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Antimicrobial Resistance Patterns of *Vibrio cholera* Strains Isolated From Afghan and Iranian Patients in Iran

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Background: The increasing prevalence of multidrug resistant strains of *Vibrio cholera*e in recent cholera epidemics across the world is a growing global public health challenge.

Objectives: This study was undertaken to identify the patterns of antimicrobial resistance in isolates collected from laboratory-confirmed cases of cholera during an outbreak occurred between August and September 2013 in Sistan and Balouchestan province, southeast of Iran.

Patients and Methods: Forty eight *V. cholera*e isolates were obtained from clinical samples. All the strains were identified as Inaba serotype. Antibiotic susceptibility of the isolates for sulfamethoxazole-trimethoprim, tetracycline, nalidixic acid, ciprofloxacin, ampicillin, ceftriaxone and erythromycin were determined. The method used for antimicrobial susceptibility testing (AST) was standard disk diffusion technique (Kirby-Bauer method). According to the criteria published by the Clinical and Laboratory Standards Institute (CLSI), the isolates were characterized as susceptible, intermediate, or resistant.

Results: A ST revealed high levels of resistance to sulfamethoxazole-trimethoprim (89.6%), tetracycline (60.4%), ampicillin (56.3%), nalidixic acid (43.7%) and erythromycin (22.9%). Intermediate susceptibility levels to erythromycin (68.8%), nalidixic acid (56.3%) and ampicillin (33.3%) were identified. All the samples were susceptible to ciprofloxacin and ceftriaxone. Resistant to erythromycin, sulfamethoxazole-trimethoprim and ampicillin dominated in Afghan patients' samples, while a greater proportion of samples from Iranian patients showed resistance to tetracycline and nalidixic acid. All the differences were statistically significant.

Conclusions: Our findings suggested a worrying increase in resistance of *V. cholerae* strains to commonly used antibiotics. Differences in patterns of resistance between Afghan and Iranian patients' samples were observed, which further emphasize a need for constant observation.

Keywords: Cholera; *Vibrio cholerae*; Antimicrobial Resistance; Iran

1. Background

Cholera is an acute diarrheal infection with epidemic and pandemic potentials, caused by the bacterium *Vibrio cholerae*. Annually, there are approximately 3-5 million cases of *V. cholera*e worldwide, causing 100 000 to 120 000 deaths (1). The world has been witnessing a striking growth in the burden of cholera during the recent years, spreading to countries that had not previously experienced this disease, reflecting the failure in implementation of control measures (2). More than 200 serogroups of *V. cholerae* have been identified, but serogroups O1 and O139 are the most common causative agents of cholera epidemics (3). The O1 serogroup has two biotypes (classical and El Tor) each of which has three serotypes, Ogawa, Inaba and Hikojima (4). The last serotype is extremely rare; *V. cholerae* O1 strains have the ability to interchange between the Ogawa and Inaba serotypes (5). Cholera is transmitted by the fecal-oral route and *V. cholerae* is sensitive to the low pH found in human stomach. Therefore, people with low stomach acid production such as young children, the elderly and patients who take antacids are at increased risks of contracting cholera (6). A potent enterotoxin (CT, also called choleragen) produced by *V. cholera*e strains is responsible for lethal symptoms of the disease, which causes massive secretion of electrolytes and water into the intestinal lumen (4). Replacement of fluid and electrolytes is the basis of cholera treatment (3). However, antibiotics are commonly administered for treatment of severe cases to reduce the duration of illness and to lessen shedding of *V. cholerae* in the stool, and hence, the risk of infection spread (7). The rapid emergence and spread of multidrug resistant *V. cholerae* with resulting outbreaks across the globe can undermine the success of antimicrobial therapy (8). However, there is a great variation in patterns of antibiotic resistance at different times and different places, with multiple antibiotic-resistant *V. cholerae* commonly found during epidemics. There are several reports of *V. cholerae* resistance against tetracy-

