



Increased Resistance to Tetracycline and *Erythromycin* in *Vibrio cholerae* Clinical Isolates Isolated from Patients with Cholera Disease during 2012-2013 Outbreaks in Iran

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ABSTRACT

Aims *Vibrio cholerae* is one of the intestinal gram-negative bacteria, causing cholera disease in developing countries; the two serogroups of O1 and O139 are the main causes of diarrhea. The bacteria resistance pattern to antibiotics varies in different countries. The aim of this study was to determine the resistance pattern of the isolates to representative antibiotics.

Materials & Methods A total of 20 *V. cholerae* clinical strains were isolated from patients with cholera in Sistan and Baluchestan province of Iran during 2012-2013 outbreaks. After being identified by biochemical and molecular techniques, antibiotic susceptibility testing was performed for 6 antibiotics according to CLSI standards. Then, minimum inhibitory concentration (MIC) was also determined for tetracycline and erythromycin, using E-Test method.

Findings All of the isolates were EL Tor biotype, O1 serogroup, and Inaba serotype. All of isolates were resistant to erythromycin and nalidixic acid, and 50% were resistant to tetracycline, while no resistance was observed against ciprofloxacin, gentamicin, and ampicillin.

Conclusion The sensitivity of all clinical isolates to antibiotics mentioned suggests that these antibiotics can likely be used in cholera disease treatment.

Keywords *Vibrio cholerae*; Resistance; Outbreak

CITATION LINKS

[1] *Vibrio cholerae* in the horn of Africa: Epidemiology, plasmids, tetracycline resistance gene amplification, and comparison between O1 and non-O1 strains [2] Lysogenic conversion by a filamentous phage encoding cholera toxin [3] Duplication and amplification of toxin genes in *Vibrio cholerae* [4] CTX genetic element encodes a site-specific recombination system and an intestinal colonization factor [5] Acidosis in a patient with cholera: A need to redefine concepts [6] Epidemiologic and drug resistant pattern of *Vibrio cholerae* O1 biotype EL Tor, serotype Inaba during the summer of 2005 outbreak in Iran [7] Antibiotic sensitivity of the *Vibrio cholerae* isolated from rectal swab of Golestan' patients [8] Simple procedure for rapid identification of *Vibrio cholerae* from the aquatic environment [9] Analysis of 16S-23S rRNA intergenic spacer regions of *Vibrio cholerae* and *Vibrio mimicus* [10] Classification of *Vibrio cholerae* (*Vibrio comma*), including El Tor vibrios, by infrasubspecific characteristics [11] Manual for the laboratory identification and antimicrobial susceptibility testing of bacterial pathogens of public health importance in the developing world: *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Salmonella* serotype Typhi, *Shigella*, and *Vibrio cholerae* [12] Methods for antimicrobial dilution and disc susceptibility testing of infrequently isolated or fastidious bacteria; Approved guideline [13] Antibiotic resistance of *Vibrio cholerae* isolates from Kashan, Iran [14] Survey of antibiogram tests in cholera patients in the 2005 epidemic in Hamadan, Islamic Republic of Iran [15] Serotyping, antibiotic susceptibility pattern and detection of hlyA gene among cholera patients in Iran [16] Antibiotic resistance pattern of Cholera epidemic in Guilan province [17] Antimicrobial resistance patterns of *Vibrio cholerae* strains isolated from Afghan and Iranian patients in Iran [18] Cholera in a developing megacity; Karachi, Pakistan [19] Long-term comparison of antibiotic resistance in *Vibrio cholerae* O1 and *Shigella* species between urban and rural Bangladesh [20] Antibiotics resistance in El Tor *Vibrio cholerae* O1 isolated during cholera outbreaks in Mozambique from 2012 to 2015 [21] Comparison of distribution of virulence determinants in clinical and environmental isolates of *Vibrio cholerae* [22] Dissemination of a single *Vibrio cholerae* clone in cholera outbreaks during 2005 in Iran [23] The epidemiology and antimicrobial resistance of cholera cases in Iran during 2013 [24] Assessing clonal correlation of epidemic *Vibrio cholerae* isolates during 2011 in 16 provinces of Iran [25] Molecular evidence of cholera outbreak caused by a toxigenic *Vibrio cholerae* O1 El Tor variant strain in Kelantan, Malaysia

