

Full Length Research Paper

Distribution of sulfamethoxazole trimethoprim constin in *Vibrio cholerae* isolated from patients and environment in Iran

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Accepted 21 May, 2020

The occurrence of drug-resistant *Vibrio cholerae* is being reported with increasing frequency worldwide. Spread of resistant strains has been attributed, in part, sulfamethoxazole trimethoprim-constin (SXT-C). Sixty *V. cholerae* isolates obtained from cholera patients from different provinces in Iran during 2004 to 2006 and thirty-seven *V. cholerae* strains from surface water sources at 5 different locations in Tehran, Iran during 2006 were subjected to antibiotic susceptibility testing and polymerase chain reaction amplification of SXT-C. In clinical isolates the highest and the least levels of antibiotic resistance were seen to SXT, streptomycin and chloramphenicol (95, 95 and 92%, respectively) and doxycycline, gentamicin and oxytetracycline (0, 3 and 3%, respectively). PCR for SXT element of clinical and environmental isolates was positive for 95 and 19% of isolates, respectively. The results of this study showed that among the clinical and environmental *V. cholerae* resistance to SXT, streptomycin and chloramphenicol could be, in part, due to wide distribution of SXT-C isolates.

Key words: Anti-microbial resistant, *Vibrio cholerae*, sulfamethoxazole trimethoprim-constin (SXT-C).

INTRODUCTION

Vibrio includes a wide spectrum of species causing pandemics to free-living aquatic strains. Besides morbidity and mortality associated with cholera, multiple drug-resistant *Vibrio cholerae* O1 has been shown as a major public health problem in Iran (Bakhshi et al., 2008a). The reports of strains resistant to commonly used antibiotics are also appearing with increasing frequency globally (Garg et al., 2000; 2001). Spread of antibiotic resistance among *V. cholerae* occurs through mobilization of a variety of genetic elements. Antibiotic resistance determinants have been detected on sulfamethoxazole trimethoprim-constin (SXT-C), a novel conjugative transposable element, which encodes resistance to SXT, chloramphenicol and streptomycin (Glass et al., 1980). In this study we determined antibiotic susceptibility testing and distribution SXT-C among the clinical strains of *V. cholerae* isolates

collected from cholera patients from different provinces in Iran during 2004 to 2006, and environmental isolates collected from surface water sources at 5 different locations in Tehran, Iran during 2006, where several cholera epidemics have occurred in the last several years (Bakhshi et al., 2008b; Pourshafie et al., 2007). Presence of SXT-C as an important antibiotic resistance genetic determinant among these clinical and environmental isolates was studied to understand the role of this element in emergence of drug-resistant *V. cholerae* isolates.

MATERIALS AND METHODS

Clinical samples

Sixty *V. cholerae* isolates obtained from cholera patients from different provinces in Iran during 2004 to 2006 were studied. Out of clinical isolates 9 (15%), 40 (67%), and 11(18%) were from 2004, 2005 and 2006, respectively. The isolates of 2004 and 2006 were collected from Zahedan and Tehran provinces, respectively. The isolates of 2005 were collected from different provinces: Tehran,

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Table 1. Clinical *Vibrio cholerae* strains studied and the number and percentage of the strains containing sulfamethoxazole trimethoprim-constin in each year (2004 to 2006).

Year of isolation	O1-Inaba	O1-Ogawa	Non-O1-non -O139	Percent (from total) (%)	No. of isolates (%)
2004	-	9 (15%)	-	9(15)	9 (100)
2005	40 (67%)	-	-	40 (67)	39 (98)
2006	9 (15%)	-	2 (3%)	11 (18)	9 (82)
Total	49 (82%)	9 (15%)	2 (3%)	60(100)	57 (95)

SXT, sulfamethoxazole trimethoprim.

Zahedan, Golestan and Qom (Table 1). *V. cholerae* ATCC 14035 type strain was used as a positive control throughout the study.

Collection and processing of environmental samples

Five hundred milliliters of surface water samples were collected from 5 different geographical locations in Tehran, Iran during a period of 3 months between June and August 2006. The samples were taken from local surface water at different locations in the west (labeled as sources 1 and 2), east (4 and 5) and center (3) of Tehran where there were no outlets from waste water treatment plants. Sampling from each surface water source was performed 3 times with the interval of 30 days. The transportation of each sample to the laboratory was performed in the sterile 500cc glass container on ice. Vacuum pressure of 15 to 20 lb/in² was used to filter water samples through 0.45 mm pore size membrane after a pre-filtration through filter paper (Wattman No.1; Maidston, UK). The membranes were then cut into 8 pieces after which they were vortexed in 2 ml of 10 mM phosphate buffered saline (PBS, pH 7.4) for 3 min, followed by addition of 20 ml of alkaline peptone water (APW) containing peptone (1% wt/vol) and NaCl (1%wt/vol,pH 8.5). The tubes were then shaken (100 rpm) for 16 h at 37°C. Each sample was streaked on thiosulfate- citrate-bile-sucrose agar (TCBS) plates and incubated overnight at 37°C. All the yellow colonies on TCBS plates which were suspected of being *V. cholerae* were further examined by 10 biochemical assays including oxidase, motility, sucrose and lactose fermentation, growth in 0% NaCl, arginine dehydrolase, ornithine decarboxylase, methyl red, Voges-Proskauer and indole test. Confirmation of identity of isolates was performed by PCR using species-specific primers (prVC-F/prVCM-R). A total of 37 *V. cholerae* strains were then isolated and subjected to further analysis.

