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Serotyping, Antibiotic Susceptibility Pattern and Detection of hlyA Gene Among Cholera Patients in Iran

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ABSTRACT

Background: Cholera is a severe diarrheal illness caused by *Vibrio cholerae*, which produces a virulence factor named El Tor hemolysin encoded by the hlyA gene.
***Objectives*:** This study meant to analyze the phenotypic characteristics and presence of *hlyA* gene in *V. cholerae* isolated from patients in Iran. The *hlyA* gene which codes for hemolysin, plays an essential role in manifestation of cholera ,and could be used to diagnose pathogenic *V. cholerae* El Tor O1 strains.
Patients and Methods: One hundred stool samples from the patients with cholera during 2002-2003 were collected from Tehran, Kashan, Kermanshah and Ahvaz cities, which were subject to diagnostic tests. Serotyping, and antibiotic susceptibility tests were applied and polymerase chain reaction (PCR) was also used to detect the *hlyA* gene.
Results: The group specific antisera identified the isolates as Ogawa, Inaba, Hikojima and NAG (Non-agglutinable) in 74%, 3%, 0% and 23% of the isolates, respectively. Antibiotic susceptibility test showed that all of the strains were sensitive to ciprofloxacin, gentamycin and doxycyclin but the isolates showed resistance to sulfamethoxazole/trimethoprim (74%), erythromycin (64%) and tetracycline (50%). *V. cholerae* El Tor isolates were 100% positive for *hlyA* gene, but hemolysis phenotype characteristics were found in 95% of the cases.
Conclusions:The results indicated that Ogawa serotype was identified as the dominant serotype which revealed multiple antibiotic resistances to sulfamethoxazole/trimethoprim, oxytetracycline, erythromycin, tetracycline and chloramphenicol. The presence of *hlyA* gene in nonhemolytic strains of *V. cholerae* O1 biotype El Tor indicated that some factors prevent expression of the hemolysin gene.

▸ *Implication for health policy/practice/research/medical education:*
Even though cholera is easily prevented and treated, it remains life- threatening in Iran , this study indicated the need for continuous recording of antibiotic resistance and status of hlyA production *Vibrio cholerae* El tor at national level.

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1. Background

Cholera is a disease unique to humans with sporadic epidemic cases, and seven pandemics have been reported that resulted in thousands of deaths and major changes in socio-economic situations worldwide, these outbreaks indicate that cholera is still among the most prevalent human diseases , especially in developing countries (1-3).

The three most commonly reported serotypes of cholera are caused by O1, O139 and non O1 - non O139 serotypes. The O1 strains are divided into two biotypes of classical and El Tor with three serotypes of Ogawa, Inaba and Hikojima (2).

In some Asian countries such as Pakistan and Afghanistan the disease has been reported as endemic. In Iran, several outbreaks with a number of mortalities in the recent years have been reported (4, 5).The genotypic characteristics of *Vibrio cholerae* are several toxin genes such as cholera toxin (*ctx*), zonula occludens toxin (*zot*), the accessory cholera toxin (*ace*) and toxin-coregulated pilus *(tcpA).*

2. Objectives

In this study, the serological and antibiotic resistance patterns of the *V. cholerae* isolates from four cities of Iran, were sought. Moreover the isolated *V. cholerae* from infected patients were phenotypically characterized and the presence of hemolysin gene (*hlyA*) which codes for a thermostable hemolysin, an important virulence factor, was studied.

3. Patients and Methods

3.1. Bactertial Isolation and Serotyping

One hundred *V. cholerae* were isolated during 2002-2003 from different cities of Iran including Tehran (in the North, 32 isolates), Kermanshah (in the West, 12 isolates), Ahvaz (in the South, 30 isolates) and Kashan (in the Center, 26 isolates).The specimens were collected on sterile swabs, placed in Cary-Blair transport medium , then were immediately transferred to alkaline peptone water (APW, pH 8.6) and incubated for 6 h at temperature of 37C. They were then cultured on thiosulfate-citrate-bile salts-sucrose (TCBS) agar medium (Difco, Sparks, MD USA) (2).

After growth of bacteria at 37 ° C for 24 h, the biochemical characteristics including triple sugar iron Agar (TSI), indole, simon citrate, methyl red, voges-proskauer, hemolytic activity on blood agar, motility, oxidase, urease tests (Difco) and inhibition by polymyxin B (50U/ml) (Difco) were studied (2, 3). Serological test using anti-sera O1, Ogawa, Inaba which were produced by Pasteur Institute of Iran was done on slide agglutination.