cline and fluoroquinolones and the disease surveillance statistics across the world show that multidrug-resistant *V. cholerae* outbreaks are on a rise (9). Nalidixic acid-resistant *V. cholerae* O-1 strains have been detected in Karachi, Pakistan (10). Antimicrobial drug resistance in *Vibrio* spp. can develop through efflux pumps, spontaneous chromosomal mutation, conjugative plasmids, trimethoprim/sulfamethoxazole (SXT) elements and integrons, and integrating conjugative elements (ICEs) (9). Many O139 and O1 isolates have acquired SXT elements or a closely related ICE (11). Improper administration of antibiotics by health professionals, misuse by unskilled practitioners and ordinary people, substandard quality of antimicrobials, poor sanitation that facilitates the spread of multidrug-resistant *V. cholera*e, and inadequate diseases surveillance are among the social and behavioral factors associated with appearance of antibiotic resistance pathogens in developing countries (12). Other factors contributing to the emergence of antimicrobial drug resistance in *Vibrio* spp. include an increase in the number of susceptible people in communities, international travel and population movements, and the breakdown of public health measures (13). Given the fact that the geographical and temporal occurrence of *V. cholerae* shows changes across the world, there is a continuing need to monitor antimicrobial resistance patterns of this potentially life-threatening pathogen (14). Periodic outbreaks of cholera have been reported from Sistan and Balouchestan province, southeast of Iran. Most of these outbreaks have been linked to the cross-border movement of populations (15). Although the predominant *V. cholerae* serotype in Sistan and Balouchestan province is Ogawa and serotype Inaba is rare, all the *V. cholerae* strains isolated during a recent outbreak that occurred between August and September 2013 were of Inaba serotype. In Pakistan, a country in which cholera is endemic (16), which has a long common border with Sistan and Balouchestan province, serotype Inaba has outnumbered serotype Ogawa and the pathogen has been resistant to sulfamethoxazole-trimethoprim and chloramphenicol, but not to ampicillin, tetracycline and ofloxacin (17). The increasing antimicrobial resistance in *V. cholera*e strains isolated from cases detected in the region and also in Sistan and Balouchestan province is a major challenge in treatment of patients with cholera (8, 18).

2. Objectives

Since the majority of patients with cholera in the 2013 outbreak were Afghans entering Iran through Pakistan border, the authors decided to investigate the possible drug resistance in isolates collected from laboratory-confirmed cases of cholera and compare the antimicrobial resistance patterns between non-Iranian and Iranian patients with cholera.

3. Patients and Methods

This study included *V. cholerae* strains recovered from stool samples of 48 patients (42 Afghans and 6 Iranians) with acute watery diarrhea. The mean age of patients was 22.1 ± 9.7 years old. All the patients were either Afghans who newly crossed the Iran-Pakistan border or Iranians that came in contact with Afghan patients. The stool samples were collected before starting the antibiotic therapy. All the strains were characterized as serotype Inaba. As recommended by the Clinical and Laboratory Standards Institute (CLSI), the standard disc diffusion technique (Kirby-Bauer method) (19) on Mueller-Hinton agar was used to determine the in vitro susceptibility of the identified *V. cholera*e isolates (19). The antibiotic discs used in this study were purchased from Iranian Padtan Teb Company (www.padtanteb.com). The antibiotics discs were used at the following concentrations: sulfamethoxazole-trimethoprim5μg, tetracycline 30 μg, nalidixic acid 30 μg, ciprofloxacin 5 μg, ampicillin 10 μg, ceftriaxone 30 μg, and erythromycin 15 μg. The zones of growth inhibition were recorded. The criteria published by the CLSI (20) were used to interpret the test results. Since the reliability of disk diffusion results for ciprofloxacin and nalidixic acid has not been validated, the interpretive criteria for Enterobacteriaceae was used as the tentative zone size standards (21). *Escherichia coli* ATCC 25922 was used as the control sample. Susceptibility levels of the *V. cholera*e isolates were determined as susceptible, intermediate, or resistant.

