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Article *in* Acta Microbiologica et Immunologica Hungarica · March 2012

DOI: 10.1556/AMicr.59.2012.1.8 · Source: PubMed

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Acta Microbiologica et Immunologica Hungarica, 59 (1), pp. 77–84 (2012)
DOI: 10.1556/AMicr.59.2012.1.8

DRUG RESISTANCE IN *VIBRIO CHOLERAE* STRAINS ISOLATED FROM CLINICAL SPECIMENS

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(Received: 18 December 2011; accepted: 3 February 2012)

Cholera is a serious epidemic and endemic disease caused by the Gram-negative bacterium *Vibrio cholerae*. SXT is an integrative conjugation element (ICE) that was isolated from a *V. cholerae;* it encodes resistance to the antibiotics chloramphenicol, streptomycin and sulfamethoxazole/trimethoprim. One hundred seven *V. cholerae* O1 strains were collected from cholera patients in Iran from 2005 to 2007 in order to study the presence of SXT constin and antibiotic resistance.

The study examined 107 *Vibrio cholerae* strains isolated from cholera prevalent in some Iranian provinces. Bacterial isolation and identification were carried out according to standard bacteriological methods. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) to four antibiotics (chloramphenicol, streptomycin, sulfamethoxazole, and trimethoprim) were determined by broth microdilution method. PCR was employed to evaluate the presence of established antibiotic resistance genes and SXT constin using specific primer sets.

The resistance of the clinical isolates to sulfamethoxazole, trimethoprime, chloramphenicol, and streptomycin was 97%, 99%, 99%, and 90%, respectively. The data obtained by PCR assay showed that the genes sulII, dfrA1, floR, strB, and sxt element were present in 95.3%, 95.3%, 81.3%, 95.3%, and 95.3% of the *V. cholerae* isolates.

The *Vibrio* strains showed the typical multidrug-resistance phenotype of an SXT constin. They were resistant to sulfamethoxazole, trimethoprime, chloramphenicol, and streptomycin. The detected antibiotic resistance genes included *dfrA* for trimethoprim and *floR, strB, sulII* and *int,* respectively, for chloramphenicol, streptomycin, sulfamethoxazole, as well as the SXT element.

Keywords: *Vibrio cholerae,* antibiotic resistance, SXT element

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Introduction

Vibrio cholerae is a Gram-negative bacterium that produces cholera toxin and responsible for life-threatening secretory diarrhea [1]. It is the causal organism of Asiatic cholera or epidemic cholera, which is actually an infectious gastroenteritis [2]. Cholera generally improves with fluid injection, and antibiotic therapy is not usually necessary. Nonetheless, antibiotic treatment is normally administered because it shortens the period of diarrhea as well as to reduce carriage rates [3, 4].

The extension of antibiotic resistance among bacteria is the most notable example of evolution that has been observed in this group over the past six decades. Indeed, antimicrobial resistance has been a hindrance to the effective therapy of infectious disease for as long as antibiotics have been used [5].

The appearance of drug-resistant *V. cholerae* strains has become increasingly frequent. The emergence of microbial resistance to multiple drugs is an earnest clinical problem in treatment [6]. SXT constin, a conjugative insertion element (ICE), is a kind of self-transferable mobile element that has beendetected early in *Vibrio cholerae* clinical isolate O139 from India [4, 7]. This genetic element of approximately 100 kb long encodes resistance to four types of antibiotics: trimethoprim, streptomycin, chloramphenicol, and sulfphonamides [8].

Materials and Methods

Bacterial strains

One hundred seven isolates of *V. cholerae* belonging to serogroup O1 were studied. All the strains were biochemically identified as *V. cholerae*. Bacterial strains were routinely grown in Luria-Bertani (LB) broth at 37°C and maintained in Luria-Bertani broth completed with 30% glycerol and pearl glass; they were stored at –70°C [9].

