



Determination of tetracycline resistance genes in *Vibrio cholerae* O1 biotype El Tor serotype Inaba strains isolated from outbreaks occurred in Iran in 2013

Azin Khany¹, Mitra salehi¹ and Marjan Rahnamaye Farzami^{2*}

¹Department of Microbiology, faculty of biosciences, Islamic Azad University, Tehran North Branch, Iran

²Reference Health Laboratory, Ministry of Health and Medical Education, Tehran, IR Iran

*Corresponding E.mail:marjan.farzami@gmail.com

ABSTRACT

V. cholerae is the causative agent of potentially life threatening diarrheal disease named as cholera. Cholera is an endemic disease in Iran. Encountered increasing resistance of *V. cholerae* to commonly used antibiotics such as tetracycline has led to major challenges in the treatment of this disease. The present study was carried out to determine the prevalence of drug resistance as well as molecular bases of resistant *V. cholerae* strains which were isolated from patients in cholera outbreaks during summer of 2013 in Iran. Susceptibility testing was performed on *V. cholerae* strains isolated from stool of patients suffering from cholera in Iranian reference health laboratory by E-test MIC method as recommended by CLSI guideline. Antibiotic strips used included Ampicillin, Ciprofloxacin, Nalidixic acid, Cefixime, Tetracycline, Erythromycin and Trimethoprim-sulfamethoxazole. Regarding observed dominant pattern of tetracycline resistance comparing to results of previous years, we decided to confirm the resistance by detecting the *tetA*, *tetB* and *tetC* by Polymerase chain reaction method. The results of antibiotic susceptibility testing revealed 100% resistance of isolated strains to tetracycline. Data obtained from PCR reaction on resistant strains for *tetA*, *tetB* and *tetC* showed that 45(44.1%), 37(36.2%) and 70(68.6%) were containing *tetA*, *tetB* and *tetC* gene respectively. Moreover, the frequency of *tetA+tetB*, *tetA+C*, *tetB+tetC*, *tetA+tetB+tetC* also were determined as 9(8.8%), 32(31.3%), 19(18.6%) and 8(7.8%) respectively. This study revealed the pattern of drug resistance distribution of isolates harboring *tetA*, *tetB*, *tetC* genes in relation to sex, age and nationality of patients and the cities where the cases were reported. A significant correlation was obtained between reported geographical incidence and drug resistant strains.

Keywords: Tetracycline, *V. cholera*, Antimicrobial resistance, *tet* genes.

INTRODUCTION

Cholera is a severe form of acute secretory diarrheal disease, caused by *Vibrio cholerae*. Cholera currently is a serious global health problem that has an incidence of 3-5 million per year that leads to 100,000 – 120,000 deaths [1]. This disease is typically characterized by frequent passage of rice watery stools. The other clinical manifestations of cholera include symptoms such as abdominal cramps, vomiting and severe dehydration [2]. *V. cholera* has more than 206 known serogroups, which only O1 and O139 can cause epidemic and pandemic Cholera [3].

Cholera, is usually treated with appropriate rehydration either orally in moderate cases or intravenously in severe form. According to current World Health Organization guidelines, antibiotic therapy is not necessary, unless in severe cases of cholera which is aimed to shorten the duration of disease and *V.*

Cholerae excretion and reduce the volume of fluid loss [4,5]. Tetracycline is the most commonly-used antibiotic recommended by world health organization as the first line therapeutic in cholera cases. After oral administration, concentrations of tetracycline become relatively high in the intestinal lumen because of partial absorption and hepatobiliary excretion [6].

There is three known mechanisms of tetracycline resistance in *V.cholerae* that mediated by more than 38 different tetracycline-resistance determinants [7]. The first is mediated by tet genes encoding ribosomal protection proteins, including Tet(M) and tetO. The second mechanism involves the tetX gene product from bacteroides and is the only known example of enzymatic inactivation of tetracycline that has been described so far. In the third mechanism, tetracycline-efflux genes, including those designated as tetA to tetE, tetG and tetH in Gram-negative bacteria and tetK, tetL and tetP in Gram-positive bacteria, encode membrane-associated proteins that transport tetracycline out of cells [8]. Jun et al. have differentiated by multiplex PCR the tetA, tetB, tetC, tetD, tetE and tetG, which are found primarily in Gram-negative bacteria [8].

