. cholerae* isolated from water in Brazil harboured only one *tcp* positive strains and carried no *ctxA*, *zot* and *ace* gene.⁴⁶ Seven isolates were found to harbour toxin-coregulated pilus, *tcp* with 295 bp gene product. So, the prevalence of percentage was 87.5%. Nany *et al.* stated that genetic sequence of the *ompW* gene remained constant among different *V. cholerae* isolates and was considered as an important genetic marker.⁴⁷ Saleh *et al.* study found that all clinical and environmental isolates harboured *ompW* genes.⁴⁸ Another study found that *ompW* gene was present in all *V. cholerae* O1 and non-O1 isolates⁴⁹ while serotypes O1, O139, and Non-O1/O139 were also

found to be positive for *ompW* gene.⁵⁰ The *ompW* gene confirmed the biochemical identification of *V. cholerae* as it acts as an internal control.^{8,51} Present study recorded that 87.5% isolates had the *ompW* gene. Cholera toxin, *ctx* and toxin co-regulated pilus, *tcp* is regulated by transcription activating factor *toxR* gene.³⁸ Present study found that 87.5% isolates had the *toxR* gene. The genes *tcp* and *ompW* were encountered at higher prevalence in genotype 3 while 25% isolates in genotype 3 carried *toxR* genes (Figure 4). NC_009456 *Vibrio cholerae* O395 chromosome 1 in genotype 2 harboured all virulence genes. Seventy-five percent isolates in genotype 1 harboured *tcp* and *ompW* genes but about 33.33% of the isolates in genotype 1 carried *toxR* genes.

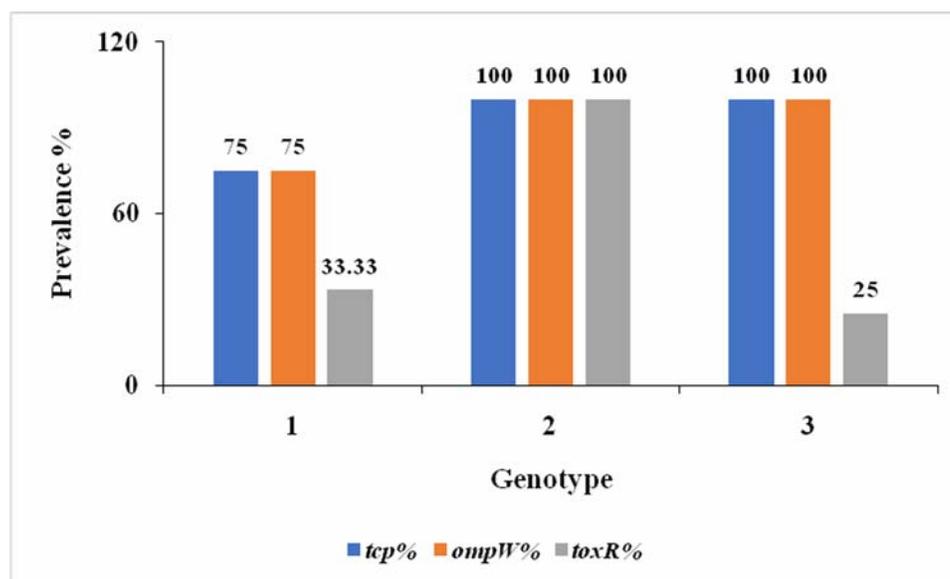


Figure 4. Genotypic distribution of *tcp*, *ompW* and *toxR* genes.

Hochhut *et al.* stated that in case of cholera treatment, oral rehydration is effective but antibiotic reduces the disease progression and decreases the severity of the symptoms in developing countries.²⁰ Many developing countries acquired resistance to trimethoprim, sulfamethoxazole and chloramphenicol antibiotics which have been used for cholera treatment.^{25,52,53} Eight *V. cholerae* isolates were screened for antibiotic resistance genes in the SXT element. Sixty-two kb conjugative, self-transmissible SXT-C encoded resistance to sulfomethoxazole,

chloramphenicol, trimethoprim and streptomycin antibiotic was documented by Waldor *et al.*²⁵ *V. cholerae* O139 serogroup harbouring SXT-C was first detected in 1992. Several countries such as India, Bangladesh, Mozambique and Laos identified this SXT element in O1 strains of *V. cholera* from 1999 to 2004.^{21,53,54} Two isolates were found to harbour SXT element and gave 1035 bp gene product in the present study. Marashi *et al.* study detected ninety-two isolates harbouring *sullI*, *dfrAI*, *strB* and *Int* gene by multiplex-PCR and they confirmed the