Introduction

Vibrio cholerae is one of the intestinal gram-negative bacteria, causing severe diarrhea with cholera toxin production [1]. The two serotypes of O1 and O139 are considered as the major serogroups in the disease development. serogroup O1 is classified into 2 classical and El Tor biotypes, each of which is important in terms of its unique genetic structure in the cholera toxin expression and production [2]. The cholera toxin production depends on the amount of *ctxB* gene expression, which is located on the CTX phage. In recent studies, El Tor and Classical biotypes have been identified as the causative agents of this disease. In El Tor biotype, several copies of CTX phage are located on each chromosome, while in classical biotype, there is one copy of CTX phage on each chromosome [3, 4]. Cholera is widespread in Asian countries such as Bangladesh, India, Pakistan, Afghanistan, and Iran. In the early stages, the disease has no specific symptoms. But, the peak time of the disease is accompanied by severe diarrhea. The cholera prevalence rate increases with the consumption of contaminated water and vegetables as well as with the weather warming.

Cholera is treated with water and electrolyte, and in severe cases, a combination of antibiotics are used. In cases, where the disease becomes severe, due to the lowering blood pressure and the resulting shock, they result in death [5]. In Asian countries, due to the increase in antibiotic resistance in *V. cholerae* strains, it is important to understand the cause of such resistance. One of the factors causing bacteria resistance to existing antibiotics is inappropriate and non-specialized antibiotics usage, which not only does not treat the disease, but also creates problems such as increased resistance [6]. *V. cholerae* resistance to antibiotics is not the same in all parts of every country [7].

The aim of this study was to investigate the antibiotic resistance patterns in *V. cholerae* clinical isolates during 2012-2013 outbreaks in Sistan and Baluchestan province.

Materials and Methods

Isolation and identification of the strains by biochemical (API 20E kit) and molecular tests: In cholera outbreaks during 2012-2013 in Sistan and Baluchestan province, 20 isolates were randomly selected from patients hospitalized in this province. An API 20E (Biometrix, France) kit was used for biochemical tests. At first, the bacteria strains were cultured on Thiosulfate citrate bile salts-sucrose agar (TCBS) medium, and, then, one of the colonies was transferred to Brain Heart Infusion agar (BHI) agar medium. After bacterial growth, similar colonies were picked up, and using a physiological serum, a suspension equivalent to 0.5 McFarland turbidity was prepared. Then, the mixture was

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inoculated into the vials included in the kit in accordance with the API 20E kit instructions and incubated at 37°C. After that, some tests were separately performed, including 27 tests, using the API web standalone V1.2.1 software, the growth on MacConkey medium, the oxidase test, observing movement on a wet slime, OF glucose test, and the growth on different salt concentrations (*Vibrio* genus specific) [8].

For molecular confirmation, a specific primer was designed for 16s-23s rDNA and conserved intergenic region was used. [9]. Polymerase Chain Reaction (PCR) was performed, using Bio-Rad Thermal cycler according to the program listed in Table 1 and primers listed in Table 2. The materials used in PCR reaction were as follow: PCR buffer, dNTP, Taq Polymerase enzyme, MgCl₂, forward and reverse primers, DNA template, and distilled water. The standard strain of ATCC14035 was used as control in all tests.

Table 1) Conditions used in PCR reaction to amplify the 16s-23s rRNA region gene for *V. cholerae* isolates identification.

Stage	Temperature (°C)	Time period (Min)
Initial denaturation	95	10
Denaturing	95	1
Annealing	55	1
Extension	72	1
stages repeated for 30 cycles	----	----
Final extension	72	5
Hold	4	----

Table 2) Primers used for *V. cholerae* strains identification

Primer Name	Sequence (5' to 3')	Amplicon Size	Reference
prVC-F	AGTCACTTAACCAT TCAACCCG	295bp	9
prVC-R	TTAAGCGTTTTCCG TGAGAATG		

Determination of *V. cholerae* strains serotypes and biotypes: Clinical isolates of *V. cholerae* were classified, using proprietary antisera. For serogroups identification, O1 and O139 polyvalent antisera were used and for serotype determination, Ogawa and Inaba antisera were used, all purchased from the Pasteur Institute of Iran. Isolates biotyping was performed, using voges-proskauer (VP) test and sheep erythrocytes hemolysis [8, 10].