In the present study, antibiotic susceptibility testing by MIC method and conventional PCR assay was carried out to analyze the pattern of emergence of resistance genes : tetA, tetB, tetC genes in tetracycline resistant *Vibrio cholerae* isolated from patients in different cities of IRAN in 2013.

MATERIALS AND METHODS

Study design and patient recruitment strategies:

During 2013 a total of 102 samples either as rectal swabs collected from patients with diarrhea and placed in Cary – Blair transport medium or isolated strains from patients suspected for Cholera were received in Reference Health Laboratory- Ministry of health. The samples were transported from different cities throughout the country and collected before starting any antibiotic therapy . Submitted rectal swab samples were processed for isolation and identification of *Vibrio* spp. Whereas a confirmatory serotyping by slide agglutination test was performed for isolated ones to recheck the reported serotype using polyvalent O1 and monovalent Ogawa and Inaba Antisera (BD DifcoTM, Becton Dickinson, Sparks, Maryland, USA). Antibiotic susceptibility testing had been carried out by MIC method using E-test strips(Liofilchem , Italy) according to manufacturer and CLSI guidelines (CLSI- M100-S22).The antibiotics tested were Ciprofloxacin, Nalidixic acid, Cefixime, Ampicillin, Tetracycline, Sulfamethoxazole and Erythromycin. The bacterial samples were inoculated in TSB plus 15% glycerol and maintained in -70°C till further genotyping tests.

Table1: Oligonucleotide sequences used as primers for polymerase chain reaction

Gene	Primer name	Primer sequence	Product size (bp)	References
<i>tetA</i>	<i>tetA-F</i>	GTAATTCTGAGCACTGTCCG	956	16-21
	<i>tetA-R</i>	CTGCCTGGACAACATTGCTT		
<i>tetB</i>	<i>tetB-F</i>	CTCAGTATTCCAAGCCTTTG	414	21-22
	<i>tetB-R</i>	ACTCCCCTGAGCTTGAGGGG		
<i>tetC</i>	<i>tetC-F</i>	GGTTGAAGGCTCTCAAGG GC	505	22
	<i>tetC-R</i>	CCTCTTGCGGGATATCGTCC		

Table2: The gradients of PCR reaction

Gene	Reaction volume (μL)	PCR master mix (μL)	Sterilized distilled water (μL)	Each primer (μL)	Template DNA (μL)
<i>tetA</i>	25	13	0	1	10
<i>tetB</i>	25	13	0	1	10
<i>tetC</i>	25	13	0	1	10

Molecular detection of antibiotic resistant genes:

Antimicrobial resistant genes were investigated using a single PCR assay. The stored isolates were subcultured in Trypticase Soy Agar with sheep blood at 35 °C for 24 hrs. and DNA was extracted using 250 QIAamp Mini spin column (Qiagen, cat No. 51306). The DNA extracted by this method was stored at -20 °C. The oligonucleotide primer sets were used to detect tetracycline resistance genes including tetA, tetB, tetC (Table 1). The primers were commercially synthesized by the Methabion Company

(Table 1). PCR conditions were applied to each assay according to table 2. The optimal thermal parameters were as follows: initial denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C (tetB) and 57°C (tetA & tetC) for 1 min and extension at 72 °C for 3 min. A final extension at 72 °C for 5 min at the end of the amplification cycles was included. The PCR products obtained from different tet genes were resolved by 2% agarose gel electrophoresis and stained with ethidium bromide. The amplification products were visualized under a UV trans-illuminator and photographed.