Determination of MIC and MBC

Susceptibility to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin (all from Sigma Chemical Company) all of the Vibrio strains were measured by using minimum inhibitory concentrations (MIC) by the broth

microdilution method . Standard powders were dissolved according to the instructions of the manufacturer to give a full concentration of 2048 µg/ml. Volumes of 100 µl of each antibiotic were added to each microplate. Standard suspensions of the test organism in Luria-Bertani broth were inoculated into a series of sterile microplates containing antibiotic dilutions (512^{-1} µg/ml) and incubated for 16–20 h at 37°C. The MICs were read as the lowest concentration that completely inhibited any visible growth (absence of turbidity) of the test organism. For minimum bactericidal concentration (MBC) determination, a 100 µl aliquot of broth from each of the microplate 96-wells that did not show any visible growth during MIC determination was subcultured onto fresh extract-free MHA (Mueller Hinton agar) plates and further incubated for 16–20 h at 37°C. The least concentration at which no visible growth was observed was noted as the MBC. All experiments were performed three times.

PCR

All *V. cholerae* strains were grown overnight at 37°C in Luria-Bertani broth. The total genome was isolated by DNA extraction kit (Bioneer, Korea) PCR was carried out in 25 µl containing 1 µl template DNA, 2.5 µl 10× concentrated PCR buffer [100 mM Tris/HCl (pH 8·3), 1 µl dNTP, 1 µl $MgCl_2$, 1 µl (5 U μl^{-1}) *Taq* DNA polymerase, and 16.5 µl sterilized distilled water. For each primer, 1 µl (10 pmol μl^{-1}) of the forward and reverse sequences was used (Table I). Each reaction was performed concurrently with the standard strain as a positive control and

Table I

Sequences of primers used for detection of int and antibiotic resistance genes

Accession number	Name of primer	Primer	Amplicon size (bp)	Reference
GQ495075	*int-F*	5'-GCTGGATAGGTTAAGGGCGG-3'	592 bp	(Bhanumathi et al. 2003)
	int-R	5'-CTCTATGGGCACTGTCCACATTG-3'		
GU570570	*dfrA-F*	5'-TGATGTTTACTTTCCTGAAATCCC-3'	121 bp	Original
	dfrA-R	5'-ATCCGTTGCTGCCACTTG -3'		
AB535680	*strB-F*	5'-CGTTGCTCCTCTTCTCCATC-3'	430 bp	Original
	srB-R	5'-TGCCTTCTGCCCTTCTCC-3'		
FJ750803	*sulII-F*	5'-TCAAGGCAGATGGCATTCC-3'	157 bp	Original
	sulII-R	5'-ACGACGAGTTTGGCAGATG-3'		
CP001486	*floR-F*	5'-TAGGCTCTGGTGCTGTATTTAT-3'	227 bp	(Hochhut et al. 2001)
	flor-R	5'-TACTGAACGACTGGCGATTAT-3'		

Int = integrase, *dfrA* = dihydrofolate reductase type I (trimethoprim) , *strB* = streptomysin , *sulII* = sulphamethoxazole, *floR* = chloramphenicol

distilled water as a negative control. The amplification conditions were 94°C for 5 min, 94°C for 1 min, 1 min at 55°C for floR and int, 57°C for dfrA and sul, 56°C for strB, and 72°C for 1 min for 35 cycles. The final extension cycles were performed at 72°C for 5 min.

Results and Discussion

Minimal Inhibitory Concentration Test (MIC)

Resistance occurred in O1 isolates tested with sulphamethoxazol (MICs at which 104 (97%) of the strains are higher than ≥350μg/ml) and streptomycin (MICs at which 97 (90%) of strains are higher than ≥64 μg/ml). Only 1 isolate was susceptible to trimethoprim; the MIC values for 106 (99%) strains were higher than ≥16. One hundred six (99.9%) of the 107 *V. cholerae* strains tested in this study were resistant to chloramphenicol, with an MIC higher than ≥32 μg/ml, and one showed an intermediate resistance with 16 μg/ml of MIC (Table II).