3.2. Detection of Hemolysis

To determine the hemolytic activity of *V. cholerae* samples isolated bacteria were grown in brain heart infusion broth (Difco) for 3 h in 37°C and then transferred onto the 5% sheep blood nutrient agar plates for 24 h at 37°C. The colonies were then examined for hemolysis (6).

3.3. Antibiotic Susceptibility

Antibiotic susceptibility test was carried out based on the standard Disk diffusion method recommended by Clinical Laboratory and Standard Institute (CLSI) on Muller Hinton agar. Antibiotic disks of chloramphenicol (30μg), erythromycin (15μg), tetracycline (30μg) and oxytetracycline (30μg), sulfamethoxazole/trimethoprim (23.75/1.25μg) and gentamycin (10μg), ciprofloxacin (5 μg) and doxycycline (30μg) (BBL, Sparks, MD, USA) were used (3, 7).

3.4. Polymerase Chain Reaction of hlyA

V. cholera chromosomal DNA was extracted by boiling method (8) and PCR was done using the following primers to detect *hlyA*:

Forward: 5'- GAGCCGGCATTCATCTGAAT-3'

Reverse: 5'- CTCAGCGGGCTAATACGGTTTA-3'

under the following conditions. Initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing 60 °C for 1 min, extension at 72°C for 1min and final extension at 72°C for 10 min (6, 8).The molecular weight marker of 100 bp and also a positive control of *V. cholerae O1* El Tor ATCC 14033 were used.

4. Results

One hundred isolates were collected from four different cities in Iran. All samples were positive for indole, simon citrate, motility, and oxidase tests, and also they were all resistant to polymyxin B. Moreover, they were all negative for urease and 3 and 54% were positive for methyl red and voges-proskauer, respectively.

Amongst the strains, 74, 23 and 3% were considered as Ogawa, NAG (Non-agglutinable) and Inaba. All isolates from Tehran and Kermanshah, and 90 and 10% of isolates from Ahvaz were serotyped as Ogawa and Inaba, respectively. Twenty six isolates from Kashan were NAG 88% and Ogawa 12% serotypes (*Table 1*).

Table 1. Serotyping of *V.cholerae* El Tor Isolated in Four Different Cities of Iran (2002-2003).

	Ogawa,%	Inaba,%	NAG,%
Tehran	32	-	-
Kermanshah	12	-	-
Kashan	3	-	23
Ahvaz	27	3	-

Ninety five percent of the strains were positive for β-hemolysin and were identified as Ogawa (70%), NAG (22%) and Inaba (3%). Out of 5 isolates which showed negative hemolysis, 4 and 1 were Ogawa and NAG, respectively.

The results from antibiotic resistance indicated that average nationwide resistances were 74, 64, 62, 50 and 27% of the isolates to sulfamethoxazole/trimethoprim, erythromycin, oxytracycline, tetracycline, and chloramphenicol, respectively. In Kashan, 84% of the isolates were resistant to erythromycin whereas the average resistant to this antibiotic nationwide in Iran was 64% (*Table 2*).

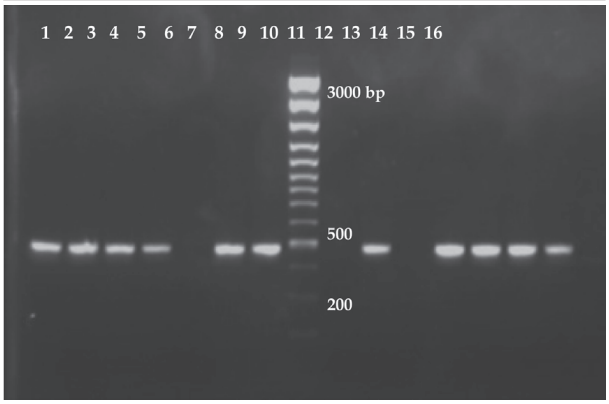
Table 2. Antibiotic Resistance Patterns of *V.cholerae* El Tor Isolated in Four Different Cities of Iran (2002-2003)

		The Percentages of Antibiotic-Resistance							
	***V.Cholrea* Isolated, No.**	**Sxt**	**Oxy**	**Te**	**E**	**C**	**Dox**	**Gm**	**Cip**
Tehran	32	100	89	98	50	32	5	0	0
Kermanshah	12	36	60	0	59	54	0	0	0
Kashan	26	0	0	0	84	0	0	0	0
Ahvaz	30	100	100	100	60	23	10	8	0

Abbreviations: C, Chloramphenicol; Cip, Ciprofloxacin; Dox, Doxcycline; E, Erythromycin; Gm, Gentamicin; Oxy, Oxytetracyclin; Sxt, Trimethoprim-Sulfamethoxazole; Te, Tetracycline

Non-hemolytic Ogawa (4 isolates) and NAG (1 isolate) showed multiple resistances to sulfamethoxazole/trimethoprim, oxytetracycline, erythromycin and tetracycline. Molecular detection of PCR indicated that all isolates were hemolysin positive (*Figure 1*), yet 95% of the isolates showed hemolysin phenotype on the blood agar.