RESULTS

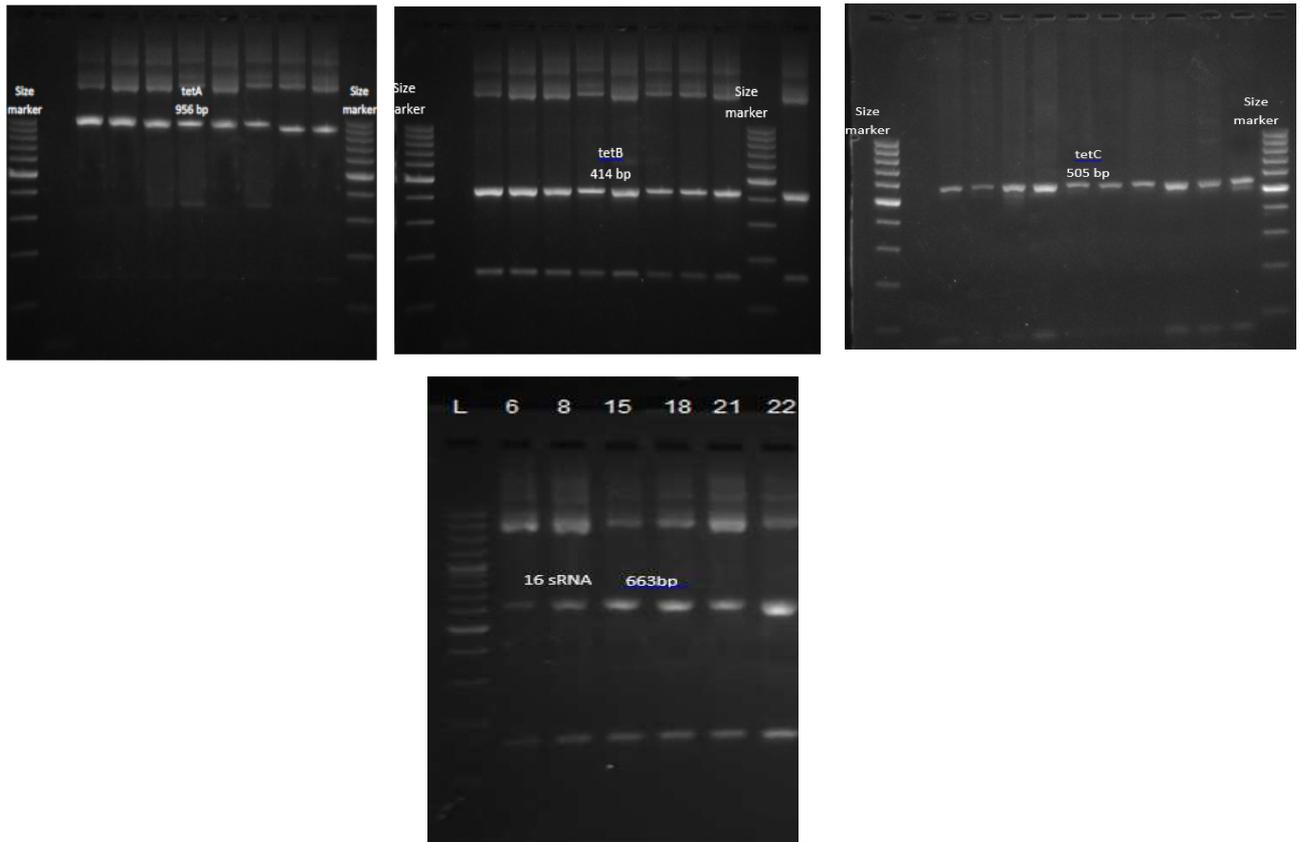


Figure 1: Illustrated results of tetA, tetB , tetC and 16s rRNA genes on agarose gel

A total of 102 *V.cholerae* positive cases were registered and confirmed by Iranian Reference Health Laboratory as *Vibrio cholerae* serotype O1 Biotype Inaba. The samples were collected from 12 out of 31 provinces in the country through August to November 2012. Thirteen (12.7 %) of cases were female and eighty nine (87.3%) of cases were male. Sixty two (61%) out of 102 specimens belonged to Iranian patients, 38(37%) and 2(2%) were from Afghanistan and Pakistan respectively. The mean age of patients was (28.6 ± 14.6%) .