resistance to streptomycin, trimethoprim and sulfamethoxazole antibiotics by disc diffusion method.²³ Rahmani *et al.* study found clinical *V. cholerae* isolates displayed 97%, 99%, 99%, and 90% resistance to sulfamethoxazole, trimethoprim, chloramphenicol, streptomycin, respectively and it was determined by broth microdilution method.² PCR amplification confirmed the presence of the resistance genes and their study reported that about 95.3%, 95.3%, 81.3%, 95.3%, and 95.3% of the *V. cholerae* isolates harboured *sulIII*, *dfrA1*, *floR*, *strB*, and *sxt* element, respectively. Several studies reported that SXT element is involved in the acquisition of antibiotic resistance gene and so it is essential to check the presence of sulfamethoxazole *sulIII*, trimethoprim *dfrA*, chloramphenicol *floR*, streptomycin *strA* and *strB* resistance genes and the SXT element in *V. cholerae*.^{14,20,30} Twenty-five percent isolates harboured *strA*, *strB*, *sulIII*, *dfrA1*, *floR* genes in the present study and they gave 862 bp, 430 bp, 157 bp, 121 bp and 627 bp gene product, respectively. These twenty-five percent isolates were also found to be positive for SXT element. Garcia-Aljaro *et al.* found a low prevalence of SXT element

in their study and concluded that other mobile genetic elements may be involved in the acquisition of resistance genes in the majority of *Vibrio* species.⁵⁵ Marashiet *al.* study reported that SXT constin harbouring the antibiotic resistance gene cassettes was widely distributed in Iran and it was essential to develop a rapid identification method of antibiotic resistance pattern among the *V. cholerae* O1 serotypes.²³ Previous study reported that the primary antibiotic oxytetracycline is used for cholera treatment.^{52,56} Only one isolate was found to harbour tetracycline resistance gene *tetA* and it produced a 957 bp gene product. A recent study found no *ermA* gene and 90% of *V. cholerae* isolates harboured the *ermB* gene.⁵⁷ The present study found that isolates showed no amplification for the *ermA* and *ermB* genes. So, erythromycin is a good choice for treating cholera infections. Antibiotic resistance genes were not found in genotype 2 (Figure 5). Twenty-five percent isolates in genotype 1 expressed all 7 antibiotic resistance genes while about 33.33% isolates in genotype 3 carried all antibiotic resistance gene except *tetA* gene.

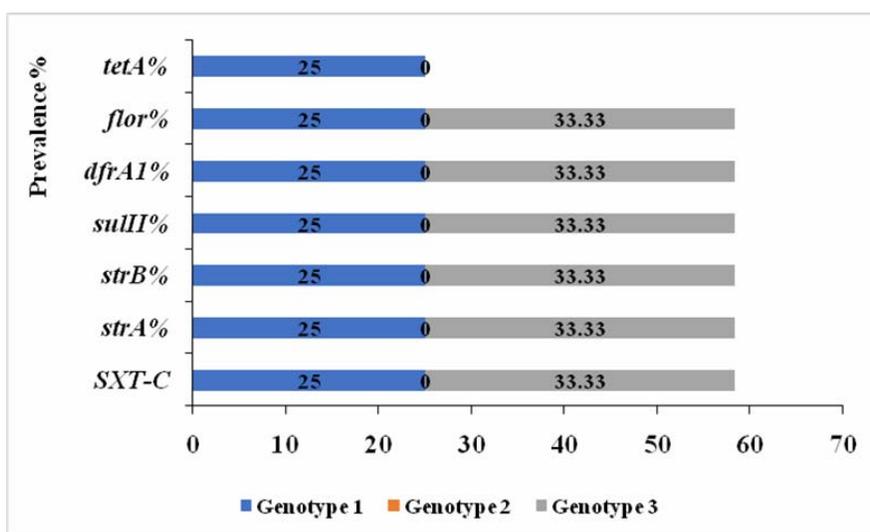


Figure 5. Antibiotic resistance genes distribution within genotypes.

CONCLUSION

Avoiding infections may be the best way to reduce the amount of antibiotics that have to be used

and to reduce the likelihood that resistance will develop during therapy. Drug-resistant infections may be prevented through immunization, safe food

preparation, hand washing, and using antibiotics - only when necessary. Preventing infections may also prevent the spread of resistant bacteria. This study can be used as an alternative for identification of toxigenic and pathogenic *V. cholerae* isolates with high specificity. The extraordinary health benefits that have so far been achieved with antibiotics have been threatened by rapidly emerging resistant bacteria. Overuse of antibiotics created this crisis. Perhaps the way antibiotics are used needs to be changed to slow down the development and spread of antibiotic-resistant infections. Stopping even some of the inappropriate and unnecessary use of antibiotics would help greatly in slowing down the spread of resistant bacteria. This study provided information to choose appropriate antibiotics for treating cholera patients. Effective antibiotic therapy reduces the chances of *V. cholera* for SXT element transfer to other hosts. Erythromycin can be considered as a good choice of antibiotics for treating cholera patients and reducing the mortality rate of cholera patients.

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