Antibiotic susceptibility testing

Antimicrobial susceptibility patterns of all clinical and environmental isolates of *V. cholerae* were tested by the standard disk diffusion technique according to CLSI guidelines (National Committee for Clinical Laboratory Standards Q1, 2001) with the following antibiotics: gentamicin (10 µg), doxycycline (30 µg), oxytetracycline (30 µg) and tetracycline (30 µg) were purchased from Difco laboratories (Detroit, MI, USA) and ciprofloxacin (5 µg), streptomycin (5 µg), ampicillin (10 µg), erythromycin (15 µg), chloramphenicol (30 µg) and cotrimoxazole (25 µg) were purchased from Becton Dickinson and Company (Sparks, MD, USA).

Polymerase chain reaction amplifications

To obtain DNA from the isolates, one isolated colony was suspended into 200 µl of sterile distilled water and boiled for 5

min after which the suspension was centrifuged at 13,000 rpm for 10 min 3 polymerase chain reaction (PCR) was carried out in 25 µl vol containing 5 µl template DNA, 2.5 µl 10 × concentrated PCR buffer [100 mM Tris = HCl (pH 8.3), 500 mM KCl and 15 mM MgCl₂], 0.25 µl (100 pmol = µl) each of appropriate primers, 0.25 µl dNTP mix (10 mM each dNTP), 0.2 µl (5U = µl) Taq DNA polymerase and 16.8 µl sterilized distilled water. Primer designation and their sequences are shown in Table 2.

RESULTS

Antimicrobial susceptibility testing of clinical samples

Twenty-one antibiotic resistance profiles were obtained among the 60 isolates. The most prevalent pattern (30%) showed resistance to co-trimoxazole, streptomycin, chloramphenicol, and erythromycin (Table 3). The highest resistance rate was seen to SXT (95%), streptomycin (95%) and chloramphenicol (92%), and the least resistance was seen to doxycycline (0%), gentamicin (3%) and oxytetracycline (3%). Most of the strains (95%) were resistant to 3 antimicrobial agents, however, with very unlike combinations of antibiotics.

Antimicrobial susceptibility testing of environmental samples

A total of 12 antimicrobial resistance profiles were observed among the total isolates (Table 4). Profile 1 was the predominant pattern (51%) and showed sensitivity to all 10 antimicrobials tested. The profile 2 constituted 11% of the isolates and represented resistance to only SXT. Among the isolates, the highest level of resistance was observed with ampicillin (27%), followed by SXT (22%) and streptomycin (16%). No resistance to gentamicin or ciprofloxacin was observed. Five isolates (13.5%) showed resistance to more than 3 antimicrobials tested.

Distribution of SXT-C among clinical and environmental samples

The majority of 60 clinical strains showed significant

Table 2. List of primers used in this study.

Primer	Sequence (5'-3')	Reference
prVC-F	AGTCACTTAACCATTCAACCCG	(Chun et al., 1999)
prVCM-R	TTAAGCGTTTTTCGCTGAGAATG	
SXT _{int} -F	GCTGGATAGGTTAAGGGCGG	(Mazel et al., 2000)
SXT _{int} -R	CTCTATGGGCACTGTCCACATTG	

Table 3. Antibiotic resistance profiling of clinical *V. cholerae* isolates with percentages of isolates showing the same profile.