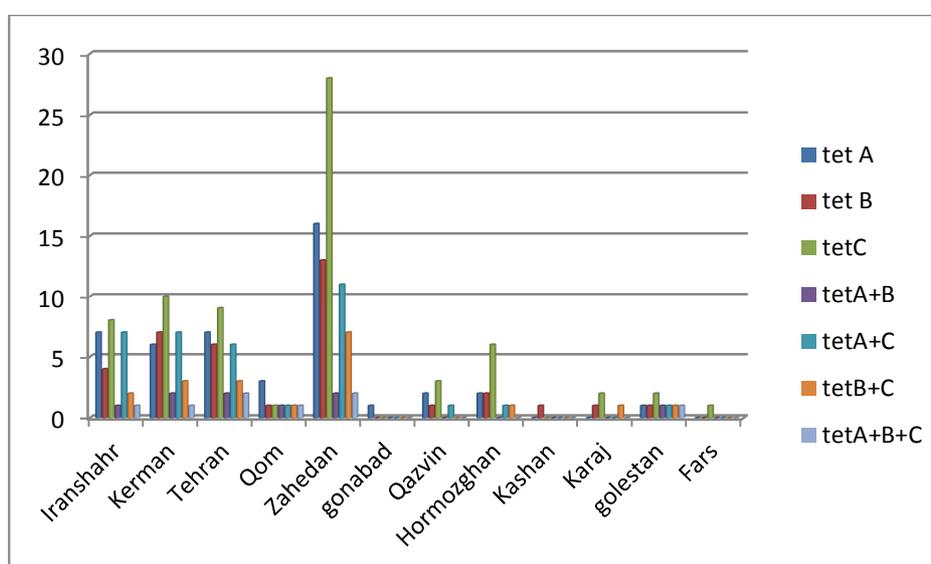
The result of antibiotic susceptibility testing using MIC test strips revealed hundred percent resistance to tetracycline. Data obtained from PCR reactions for each of resistance genes illustrated that 45(44.1%), 37(36.2%) and 70(68.6%) of strains had tetA, tetB and tetC gene respectively.

Moreover, the frequency of tetA+tetB, tetA+tetC, tetB+tetC , tetA+tetB+tetC genes coexistence was 9(8.8%), 32(31.3%), 19(18.6%) and 8(7.8%) respectively. Frequency of tetA, tetB and tetC in the samples regarding gender, age and nationality of patients are demonstrated in table 1. The present study revealed that tetC was the predominant antibiotic resistant gene among patients.

Table 1: The frequency of *tet* genes in relation with sex, age and nationality

Factors	tetA	tetB	tetC	tetA+tetB	tetA+tetC	tetB+tetC	tetA+tetB+tetC
Sex							
Male	37	33	63	7	26	17	6
Female	7	3	7	1	5	1	1
Age							
<10	3	2	3	2	3	2	2
10-20	11	7	12	1	4	3	1
20-30	14	12	24	2	13	7	3
30-40	11	7	12	3	7	3	2
40-50	1	5	8	0	3	1	0
50-60	1	3	7	0	1	2	0
60-70	2	1	3	0	1	0	0
Nationality							
Iranian	26	24	41	7	19	11	6
Afghan	17	12	28	1	12	7	1
Pakistan	2	1	1	1	1	1	1

Figure 2 shows the frequency of *tet* genes in different cities if Iran. According to the figure, the highest frequency of *tet* genes has been found in Zahedan.

Figure 2: Distribution of *tet* genes in the samples isolated in different cities of country

DISCUSSION

Acute secretory diarrhea caused by *Vibrio cholerae* is currently an important health challenge mostly in developing countries with low economic conditions and poor hygiene. Cholera is routinely treated by appropriate rehydration but in severe cases usage of recommended antibiotics are indicated to shorten the duration of symptoms and decrease the volume of water and electrolytes reduction. Studies conducted in recent years on the antibiotic susceptibility pattern of *V. cholerae* isolated in different outbreaks have revealed significant increases in the resistance of this bacteria to antibiotics such as tetracycline or fluoroquinolones. Infections with drug-resistant *V. cholerae* can result in higher case-fatality rates, prolonged hospitalization, more secondary infections, and increased health care costs. Considering that tetracycline as an affordable oral antibiotic with acceptable efficacy is the first line of drugs recommended by WHO for treating cholera, this trend by producing new challenges in providing appropriate antibiotics particularly for countries with low socioeconomic conditions would be a global health concern and needs special attention.

