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Review Article

Antimicrobial Resistance in *Vibrio cholerae* from Aquatic Environment

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Abstract

Vibrio cholerae is an enteric pathogen causing an acute diarrheal disease known as cholera. The disease is acquired through the consumption of food or water contaminated by this microorganism. Antibiotic therapy is recommended in specific situations to significantly reduce the volume of watery faeces and duration of diarrhoea, reducing the transmission of infection. The wide use and abuse of antibiotics in human and veterinary medicine, agriculture, and aquaculture systems have caused the emergence of antimicrobial resistance (AMR) in *V. cholerae.* Several epidemics worldwide were caused by multidrug-resistant (MDR) *V. cholerae*. Various resistance patterns are reported among clinical strains isolated from different parts of the country and across the globe. The drug-resistant clinical strains are dispersed into the aquatic environment through faeces/ excreta of humans, discharge from health care facilities or contaminated groundwater. In the aquatic environment, *V. cholerae* that are susceptible to antibiotics acquire resistance either by frequent exposure to antibiotics over a period of time or through the transfer of resistant genes from other resistant bacteria. In the aquatic system, genetic exchange between bacteria is readily facilitated resulting in the higher frequency of AMR *V. cholerae* and more commonly exhibited multiple antibiotic resistance.

Keywords: *Vibrio cholerae,* Aquatic environment, Antibiotic resistance, Multidrug resistance

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Introduction

Vibrio cholerae is the causative agent of cholera and the disease is acquired through the ingestion of food or water contaminated with the organism. *V. cholerae* is an ubiquitous bacteria in the aquatic environment. It enters the food chain through water or food contamination. Although there are more than 200 serogroups of *V. cholerae* identified, only 2 serogroups, O1 and O139, cause the vast majority of the disease (Kaper et al., 1995; Sack et al., 2004). Serogroup O1 has two biotypes namely El Tor and classical. Each biotype has been further classified into major serotypes namely Inaba and Ogawa, as well as one minor serotype, Hikojima (WHO, 2017). During the epidemics, *V. cholerae* O1 serogroup has dominated in the majority of the countries. However, a new serogroup *V. cholerae* O139 Bengal emerged in 1992 and caused severe epidemics in India and Bangladesh and subsequently spread to several Asian countries (Nair et al., 2013). The classical biotype was implicated in the first six recorded pandemics which are assumed to have started in the Ganges River delta. The El Tor biotype is responsible for the ongoing seventh worldwide pandemic of cholera which started in 1961 in Sulavesi, Indonesia (Mutreja et al., 2011). In recent years, infections due to *V. cholerae* non-O1/non-O139 have been increasingly reported from many countries (Baker-Austin et al., 2016). In 2017, a total of 1,227,391 cases including 5654 deaths were reported globally (WHO, 2018). Cholera is characterized by profuse watery diarrhoea leading to dehydration rapidly and often death. In addition to rehydration and repletion of electrolytes, therapy with effective antimicrobial agents significantly reduces the volume of watery faeces and duration of diarrhoea, reducing transmission of infection to family members, as well as nosocomial infections (WHO, 2017).

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Antibiotics used for the treatment of cholera

Tetracycline (eg; doxycycline), chloramphenicol, trimethoprim/ sulfamethoxazole, fluoroquinolones (eg; ciprofloxacin), macrolides (eg., erythromycin) are the antibiotics commonly employed for the treatment of cholera (WHO, 2004). Ciprofloxacin, norfloxacin, doxycycline and extended spectrum cephalosporins are used for the treatment of *V. cholerae* non-O1/non-O139 infections (Daniels & Shafaie, 2000). However, treatment failures occurred with the recurrent emergence of antimicrobial resistance (AMR) in *V. cholerae* (Clemens et al., 2017).