No. of profile	Year (location of isolation)	No. (percent of total isolates) (%)	Antibiogram										
			SXT	S	C	T	TE	AP	E	DO	GM	CIP	
1	2004 (1), 2005 (1, 2), 2006 (2)	18 (30)	R	R	R	S	S	S	R	S	S	S	
2	2004 (1)	3 (5)	R	R	S	S	S	S	R	S	S	S	
3	2005 (4)	2 (3.3)	R	R	R	S	R	R	R	S	S	S	
4	2005 (1, 3, 4)	4 (6.7)	R	R	R	S	S	R	R	S	S	S	
5	2005 (1, 3, 4)	5 (8.3)	R	R	R	S	R	S	R	S	S	S	
6	2005 (2, 3, 4)	3 (5)	R	R	R	S	R	S	S	S	S	S	
7	2005 (2, 3, 4)	5 (8.3)	R	R	R	S	S	S	R	S	S	R	
8	2005 (2), 2006 (2)	6 (10)	R	R	R	S	S	S	S	S	S	S	
9	2005 (2)	2 (3.3)	R	R	S	S	R	S	S	S	S	S	

The antibiotic resistance profiles of the isolates with less than 3% were not included in the table. Samples were collected from 1, Zahedan; 2, Tehran; 3, Golestan; 4, Ghom. SXT, sulfamethoxazole trimethoprim; S, streptomycin; C, chloramphenicol; T, oxytetracycline; TE, tetracycline; AP, ampicillin; E, erythromycin; DO, doxycycline; GM, gentamicin; CIP, ciprofloxacin.

Table 4. Antibiotic resistance profiling of environmental *V. cholerae* isolates with percentage of isolates showing the same profile.

No. of profile	Year (location of isolation)	No. (percent of total isolates)	Antibiogram										
			SXT	ST	CIP	DO	GM	AP	T	C	TE	E	
1	2006 Tehran	19 (51.3)	S	S	S	S	S	S	S	S	S	S	S
2	2006 Tehran	4 (10.8)	R	S	S	S	S	S	S	S	S	S	S
3	2006 Tehran	4 (10.8)	S	S	S	S	S	R	S	S	S	S	
4	2006 Tehran	2 (5.4)	S	R	S	S	S	R	S	S	S	S	
5	2006 Tehran	1 (2.7)	R	R	S	R	S	R	R	R	R	R	
6	2006 Tehran	1 (2.7)	S	R	S	S	S	S	S	S	S	S	
7	2006 Tehran	1 (2.7)	S	S	S	R	S	S	R	S	R	S	
8	2006 Tehran	1 (2.7)	S	S	S	R	S	R	R	S	R	R	
9	2006 Tehran	1 (2.7)	R	R	S	S	S	S	R	S	S	S	
10	2006 Tehran	1 (2.7)	R	S	S	S	S	R	S	S	S	S	
11	2006 Tehran	1 (2.7)	R	R	S	S	S	S	S	S	S	S	
12	2006 Tehran	1 (2.7)	S	S	S	R	S	R	R	R	R	R	

SXT, sulfamethoxazole trimethoprim; S, streptomycin; C, chloramphenicol; T, oxytetracycline; TE, tetracycline; AP, ampicillin; E, erythromycin; DO, doxycycline; GM, gentamicin; CIP, ciprofloxacin.

resistance to sulfonamide. The presence of the SXT_{int} gene was then verified in 95% of the isolates conferring resistant to SXT (Figure 1). Three isolates (5%) carried

no SXT-C, two of which were non-O1-non-O139 *V. cholerae* and were susceptible to erythromycin, oxytetracycline, doxycycline, ampicillin, gentamicin and