In Iran the first study on the antibiotic susceptibility of *V. cholerae* is performed by Rahbar et al in 2005 showing no resistance of isolated strains to tetracycline (9). Results obtained in another study in South

Africa revealed same susceptibility pattern (10). Since then several consecutive studies mostly performed in the Iranian reference laboratory-Ministry of Health, have shown emergence of resistant strains and gradual increase in resistance to 100% in 2013. Study performed by Barati et al on resistance pattern of 244 positive cases caused by *V.cholerae* serotype O1 biotype ogawa in outbreaks happened during 2011 in Alborz province shows increased resistance of this strain to tetracycline in comparison to previous study in same region.(11-12)

In this study we analyzed the results of 102 diagnosed Cholera cases caused by *V.cholerae* biotype El Tor serotype Inaba that were confirmed as tetracycline resistant. Antibiotic susceptibility testing was performed to determine the prevalence of most common genes responsible for resistance of *V. cholerae* ;i.e .tetA, tetB, tetC genes considering residence area, sex, age and nationality of patients.

The results show that 92% of isolates were obtained between August and September. Considering the known ecology of *V. cholerae* the seasonality incidence of disease is comparable to previous years (11).The results indicated that tetracycline resistance is significantly higher in men (87.3%), most of the cases were in the age group 20- 30 (mean age 28.6±14.6).

Regarding that in the summertime the frequency of camping, trips or similar activities is higher between young aged individuals and due to probable limited access to clean and healthy drinking water, increased incidence in the age group 20- 30 years old can be predictable

The results of PCR revealed predominance of tetC gene (68.6%) alone or in combination with tetA and tetB among the cases. In 66.5% of cases study results showed more than one gene in PCR i.e. tetA+tetB (8.8%). tetA+tetC (31.3%), tetB+tetC(18.6%) and combination of tetA, tetB and tetC (7.8%),. Most of the samples were reported in Zahedan (44%) followed by Kerman, Iranshahr and Tehran. and tetC was the dominant gene in all cities as well. As there is no previous study on the pattern of emerged genes responsible for tetracycline resistance in the country, it is not possible to compare the present results but in regard with broad common borders between Iran and neighboring countries i.e., Afghanistan and Pakistan that have similar incidence of cholera outbreaks, and the large number of travelers between these countries, the meaningful relation between geographical distribution of resistant genes is questionable. Further studies by molecular methods such as Pulsed Field Gel Electrophoresis(PFGE) are necessary for assessing the source of infections occurred in these countries.

CONCLUSION

Antibiotic resistance is one of the largest threats to future global health and these data highlighted the need for revising the recommendations of WHO for treating cholera to help choosing appropriate antibiotics. On the other hand the changing pattern of antibiotic resistance throughout the world due to over and misusing antibiotics in health system not only in human branch but also animal husbandry needs serious attention. By establishing certain trends regarding tetracycline resistance, we can initiate to uncover underlying causes for the rise in the frequency of tetracycline resistance determinants.

Acknowledgements

We would like to thank Dr Amir Sohrabi, Dr Mohammad Rahbar, miss Roghieh Saboorian and most importantly Dr Alireza Dolatyar for their advices and technical support. We should also appreciate the constant efforts of health care personnel involved in foodborne disease diagnosis and management and their important role for providing appropriate specimens used in this Study

REFERENCES

- [1] World Health Organization , Report on Cholera 2012
- [2] Weir, E., and Haider, S. (2004) Cholera outbreaks continue. CMAJ 170: 1092-1093
- [3] Shah M. Farque, and G. Balakrish Nair (2002). Molecular Ecology of Toxigenic Vibrio Cholerae. Microbiol, Immunol. 46(2), 59-66.
- [4] Lindenbaum, J., W. B. Greenough, and M. R. Islam.1967. Antibiotic therapy of cholera. Bull. W. H. O. 36:871–883.