Antimicrobial resistance in *Vibrio cholerae* from the aquatic environment

V. cholerae is a natural inhabitant of aquatic environments including freshwater, marine, and brackish water bodies. The organism can also be recovered from freshwater reaches of estuaries (Desmarchelier, 1997), where it can also be introduced by faecal contamination. During the outbreak, the pathogen released from the point and diffuse sources get transported from upstream sources to estuarine and coastal waters and contaminate the aquatic environment. *V. cholerae* is capable of growth and long term survival in aquatic ecosystems due to its varied adaptive responses to stressors. The first and primary route of transmission of *V. cholerae* is through the aquatic environment. It is well known that the wide use and abuse of antibiotics in human and veterinary medicine, agriculture, and aquaculture systems have caused the emergence of AMR in pathogenic bacteria (Cabello et al., 2013). The asymptomatic carriers and the patients with symptoms of cholera in the early epidemic period will shed pathogenic *V. cholerae* and strongly adapted, highly virulent epidemic clones in their stools (Faruque et al., 2004), enriching the water sources and get dispersed into the aquatic environment. In the aquatic system, the resistant *V. cholerae* strains from clinical settings and patients get widely disseminated and genetic exchange between bacteria is readily facilitated resulting in the higher frequency of AMR *V. cholerae* (Krumperman et al., 1983; Noorils et al., 2011).

V. cholerae are intrinsically resistant to polymixin B, erythromycin, azithromycin and rifamycin due to reduced cell membrane permeability (Das et al., 2019). The clinical strains of *V. cholerae* were sensitive to several antimicrobials for a long period with a very low resistance rate (O'Grady et al., 1976). However, the extensive, indiscriminate use of antimicrobials for prophylactic and therapeutic purposes has changed the scenario. Antimicrobials are essential clinical tools used to kill or inhibit *V. cholerae*, yet AMR *V. cholerae* continues to emerge, diversify, and spread rapidly and is becoming increasingly common (Kitaoka et al., 2011). Drug resistance usually varies from one place to another. The resistance patterns observed among clinical strains isolated varied across the country and globe. Tetracycline resistance was common in epidemic strains of classical and the El Tor biotypes of *V. cholerae* O1 from Latin America, Tanzania, Bangladesh and Zaire (Garg et al., 2000; Roychowdhury et al., 2008; Kitaoka, et al., 2011); *V. cholerae* O139 from Pakistan (Nizami & Farooqui, 1998) and intermittent appearance of tetracycline resistance in several Asian countries (Glass et al., 1980; Ranjit & Nurahan, 2000; Kondo et al., 2001; Roychowdhury et al., 2008, Bhattacharya et al., 2011). Nowadays, tetracycline is limited in usage as a drug of choice for cholera treatment due to the emergence of tetracycline-resistant *V. cholerae*. Resistance to co-trimoxazole in *V. cholerae* O1 clinical strains were detected in India (Jesudason & Saaya, 1997) and Nepal (Karki et al., 2010; Gupta et al., 2016); co-trimoxazole and nalidixic acid in altered *V. cholerae* O1 clinical isolates from Delhi during 2008-2012 (Sharma, 2015).

An increase in resistance to ampicillin, nalidixic acid, norfloxacin, furazolidone and ciprofloxacin in *V. cholerae* O1 El Tor (Garg et al., 2000; Dutta et al., 2006; Roychowdhury et al., 2008; Uppal et al., 2017) and O139 in India (Ghosh et al., 2016). Resistance to fluoroquinolones was increasingly reported from clinical samples of *V. cholerae* in Kolkata (Mukhopadhyay et al., 1998) and in South India (Krishna et al., 2002). The *V. cholerae* O139 isolates were resistant to cotrimoxazole and neomycin (Mukhopadhyay et al., 1998; Mitra et al., 1998; Faruque et al., 2004) and the antibiotic resistance profile is found to be changing in India.

In aquatic environments, *V. cholerae* can survive and acquire resistance to antibiotics due to the release of antibiotic residues from clinical/ veterinary/ aquaculture/ industry facilities by frequent and extensive exposure to antibiotics. Moreover, the remarkable genetic plasticity allows *V. cholerae* to respond to a wide variety of environmental stresses, including antimicrobials (Escudero & Mazel, 2017).

Non-O1/non-O139 strains resistant to ampicillin were isolated from environmental samples in different countries (Choury et al., 1999; Dalsgaard et al., 2000; Melano et al., 2002; Petroni et al., 2004, Lepuschitz et al., 2019).