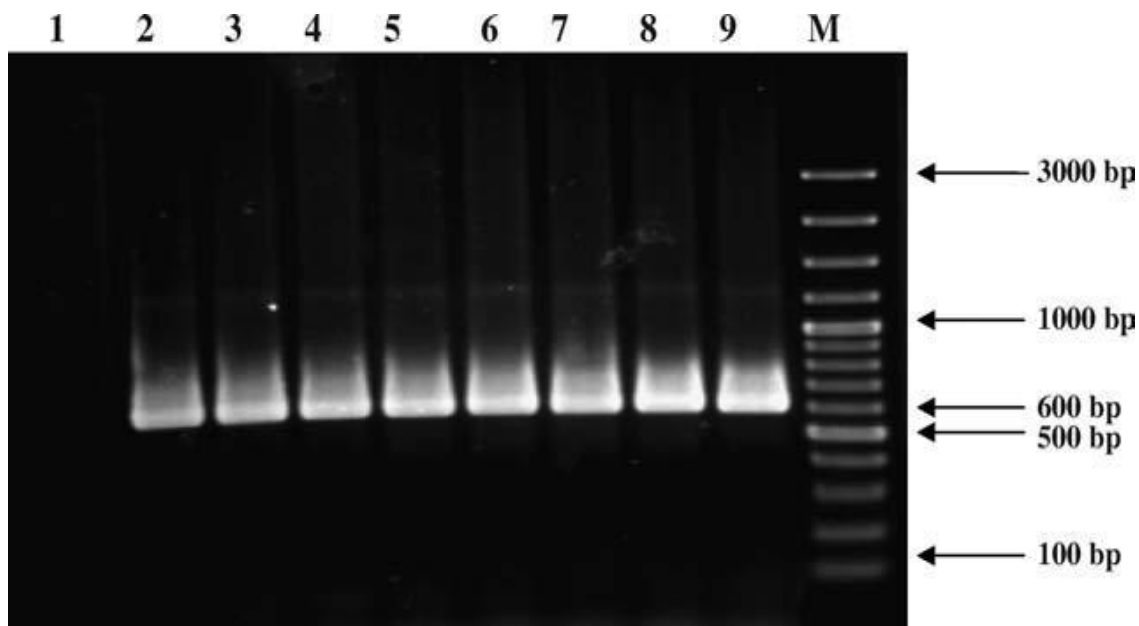


Figure 1. PCR amplification of the *SXTint* gene. Lane 1 shows negative control, Lanes 2 to 9 shows the amplified sulfamethoxazole trimethoprim-constin. M is 100 bp DNA size marker.

ciprofloxacin. The occurrence of SXT-C among the clinical isolates is depicted in Table 1. The occurrence of integrase gene of SXT element (*SXTint*) in the environmental isolates was 19%.

DISCUSSION

In this study, we analyzed one important genetic determinant responsible for drug susceptibility pattern of clinical *V. cholerae* strains isolated from four different provinces in Iran over a 3 year period between 2004 and 2006 and environmental *V. cholerae* strains from surface water sources at 5 different locations in Tehran, Iran during 2006.

The pattern of antibiotic resistance among clinical isolates was similar throughout the 3 years period, observing that the time of sample collection had no effect on the drug susceptibility. During the 3 years period only a few isolates, obtained during 2005, were resistant to ciprofloxacin, gentamicin, oxytetracycline and tetracycline. This trend of antibiotic sensitivity, however, was changed, and clinical isolates become sensitive during 2006. The results showed that the trend of ampicillin resistance had significantly increased from 2004 to 2006, whereas erythromycin resistance was decreased. Such change could be an indication of the antibiotic usage by the public in Iran, which further signifies the need to further investigate the genetic mechanisms involved. PCR assay for *SXTint* revealed that 95% of our total clinical isolates in 3 years contained SXT-C responsible for resistance to cotrimoxazole (95%),

streptomycin (95%) and chloramphenicol (92%). The appearance of SXT-C has been reported by Iwanaga et al. (2004) to have occurred in El Tor O1 strains isolated in Laos after 1997, which has replaced the class I integron with an *aadA1* gene cassette in pre-1997 *V. cholerae* isolates. Similarly, Amita et al. (2003) have reported that class I integron with an *aadA1* gene cassette was widely distributed among clinical O1 strains before emergence of pre-O139 in India, which eventually disappeared and replaced with SXT-C. The results may suggest that the SXT-C has turned into the dominant antibiotic-resistant element among clinical *V. cholerae* O1 isolates in Iran.

Increasing *V. cholerae* antibiotic resistant strains of clinical origin poses the need for assessing mobile nature of resistance gene content of the environmental isolates (Sack et al., 2003). Among the antibiotic resistance profiles, significant resistance to SXT and streptomycin was seen in comparison to other antibiotics among our clinical isolates. We previously found resistance to same antibiotics in the clinical isolates obtained in Iran (Pourshafie et al., 2007), which suggests the presence of the resistant genetic determinants in the environmental isolates even during non-endemic cholera periods. Presence of the SXT element (19%) among our environmental isolates supports this supposition. Two of our isolates (5.4%) were found to be resistant to 8 antibiotics. Among the *SXTint* environmental isolates (19% of the total isolates), 57 and 71% were resistant to cotrimoxazole and streptomycin, respectively. The resistant genes for these two antibiotics are known to be encoded by SXT element. The reason that not all *SXTint* isolates were resistant to SXT and streptomycin could be the

finding that the SXT element may acquire the resistance genes through horizontal transfer (Miyazato et al., 2004). Therefore, the presence of SXT element should be monitored even in drug susceptible *V. cholerae* isolates. Miyazato et al. (2004) have indicated that no mobile genetic elements such as plasmid, class I integron or SXT element was detected in 22 environmental non-O1, non-O139 strains which were isolated from 13 distant sampling sites in Vietnam. In conclusion, the data presented in this study showed that the SXT-C element intrinsically harboring several different antibiotic resistance gene cassettes is widely distributed among different clinical and in acceptable number, in environmental *V. cholerae* in Iran. Although the resistance element found in this study may be responsible for the antibiotic resistance seen in this study, further global investigations are needed to determine the importance of SXT element in particular in the horizontal acquisition and dissemination of antibiotic resistance genes among *V. cholerae* and other microbes. Also the limited information on the epidemiological linkage between the clinical and environmental isolates of *V. cholerae* pose the need to further monitor the environmental isolates.

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