Determination of antibiotic resistance profiles: Antibiotic susceptibility testing was performed for 6 antibiotics, including ciprofloxacin (5 µg), gentamicin (10 µg), ampicillin (10 µg), nalidixic acid (30 µg), erythromycin (15 µg), and tetracycline (30 µg) according to CLSI standards and Kirby Bauer methods. All of the antibiotics were purchased from MAST (Mast Diagnostics Ltd, Bootle, Merseyside, UK) company [11]. The *Escherichia coli* ATCC25922 standard strain was used as positive control.

Determination of minimum inhibitory concentration (MIC): To confirm the antibiotic susceptibility testing results, MIC test was performed for erythromycin and tetracycline antibiotics, using E-test method.

Findings

Biochemical and molecular confirmation of *V. cholerae* clinical isolates under study: In this study, 20 isolates were examined. According to the API 20E kit software, the existing samples were approved as *V. cholerae* strains with 99.9% likeness (an example of API 20E kit results and API 20E kit software profiles are shown in Figures 1 and 2, respectively). The results of other supplementary tests are listed in Table 3, including oxidase test, observing movement on a wet slime, the growth on the MacConkey medium, OF glucose test, and the growth on different salt concentrations.



Figure 1) An example of API 20E kit results for *V. cholerae* strains identification

PRESUMPTIVE IDENTIFICATION	
Strip	API 20 E V4.1
Profile	534712457
Note	

Significant taxa	% ID	T	Tests against
<i>Vibrio cholerae</i>	99.9	1.0	

Figure 2) An example of an acceptable profile related to API 20E kit for *V. cholerae* strains identity confirmation

Table 3) Additional biochemical tests used for *V. cholerae* clinical strains identification

NO2	N2	OX	MOB	McC	OF/O	OF/F
+	-	+	+	+	+	+

The PCR results showed the 295 bp band for all the strains, using primers specific for 16S-23S rDNA intergenic region. All the isolates were confirmed as *V. cholerae* strains by band sequencing, using the Sanger test (Macrogen, Seol, Korea method) and by comparing the resulting sequences with the sequences in National Center for Biotechnology Information (NCBI) gene bank, using BLAST software (Figure 3). Biotyping and serological grouping methods placed 20 *V. cholerae* isolates into O1 serogroupe, Inaba serotype, and El Tor biotype.

Antibiotic resistance profile: Antibiotic resistance patterns showed 100% resistance to erythromycin and nalidixic acid antibiotics. About 50% of the strains were susceptible to tetracycline, while no resistance was observed against ciprofloxacin, gentamicin, and ampicillin. The antibiotic resistance patterns are shown in Table 4 and Diagram 1. All of

the strains showed MIC 32 µg.mL⁻¹ for erythromycin. The MIC for tetracycline was 32 µg.mL⁻¹ in 50% of the isolates and 2 µg.mL⁻¹ (sensitive) in other 50% strains.

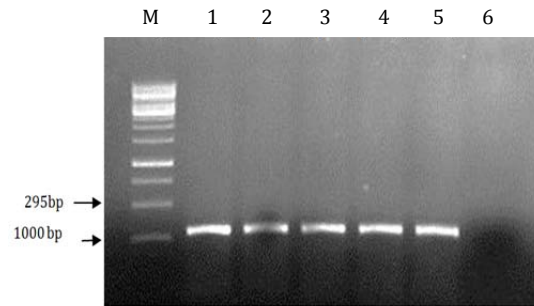


Figure 3) PCR of 16s-23s rRNA region gene identification; Well No. 1: Positive Control (*V. cholerae* ATCC14035 standard strain), Wells No. 2 to 5: Isolates containing 16s-23rRNA region gene. Well No. 6: Negative control. M: 1kb size marker