An increase in resistance to streptomycin was observed in *V. cholerae* isolates from cultured shrimp (He et al., 2015) and four commonly consumed freshwater fish (Xu et al., 2019) in China. Resistance to ampicillin was recorded in *V. cholerae* isolates from freshwater fish in China (Xu et al., 2019) and in clinical and water samples in Nepal (Thapa Shrestha et al., 2015). In France, resistance to streptomycin and ampicillin was reported among 99 strains of *V. cholerae* strains (nonO1/nonO139) from wastewater and shellfish in the La Rance estuary (Baron et al., 2017). Resistance to furazolidone, bacitracin, and vancomycin (100%) was found in *V. cholerae* isolates of freshwater fish from the hypermarkets in Malaysia (Noorlis et al., 2011). They also observed resistance to tetracycline (88%), cephalothin (77%) and erythromycin (63%) in those isolates and lower resistance recorded to streptomycin (25%), chloramphenicol (25%), ceftazidime, ciprofloxacin and kanamycin (13% each).

Variations in resistance to ampicillin, co-trimoxazole, nalidixic acid, polymyxin-B, streptomycin, ciprofloxacin and tetracycline were identified in *V. cholerae* strains (both O1 and non O1/non-O139) originated from the aquatic environment in Assam (India) including river water, canal water, pond water and hand-pump water (Bhuyan et al., 2016). High resistance to ampicillin (50.4%) was reported in *V. cholerae* nonO1/nonO139 isolates from water and fish samples collected from inland saline aquaculture areas in Punjab, India (Singh et al., 2018).

In China, high resistance to erythromycin, streptomycin and polymyxin B was reported among toxigenic (clinical) and non-toxigenic (environmental-water and seafood) *V. cholerae* O139 strains during 1993-2009 (Yu et al., 2012). Laviad-Shitrit et al. (2018) reported that the majority of nonO1/O139 *V. cholerae* isolates from fish intestines in Israel showed high minimum inhibitory concentration (MIC_{90} of 16 μg ml^{-1}) to doxycycline. Widespread resistance of *V. cholerae* non-O1/non-O139 strains was reported against the "old antibiotics" such as sulfonamides, streptomycin and ampicillin which had been widely used before the 1970s in the Baltic Sea and the North Sea (Bier et al., 2015) and the Chesapeake Bay (Ceccarelli et al., 2015). Fish contaminated with AMR bacteria serve as an international vehicle of antibiotic-resistant bacteria (Duran & Marshall, 2005). In the aquatic environment, *V. cholerae* that are susceptible to antibiotics acquire resistance either by frequent exposure to antibiotics over a period of time or through the transfer of resistant genes from other resistant bacteria. Zulkifli et al. (2009) have stated that food contaminated with antibiotic-resistant bacteria is a threat to public health as the antibiotic resistance determinants may be transferred to bacterial pathogens of clinical significance. The definitions proposed by European Centre for Disease Control (ECDC) and Centre for Disease Control & Prevention (CDC), Atlanta for resistance in bacteria are multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR). These definitions are meant for public health use and epidemiological purposes only (Magiorakos et al., 2012). MDR acquired resistance to at least one agent in three or more antimicrobial categories while XDR have shown resistance to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and PDR is defined as resistance to all agents in all antimicrobial categories.

Rapid dissemination of resistance in *V. cholerae* towards three or more than three different classes of antibiotics is now a major threat to public health as it leads to treatment failures during epidemics and an increase in morbidity and mortality rates (Dalsgaard et al., 2000; Ghosh & Ramamurthy, 2011). Multidrug resistant *V. cholerae* has been implicated in many cholera cases reported from all around the globe (Mandal et al., 2011) and in sporadic cholera cases frequently reported from India (Nair et al., 2010, Gupta et al., 2016). MDR *V. cholerae* O1 strains were first isolated in 1964-1965 in Philippines (Kobari et al., 1970) and circulation of these strains resistant to tetracycline, streptomycin and chloramphenicol was first reported in 1970 (Mhalu et al., 1979). Since the mid-1980s, there was a considerable increase in MDR toxigenic *V. cholerae* (Kitaoka et al., 2011) in cholera cases reported from Africa, Asia, America and China (Maimone et al., 1986; Jesudason & John, 1990; Wang et al., 2012).