4. Results

The results for antimicrobial susceptibility testing using the standard disk diffusion technique (Kirby-Bauer method) for *V. cholera*e isolates are presented in Table 1. About 89.6% of *V. cholera*e isolates exhibited resistance to sulfamethoxazole-trimethoprim, while the proportions of intermediate and sensitive strains were 2.1% and 8.3%, respectively. For tetracycline, 60.4% of the isolates were resistant, while 39.6% were sensitive. Resistant and intermediate outcomes for ampicillin were identified in 56.3% and 33.3% of samples, respectively. Only 10.4% of the isolates were sensitive to ampicillin. None of the samples were sensitive to nalidixic acid and intermediate outcome was found in 56.2% of isolates and 43.8% were resistant. The majority of isolate were resistant (22.9%) or intermediate (68.8%) against erythromycin and 8.3% were sensitive. All the samples were resistant to ciprofloxacin and ceftriaxone. The pattern of antimicrobial resistance greatly differed between the *V. cholerae* strains isolated from Afghan patients and the bacteria isolated from Iranian patients with cholera (Table 2). The isolates from Afghan patients were more likely to be resistant to erythromycin, sulfamethoxazole-trimethoprim and ampicillin. Only 2.4% of isolates form Afghan patients were sensitive to erythromycin, while 50% of isolates from Iranian patients were sensitive. In comparison with Iranian isolates, a greater proportion of Afghan patients' isolates showed

intermediate resistance (73.8% versus 33.3%) or resistance (23.8% versus 16.7%)against erythromycin and the difference was statistically significant (P value < 0.006). Almost all the samples obtained from Afghan patients (97.6%) were resistant to sulfamethoxazole-trimethoprim, while 3.3% of Iranian samples were resistant and 50% were sensitive (P value < 0.0001). For ampicillin, 61.9% of *V. cholera*e strains from Afghan patients were resistant and this proportion was 16.7% for Iranian samples (P value < 0.0001). However, a greater proportion of *V. cholerae* strains obtained from Iranian patients showed antimicrobial resistance to tetracycline (83.3% versus 57.1%) and nalidixic acid (66.7% versus 40.5%). All of the abovementioned differences were statistically significant. Isolated *V. cholerae* strains from both Iranian and Afghan patients were 100% sensitive to ciprofloxacin and ceftriaxone.

5. Discussion

Our findings showed that strains of *V. cholerae* have become resistant to commonly used antibiotics and multidrug resistance has been on a rise. We also found different patterns of antimicrobial susceptibility for *V. cholerae* isolates obtained from Iranian and non-Iranian patients with cholera. Our findings were consistent with the results from studies that have documented an increase in emergence of multidrug-resistant *V. cholerae* strains during the outbreaks occurred in the past decade in Iran. Comparing the results from the present study with a similar study on antimicrobial susceptibility patterns of *V. cholerae* strains in Sistan and Balouchestan province during 2008-2010, showed a similar pattern of resistance for sulfamethoxazole-trimethoprim and no resistance to ciprofloxacin. However, the dominated pattern for ampicillin and tetracycline changed from intermediate to resistant, that of nalidixic acid shifted from resistant to intermediate, and for erythromycin it altered from sensitive to mostly intermediate (18). In 2005 in a cholera outbreak in Hamedan province, Iran, resistance

Table 1. Patterns of Antimicrobial Susceptibility in *Vibrio cholerae* Strains Obtained From Cholera Patients [a]

Susceptibility	Frequency	Disc Content, μg	Inhibitory Zone, mm
Erythromycin		15	21.2 ± 4.8
Intermediate	33 (68.8)		
Resistant	11 (22.9)		
Sensitive	4 (8.3)		
Ampicillin		10	12.9 ± 3.2
Intermediate	16 (33.3)		
Resistant	27 (56.3)		
Sensitive	5 (10.4)		
Sulfamethoxazole-Trimethoprim		5	10.5 ± 3.6
Intermediate	1 (2.1)		
Resistant	43 (89.6)		
Sensitive	4 (8.3)		
Tetracycline		30	22.2 ± 10.3
Resistant	29 (60.4)		
Sensitive	19 (39.6)		
Nalidixic acid		30	12.4 ± 6.4
Intermediate	27 (56.3)		
Resistant	21 (43.8)		
Ciprofloxacin		5	24.6 ± 5.3
Sensitive	48 (100.0)		
Ceftriaxone		30	29.5 ± 1.1
Sensitive	48 (100.0)		

[a] Data are presented as mean ± SD or No. (%).