Figure 1. PCR Analysis of *V.cholerae* Genes in Patients Isolates and Simultaneous Use of Bacteria *Staphylococcus aureus, Escherichia coli ,Aeromonas hydrophila*, as Negative Controls and (*V.cholerae O1* El Tor, ATCC: 14033) as a Positive Control, a Molecular Weight Marker 100 bp, Amplified Product of the hlyA Gene is 481bp.



1, 2, 3, 4, 6, 7, 10, 12, 13, 14: V.cholerae,5: S.aureus,9: E.coli, 11: *A.hydrophila*, 15: positive control, 16: negative control.

5. Discussion

The present study was performed on 100 strains of *V. cholerae* El Tor collected from different of Iran. It, it was found that 95% of the strains showed hemolysis on blood agar medium, whereas all were positive PCR for hylA gene. The *hlyA* gene is a pathogenic factor involved in the cholera disease process, causing host tissue damage and releasing a variety of cellular components, Unlike non-hemolytic strains of the classical biotype, the El Tor strains can produce and secrete hemolysin toxin into the medium (6, 9, 10). This is in accordance with other reports that non-hemolysin producing El Tor may be found amongst the *V. cholerae* isolates (8).

Comparing the present study results with the other published reports (6) indicated that the ratio of non-hemolytic to hemolytic isolates could vary from 5% to 15%. On the other hand, it has been reported in Latin America that *V.cholerae* carrying hemolysin characteristics but lacking toxin genes such as *ctx*, *zot, tdh, tcp* may still cause epidemic (11).

In the present study, 74, 23 and 3% of all isolates were Ogawa, NAG and Inaba serotypes, suggesting that the Ogawa serotype was the predominant serotype in 2002. However, in the early 1990, the predominant serotype in Iran was Inaba (12), indicating a serotype switch from Inaba to Ogawa which still persists in Iran. Multiple antibiotic resistances to chloramphenicol (27%), tetracycline (50%), oxytetracycline (62%), erythromycin (64%) and sulfamethoxazole/trimethoprim (74%) were observed in the current study. Various studies in Iran and elsewhere have reported resistance of *V. cholerae* to chloramphenicol, erythromycin, kanamycin, tetracycline and sulfamethoxazole/trimethoprim (7, 13). Results of antibiotic susceptibility testing in 2005 in Iran showed that 86, 84, 84 and 82% of isolates were resistant to streptomycin, chloramphenicol, sulfamethoxazole/trimethoprim and tetracycline, respectively (7). Comparing the results of the two studies in different periods indicates significant drug resistant of *V. choerlae* isolates obtained in 2002 and 2005 in Iran. Such increase in antibiotic resistance among the *V. choerlae* isolates is alarming, and may indicate that constant monitoring of the drug susceptibility must be conducted.

The study from 1991 to 1994 in Angola showed multiple antibiotic resistances of *V. cholerae* to chloramphenicol, tetracycline, erythromycin and co-trimoxazole. This antibiotic resistance clone remained stable during the 4-year epidemic period in Angola (14). Collectively, these studies suggest that the multiple antibiotic resistant strains of *V. cholerae* are highly stable and may remain endemic regardless of geography. The resistance patterns in this study, indicate that ciprofloxacin for the treatment phase, and then gentamicin and doxycycline are recommended.

In this study, multiple resistance to sulfamethoxazole/trimethoprim, oxytetracycline, erythromycin, tetracycline and chloramphenicol were found among *V. cholerae* isolates in Tehran, Kermanshah and Ahvaz. On the other hand in Kashan, except all of the isolates were susceptible to all antibiotics examined, except erythromycin**.** Such difference in antibiotic susceptibility pattern among the isolates could be due to eco-system of different provinces and/or extensive use of antibiotics in Iran. Kashan as an arid

city could affect the existence of *V. cholerae* with certain phenotypic and genotypic characteristics which needs to be studied. Therefore Monitoring serotype switching, antibiotic resistance and presence of toxin and virulence factors in *V. cholerae* is essential in limiting the dissemination of cholera at the local, regional and international levels.

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Authors' Contribution

None declared.

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