Table II

MICs for *V. cholerae* O1 isolates from the cholera outbreaks in Iran in 2005 and in 2007

No. of strains *V. cholerae*	Antimicrobial agent	MIC (μg/ml) Interpretive standard		
		S	I	R
n = 107	Trimethoprim	≤8 n = 1	–	≥16 n = 106
n = 107	Sulfamethoxazole	≤100 n = 3	–	≥350 n = 104
n = 107	Chloramphenicol	≤8 n = 0	16 n = 1	≥32 n = 106
n = 107	Streptomycin	≤32 n = 10	–	≥64 n = 97

Presence of the SXT element and antibiotic resistance genes

Vibrio cholerae O1 El Tor strains were tested for the presence of antibiotic resistance genes in the SXT element. All *V. cholerae* isolates were analyzed by PCR for the presence of *int, sulII, floR, strB,* and *dfr* genes.

The data obtained by PCR assay showed that 102 out of 107 (95.3%) *V. cholerae* isolates for the *int, sulII, dfr,* and *strB* genes, and 87of 107 (81.3%) were positive for the *floR* gene and amplified a 157 bp fragment for *sulII*, a 121 bp

fragment for *dfrA1*, a 430 bp fragment for *strB*, a 592 bp fragment for the *int,* and a 227 bp fragment for *floR* (Figures 1, 2, 3 and Table III).

Antibiotic therapy shortens the duration of diarrhea caused by cholera, but drug resistant pathogens usually appear after the use of antibiotics to treat *V. cholerae* infections [4, 11]. In this study, we have investigated the genetic determinants responsible for the changes in the drug susceptibility pattern of *V. cho-*

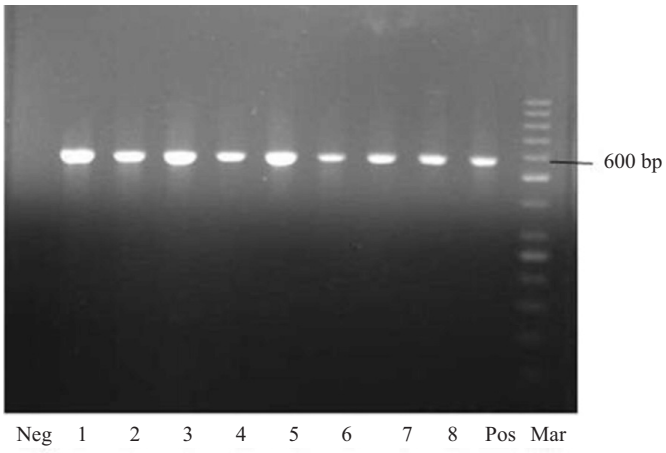

Figure 1. PCR amplification of the SXTint gene(592 bp)
Lanes 1–8 show the amplified sulfamethoxazole-trimethoprim constin. Neg = negative control. Pos = p ositive control. Mar is 50 bp DNA size marker

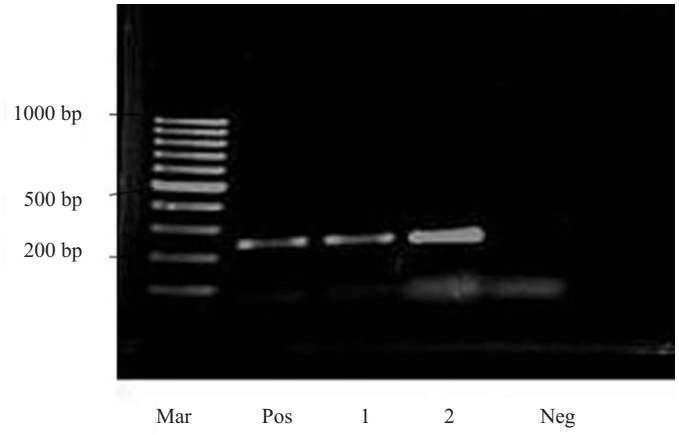

Figure 2. PCR amplification of the flor gene (227 bp)
Lanes 1–2 show the amplified flor. Neg = negative control. Pos = positive control. Mar is 100 bp DNA size marker