Table 2. Comparison of Antimicrobial Susceptibility Patterns Between *Vibrio cholerae* Strains Isolated From Afghan and Iranian Patients [a]

Susceptibility	Nationality		P Value [b]
	Afghan	Iranian	
Erythromycin, 15 µg			0.006
Sensitive	1 (2.4)	3 (50.0)	
Intermediate	31 (73.8)	2 (33.3)	
Resistant	10 (23.8)	1 (16.7)	
Sulfamethoxazole-Trimethoprim, 5 µg			< 0.0001
Sensitive	1 (2.4)	3 (50.0)	
Intermediate	0 (0.0)	1 (16.7)	
Resistant	41 (97.6)	2 (33.3)	
Ampicillin, 10 µg			< 0.0001
Sensitive	1 (2.4)	4 (66.7)	
Intermediate	15 (35.7)	1 (16.7)	
Resistant	26 (61.9)	1 (16.7)	
Tetracycline, 30 µg			0.223
Sensitive	18 (42.9)	1 (16.7)	
Resistant	245 (7.1)	5 (83.3)	
Nalidixic acid, 30 µg			0.220
Intermediate	25 (59.5)	2 (33.3)	
Resistant	17 (40.5)	4 (66.7)	
Ciprofloxacin, 5 µg			
Sensitive	42 (100.0)	6 (100.0)	
Ceftriaxone, 30 µg			
Sensitive	42 (100.0)	6 (100.0)	

[a] Data are presented as No. (%).
[b] P value for Fisher's exact test.

to furazolidone, trimethoprim-sulfamethoxazole and erythromycin was 100%, 98% and 62%, respectively (22). Antibiotic susceptibility testing of 60 clinical *V. cholerae* isolates isolated from four different provinces of Iran during 2004-2006, showed that 95% of samples were resistant to trimethoprim-sulfamethoxazole (23). The majority of patients with cholera in the 2013 cholera outbreak in Sistan and Balouchestan province were linked to Pakistan. Since 1988, cholera has become a significant cause of gastroenteritis in both adults and children in Pakistan (24). The socio-cultural and environmental circumstances including poor sanitation, massive religious gatherings and some natural disasters such as severe flooding, facilitates the continued presence as well as the spread of the disease in Pakistan (16,25). When facing repeated cholera epidemics, the emergence of multidrug-resistant strains of *V. cholera*e is a major public health problem in Pakistan. A review of reported antimicrobial sensitivity levels of the *V. cholerae* isolates from 1990 to 1996 in Pakistan showed that most sensitive strains became resistant to commonly used antibiotics such as tetracycline (91%), trimethoprim-sulfamethoxazole (96%) and erythromycin (66%), but remained sensitive to nalidixic acid (16). In 1994, the *V. cholerae* isolates from patients with cholera admitted to the pediatric ward of Aga Khan University Hospital, Karachi, were resistant to tetracycline, ampicillin and erythromycin, but sensitive to ceftriaxone, cefixime, ofloxacin and nalidixic acid (24). However, some studies have revealed that susceptibility patterns fluctuate from year to year. For instance, in comparison with the antimicrobial sensitivity patterns of *V. cholerae* isolates obtained during 1993-1994 which were resistant to sulfamethoxazole-trimethoprim (99%) and chloramphenicol (35%), the samples collected during 2000-2001 were almost 100% sensitive (26). Similar antimicrobial resistance patterns have also been reported from other cholera endemic areas such as African countries and the Indian subcontinent. A significant increase in the trend of antimicrobial resistance of *V. cholerae* 01 strains isolated during two cholera epidemics in 1997 and 1999 in Dar es Salaam, Tanzania, was reported. The greatest increase was 37%,