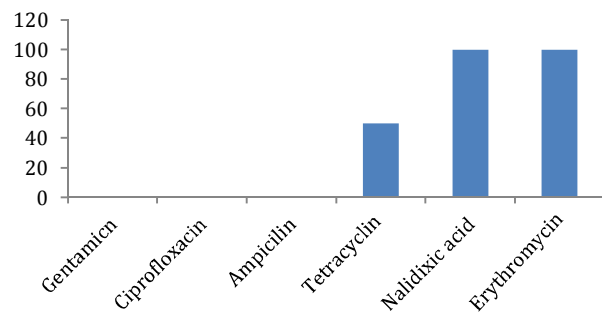


Diagram 1) Frequency of *V. cholerae* isolates resistant to antibiotics under study

Table 4) Antibiotic susceptibility test of *V. cholerae* clinical isolates

Isolates	Cip	Tet (MIC)	Tet	Ery (MIC)	Ery	NA	GEN	AMP
1	S	2	S	32	R	R	S	S
2	S	2	S	32	R	R	S	S
3	S	2	S	32	R	R	S	S
4	S	2	S	32	R	R	S	S
5	S	2	S	32	R	R	S	S
6	S	2	S	32	R	R	S	S
7	S	2	S	32	R	R	S	S
8	S	2	S	32	R	R	S	S
9	S	2	S	32	R	R	S	S
10	S	2	S	32	R	R	S	S
11	S	32	R	32	R	R	S	S
12	S	32	R	32	R	R	S	S
13	S	32	R	32	R	R	S	S
14	S	32	R	32	R	R	S	S
15	S	32	R	32	R	R	S	S
16	S	32	R	32	R	R	S	S
17	S	32	R	32	R	R	S	S
18	S	32	R	32	R	R	S	S
19	S	32	R	32	R	R	S	S
20	S	32	R	32	R	R	S	S
Total resistant	0%	50%	50%	100%	100%	100%	0%	0%

Discussion

These results of the present study are consistent with the standard results reported in CLSI [12].

Cholera is the leading cause of annual epidemics in developing countries, which is caused by *V. cholerae* [13]. The incidence of this disease varies among people with different age groups. Children are more likely to suffer from cholera due to their weak immune system. It is more common in warm months of the year [14]. After water and electrolytes, antibiotics are used to reduce the severity of the disease and to prevent the bacteria spread in the community [7]. In the present study, after identification and serotype and biotype determination, 20 *V. cholerae* clinical isolates were examined in terms of antibiotic resistance patterns. Of 20 *V. cholerae* isolates, 100% (20 strains) were resistant to nalidixic acid and erythromycin, and 50% were resistant to tetracycline, while no resistance was observed against gentamicin, ciprofloxacin, and ampicillin antibiotics.

Erythromycin resistance: In this study, 100% of the isolates under study were resistant to erythromycin. Pourshafie *et al.* have studied 100 isolates collected from several cities during 2002-2003 and 64% of the strains were resistant to erythromycin [15].

Rahbar *et al.* have studied 142 isolates associated with 2011 outbreak and 10% of the isolates were resistant [16]. In another study by Rahbar *et al.* conducted on 118 clinical isolates associated with 2005 outbreak, 100% of the isolates were susceptible to erythromycin. In their study, the strains susceptibility was also reported by MIC test ($1-1.5 \mu\text{g.mL}^{-1}$) [6].

In another study conducted on 48 *V. cholerae* clinical isolates in Iran and Afghanistan in 2013 outbreak, 22.9% of the isolates were resistant to erythromycin [17]. Totally, Due to an increase in antibiotics resistance, performing antibiotic susceptibility testing is effective in the cholera treatment process and can prevent unnecessary antibiotics prescription and consumption [16].

The results of another research conducted in Pakistan during 1990-1996 indicated that all of the strains (888 isolates) were susceptible to nalidixic acid, but resistant to tetracycline (91%), sulfamethoxazole (96%), and erythromycin (66%) [18]. A serious problem in cholera treatment process is fluctuation in antibiotic resistance patterns. For example, in a study conducted in Bangladesh during 2004-2005, resistance to erythromycin was reported to be increasing over a year [19].