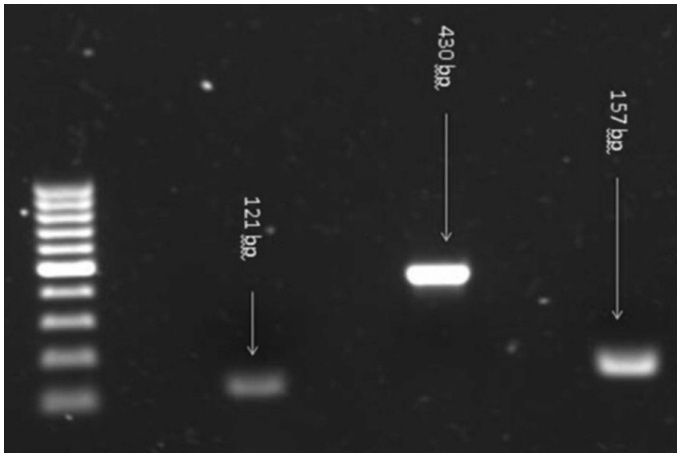

Figure 3. PCR amplification of the dfra, strb, and sulii genes
Lane 1 is the amplified dfra (121 bp). Lane 2 is the amplified strB (430bp). Lane 3 is the amplified sulII (157 bp). Mar is 100 bp DNA size marker. Neg = negative control

Table III

Results of analyses employing PCR to study *V. cholerae* O1

Year of isolation	Presence of					
	O1 – Inaba	*SXT int*	*strB*	*sul*	*dfr*	*floR*
2005	71 (66.3%)	66 (64.7%)	66 (64.7%)	66 (64.7%)	66 (64.7%)	52(59.7%)
2007	36 (33.6%)	36 (35.2%)	36(35.2%)	36 (35.2%)	36 (35.2%)	35(40.2%)
Total	107 (100%)	102 (100%)	36(35.2%)	102 (100%)	102 (100%)	87(100%)

lerae strains isolated from five different provinces in Iran over the 2 year period between 2005 and 2007, and we found notable variations in drug susceptibility and in the genes of the organisms. However, in our study, antibiotic resistance patterns for the investigated antibiotics were extending.

Our results showed that the *V. cholerae* strains isolated from Iran were resistant to sulphamethoxazole, trimethoprime, streptomycin and chloramphenicol and were positive by PCR for the *flor, strB, sulII, dfr,* and *int* genes. The resistance genes for these antimicrobial agents are carried on by an approximately 100-kb long, self-transmissible, chromosomally integrating genetic element, the SXT element. SXT constin is present in many recent clinical *V. cholerae* isolates from Iran. The properties of the SXT element include self-transferable, site-specific excision and integration, as well as linked antibiotic resistances [7].

Ramachandran et al. reported that *V. cholerae* O1 strains isolated from Kottayama and Trivandrum were resistant to ampicillin, nalidixic acid, chloram-

phenicol, sulfamethoxazole/trimethoprim, and streptomycin and positive by PCR for the *dfrA1, SulII*, and *strB* genes and for the SXT element [12].

In the study by Goel and Jiang [13] the authors reported that all the strains they examined were PCR positive for the SXT integrase gene, suggesting the presence of STX constin. These strains were resistant to sulphamethoxazole, streptomycin, and trimethoprim but were sensitive to chloramphenicol . However, in the study of Jain et al. [14] all the isolates were susceptible to chloramphenicol but PCR negative for the *floR* gene [13, 14]. In contrast, in our study, 99.9% of the *V.cholerae* strains tested were resistant to chloramphenicol, and 81.3% were positive for *floR* gene.

We detected five drug resistant genes within the SXT constin, results which were compatible with the broth microdilution assay of the strains.

In conclusion, we have demonstrated that the SXT constin comprised of different antibiotic resistance gene cassettes is dispensed among the clinical strains of *V. cholerae* O1 isolated in Iran. The broth microdilution method and PCR of the SXT constin can be helpful epidemiological tools for studying the dissemination of antibiotic resistance genes among *V. cholerae* strains.

Acknowledgement

We thank Dr. Barbara Lee Smith Pierce (University of Maryland University College Scientific and Medical Editing Baltimore, USA) for editorial work in the preparation of this manuscript.

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