- [5] Greenough WB, III, Gordon RS, Jr., Rogenberg IS, Davies BI, Benenson AS. Tetracycline in the treatment of cholera. *Lancet* 1964;1:355-7.
- [6] Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG, editors. Goodman & Gilman's The pharmacological basis of therapeutics. 9th ed. New York: McGraw-Hall, 1996:1124-31.
- [7] Roberts MC (2005) Update on acquired tetracycline resistance genes. *FEMS MicrobiolLett* 245:195–203 Roberts MC (2005) Update on acquired tetracycline resistance genes. *FEMS MicrobiolLett* 245:195–203.
- [8] Chopra I, Roberts MC (2001) Tetracycline antibiotics: mode of action, applications, molecular biology and epidemiology of bacterial resistance. *MicrobiolMolBiol Rev* 65:232–260.
- [9] Jun LJ, Jeong JB, Huh MD, Chung JK, Choi DL, Lee CH, Jeong HD (2004) Detection of tetracycline-resistance determinants by multiplex polymerase chain reaction in *Edwardsiellatarda* isolated from fish farms in Korea. *Aquaculture* 240:89–100
- [10] Rahbar M, Sabourian R, Saremi M, Abbas IM, Masoumi AH, Soroush M. Epidemiologic and drug Resistant Pattern of *Vibrio Cholerae* O1 Biotype EL Tor, Serotype Inaba during the summer of 2005 outbreak in Iran. *J Ardabil Univ Med Sci.* 2007;7(1):41–5.
- [11] Ngandjio A, Tejiokem M, Wouafo M, Ndome I, Yonga M, Guenole A, et al. Antimicrobial resistance and molecular characterization of *Vibrio cholerae* O1 during the 2004 and 2005 outbreak of cholera in Cameroon. *Foodborne Pathog Dis.* 2009;6(1):49–56. doi: 10.1089/fpd.2008.0127. [PubMed: 18991540]
- [12] Sack DA., Lyke Ch., McLaughlin C. and Suwanvanichkij V.(2001) Antimicrobial resistance in shigellosis, cholera and campylobacteriosis WHO/CDS/CSR/DRS/2001.8
- [13] Barati HA, Golmohmmadi A, Momeni I, Mpradi G. A cholera outbreak investigation in Karaj district in 2008. *Iran J Epidemiol.* 2010;6(3):28–34
- [14] Barati H., Moradi Gh., Aziz Rasouli M, and Mohammadi P. Epidemiologic and Drug Resistance Pattern of *Vibrio cholerae* O1 Biotype El Tor, Serotype Ogawa, in the 2011 Cholera Outbreak, in Alborz Province, Iran Jundishapur J Microbiol. 2015 November; 8(11): e23477.
- [15] Lipsitch M, Singer R, Levin B: Antibiotics in agriculture: When is it time to close the barn door? In Proceedings of the National Academy of Sciences of the United States of America. PNAS 2002, 99:5752–5754. Soo K: USDA invites comments on Use of tetracycline, other substances in organic agriculture. [http://www.ams.usda.gov]
- [16] Santanu Kumar Kar, Bibhuti Bhusan Pal, Hemanta Kumar Khuntia, K. Gopinath Achary, Chinmaye Priyadarshini Khuntia (2015) Emergence and Spread of Tetracycline resistant *Vibrio cholerae* O1 El Tor variant during 2010 cholera epidemic in the tribal areas of Odisha, India
- [17] Masaaki Iwanaga, Claudia Toma, Tomoko Miyazato, Sithat Insiengmay, Noboru Nakasone, and Masahiko Ehara (2004) Antibiotic Resistance Conferred by a Class I Integron and SXT Constin in *Vibrio cholerae* O1 Strains Isolated in Laos
- [18] Tabatabaei SM., Salimi Khorashad A., (2014) Antimicrobial Resistance Patterns of *Vibrio cholera* Strains Isolated From Afghan and Iranian Patients in Iran
- [19] Jharna Mandal, Dinooop K.P., and Subhash Chandra Parija (2012) Increasing Antimicrobial Resistance of *Vibrio cholerae* O1 Biotype El Tor Strains Isolated in a Tertiary-care Centre in India
- [20] Boughattas S., Salehi R. (2014) Molecular approaches for detection and identification of foodborne pathogens
- [21] Salimi-Khorashad A., Tabatabae M., Amirabadi A., Roudbar-Mohamadi Sh. (2012) *Vibrio cholerae* and Changing of Microbial Resistance Patterns in Sistan and Baluchestan Province