Tetracycline resistance: In this study, tetracycline resistance was reported to be 50%. These results were confirmed by MIC determination, using E-test method. In another study by Rahbar *et al.*, 20% of the strains were resistant to tetracycline [16]. In a study conducted by Pourshafie *et al.* in 2002-2003, 50% of the isolates were resistant, consistent with the present study results [15]. In another study by Tabatabaei and Khorashad, 60.4% of the isolates (48 numbers) were resistant [17]. In a study by Rahbar *et*

al. conducted in 2005, all of the isolates were susceptible to tetracycline [6].

Nalidixic acid resistance: In this study, resistance to nalidixic was reported as 100%. In Rahbar's study, the results showed that all of the isolates associated with 2005 outbreak were resistant to nalidixic acid [6]. In another studies by Niknezhad *et al.* conducted on 95 strains [7] and Tabatabaei and salimi Khorashad conducted in 2013 outbreak [17], 84.5 and 56.4% of the isolates were resistant to nalidixic acid, respectively.

In another study conducted abroad during 2012-2015, all of the isolates were resistant to nalidixic acid [20]. The results of above studies are almost consistent with the results of the present study. Therefore, the resistance rate to nalidixic acid can be considered as a serious risk for cholera treatment process. No compliance with laboratory standards, bacterial resistance indices, uncontrolled antibiotic consumption, and contact with neighboring countries are considered among the possible causes of high resistance to therapeutic antibiotics.

Gentamicin and Ciprofloxacin resistance: In the present study, no resistance was observed to gentamicin and ciprofloxacin. In a study by Pourshafie *et al.* conducted on 100 *V. cholerae* clinical isolates associated with 2002-2003 outbreak and in a research by Bakhshi *et al.* performed on 25 clinical isolates in 2005 outbreak in Iran, none of the clinical isolates were resistant to gentamicin and ciprofloxacin [15, 21].

Pourshafie *et al.* have studied 50 clinical isolates in summer 2005 in Iran, 14% of which were susceptible to gentamicin [22]. Masoumi asl *et al.* have studied 60 *V. cholerae* isolates in 2013 and Hajia *et al.* have studied 61 *V. cholerae* clinical isolates in 2011, all of the strains were susceptible to ciprofloxacin [23, 24]. In another study by Rahbar *et al.*, they examined 142 isolates in 2011 outbreak, a slight resistance (1.2%) was observed against to ciprofloxacin [16].

Ang *et al.* have examined 50 clinical isolates in 2012-2015 outbreak, all of the strains were susceptible to ciprofloxacin [20]. In another study conducted in Malaysia, all of the *V. cholerae* isolates (25 strains) were susceptible to ciprofloxacin [25]. Comparing the results of these studies shows that fortunately, gentamicin and ciprofloxacin could still be prescribed among as the therapeutic antibiotics for cholera treatment.

Ampicillin resistance: In this study, all of the strains were susceptible to ampicillin. In another study by Rahbar *et al.*, all of the strains were susceptible to ampicillin, consistent with the results of the present study [6]. In another study performed on 96 strains isolated from 1996-2013 [13] and in Tabatabaei and Khorashad's study [17], 33 and 33.3% of the isolates were resistant to ampicillin, respectively. In Tabatabaei and Khorashad's study, a number of isolates were collected from Iranian and

Conclusion

It should be noted that there is the possibility of transferring resistance from the neighboring countries, where they can be considered as one of the factors influencing the antibiotic resistance pattern in Iran. In this study, the isolates were resistant to 2 antibiotics, depending on different factors such as geographical location, antibiotic consumption patterns, the study time, and migration to neighboring countries. In addition, microorganisms with various capabilities such as the ability to carry factors effective in resistance transfer can have a part in changing resistance patterns and creating resistance to several antibiotics.

Microorganisms' intrinsic and acquired characteristics such as mutations can also take part in changing bacteria resistance pattern. Due to the high incidence of permanent mutations in bacteria, clinical and paraclinical examinations are very important. New strains produced by mutations differ from maternal strain in many ways, including the antibiotic susceptibility. Therefore, in order to prevent from the error in antibiotic susceptibility testing results, the quality control of culture media and used disks is important [7]. It is also necessary to note that considering precise laboratory techniques and principles are very important for obtaining accurate results from antibiotic susceptibility tests.

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