observed to ampicillin, followed by 35.9% increase in resistance against erythromycin, 35% to tetracycline and 16.7% to nalidixic acid (27). However, no change for susceptibility to ciprofloxacin and trimethoprim/sulfamethoxazole was identified. Similarly, antibiogram results of *V. cholerae* isolates during cholera epidemics in North West Ethiopia from August 2006 to September 2008 identified high levels of resistant to trimethoprim/sulfamethoxazole and ampicillin. The majority of samples tested were susceptible to erythromycin, tetracycline and ciprofloxacin (28). All the isolated strains of *V. cholerae*, obtained from patients during a series of cholera outbreaks occurred in the West African country, Senegal, between October 2004 and March 2006, were susceptible to doxycycline and fluoroquinolones, with majority of isolates being resistant to sulfamethoxazole-trimethoprim (29). The continuous emergence of multidrug-resistant *V. cholerae* strains in the Indian subcontinent has been well documented. Antibiotic susceptibility testing of the *V. cholerae* isolates collected from different cholera outbreaks in India between 2004 and 2010 revealed high rates of resistance towards trimethoprim/sulfamethoxazole, nalidixic acid and susceptibility to tetracycline. *V. cholerae* isolates from the latest outbreak in Eastern India were also resistant to tetracycline (30). Resistance to antibiotics such as nalidixic acid, furazolidone , trimethoprim/sulfamethoxazole and ciprofloxacin was constantly high (100%) among *V. cholerae* O1 Inaba isolates collected from cholera patients admitted to a hospital in East Delhi during 2001-2006 (31). Monitoring of multi-drug resistance patterns of *V. cholerae* isolates in India during 2000-2004 showed that 25% to 75% of isolates that were susceptible to trimethoprim/sulfamethoxazole, ciprofloxacin and ampicillin in 2000, developed resistance in 2004 (32). Resistance to tetracycline in *V. cholerae* strains isolated from patients with cholera in Sevagram, India, over 16 years (from January 1990 to December 2005), also varied between 2% and 17% (8). In contrary with our findings, the study on the trends of antimicrobial resistance patterns of *V. cholerae* in Thailand between 2000 and 2004 showed no increase in resistance during the study period (33). The strains investigated showed no resistance to norfloxacin. Antimicrobial resistance was observed with a higher frequency in Ogawa isolates with 16.3% and 60.5% resistant to tetracycline and trimethoprim/sulfamethoxazole, respectively. The proportion of resistant Inaba serotypes against these two antibiotics was less than 1%. Similarly, during the 2004 cholera epidemic in Douala, Cameroon, the antimicrobial susceptibility patterns of isolated *V. cholerae* strains showed no changes (34). One of the major challenges in antimicrobial treatment of cholera is the rapid fluctuations in the resistance patterns identified in *V. cholera* (35). An abrupt increase in resistance levels of *V. cholerae* to tetracycline with some degrees of resistance to erythromycin was reported during 2004-2005 in Bangladesh. However, this pattern of resistance was followed by some fluctuations in the following years (36). Likewise, chloramphenicol and trimethoprim/sulfamethoxazole-resistant strains of *V. cholerae* O139 were isolated with high frequencies from patients with cholera admitted to the Infectious Diseases Hospital, Calcutta, India, during 1994-1995. This was followed by a sharp decline in the proportion of resistant strains in succeeding years (37). This could be partly explained by the fact that *V. cholera*e microorganisms are not able to steadily carry resistance-causing plasmids (35, 38). Resistance patterns for *V. cholerae* strains can vary greatly depending on geographical location, patterns of antibiotic consumption among the studied population, and the time of study. In addition to the factors related to the microorganism itself, lacking or inadequate regulation of antimicrobials distribution and consumption, inappropriate implementation of existing laws, and international travels and population movements are among social and behavioral factors related to emergence of multidrug-resistant microorganisms in developing countries (39). In summary, according to the results from different epidemiological studies across the globe, *V. cholerae* strains have become resistant to several antibiotics and multidrug resistance is increasing. The spread of *V. cholerae* strains related to human behavior can be more easily targeted than complex environmental factors, especially in developing countries. Therefore, enhancing the infectious diseases surveillance systems with a special focus on early detection of possible cholera outbreaks is crucial for better understanding of the dynamics of the disease and improving cholera preparedness and responses (13). Antimicrobial susceptibility tests for *V. cholerae* strains should be an integrated part of the diseases surveillance system. Monitoring the changing patterns of susceptibility to antimicrobials should be specially considered when responding to cholera outbreaks. Surveillance of antimicrobial resistance could be used as a guide to select appropriate antimicrobial treatments in response to different settings and circumstances. These interventions need to be coupled with promotion of prudent use of antibiotics via precise regulations and effective disease control programs.

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Authors' Contributions

Seyed Mehdi Tabatabaei planned the study design and coordinated the conduct of the study. He also carried out statistical analysis and interpretation of the data and drafted this paper. Alireza Salimi Khorashad supervised

all the laboratory tests, collected the study data and participated in drafting the manuscript. All the authors approved the final draft of the manuscript.

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