V. cholerae O1 El Tor Ogawa resistant to sulfamethoxazole, trimethoprim and streptomycin emerged in a cholera outbreak at Alappuzha district

of Kerala (India) (Radhakutty et al., 1997). MDR *V. cholerae* O1 El Tor Ogawa strains (resistant to nalidixic acid, ciprofloxacin, co-trimoxazole, chloramphenicol, tetracycline, cephalexin and ampicillin) were resurfaced and implicated in cholera outbreaks documented from various districts namely Kottayam, Alappuzha and Trivandrum (Sabeena et al., 2001; Kumar et al., 2009) and later the same resistance pattern was reported in *V. cholerae* isolates from cholera outbreaks in Madhya Pradesh, India (Kingston et al., 2009). Bhanumathi et al., (2002) reported *V. cholerae* O139 strains resistant to ampicillin, cefotaxime, furazolidone, nalidixic acid and streptomycin from a cholera outbreak in Alappuzha district, Kerala (India) and resistance was also reported in *V. cholerae* O139 strains to trimethoprim, sulfamethoxazole and streptomycin, similar to the El Tor vibrios that emerged in several Asian countries during early 1990s (Ramamurthy & Bhattacharya, 2011). In 2007, MDR *V. cholerae* O1 El Tor biotype Inaba serotype resistant to co-trimoxazole, nalidixic acid, polymyxin B, trimethoprim, ampicillin and streptomycin was isolated from a cholera patient from Piravom, Kottayam, Kerala (Sabu et al., 2007) and in Kolkata, high resistance to co-trimoxazole, furazolidone and nalidixic acid was reported in the newly emerged El Tor biotype Inaba serogroup (Roychowdhury et al., 2008). In *V. cholerae* O1 El Tor strains from cholera outbreaks in Chennai, Madhya Pradesh (Inaba strains) and Delhi (Inaba and Ogawa) (Kingston et al., 2009), resistance to nalidixic acid, polymyxin-B, furazolidone, cloxacilin, trimethoprim-sulfamethaxazole was reported. Among the 443 *V. cholerae* clinical strains isolated during 2008 to 2015 from Kolkata and Delhi in India, MDR was found in 17.2% of the isolates (resistant against ≥10 antibiotics) and XDR in 7.5% isolates (resistant against ≥14 antibiotics) and among these, one XDR and one MDR *V. cholerae* isolates belong to the O1 and O139 serogroups, respectively (Varma et al., 2019). They found high resistance to sulfamethaxozole, nalidixic acid, trimethoprim and streptomycin in the isolates and variation in the resistance diversity of *V. cholerae* O1 and nonO1-nonO139 strains. Significantly higher resistance to streptomycin, trimethoprim, nalidixic acid, tetracycline, and chloramphenicol was reported in O1 isolates while resistance to polymyxin B, rifampicin, and erythromycin was observed to be higher in nonO1-nonO139 isolates. Yu et al. (2012) have reported a higher rate of MDR in toxigenic strains of *V. cholerae* O139 than non-toxigenic strains of *V. cholerae* O139 from China. There are reports of diversity and variation in the prevalence of causative serovars as well as changing antibiogram of *V. cholerae* isolates in many parts of the world (Nair et al., 2013; Uppal et al., 2017).

In the aquatic environment, high frequency of MDR *V. cholerae* is reported (Krumperman et al., 1983). Several antibiotics are commonly used in fish/shrimp culture farms either as growth promoters or as therapeutics/prophylactics. In Malaysia, multidrug resistance was reported in *V. cholerae* isolates from fish (Noorlis et al., 2011) and resistance was found to 3-8 antibiotics tested with MAR indices ranging from 0.2 to 0.53. MAR index values of more than 0.2 were considered to have originated from the higher risk sources of contamination such as humans, commercial poultry farms, swine and dairy cattle farms where antibiotics are often used. In China, 30% of the 400 *V. cholerae* isolates recovered from four fish species with 42 resistant profiles (Xu et al., 2019). Thapa Shrestha et al. (2015) demonstrated multidrug resistance (resistance to ampicillin, nalidixic acid, cotrimoxazole and erythromycin) in environmental *V. cholerae* isolates in Nepal. In Egypt, multidrug resistant (5-13 drugs) *V. cholerae* were isolated from fish and shrimp (Ahmed et al., 2018). In India, Singh et al. (2018) reported MDR in *V. cholerae* non O1/non O139 isolates from water and fish samples from inland saline aquaculture areas in Punjab. In the same study, they recorded a very high MAR index value of 0.85 and suggested that these MDR strains could serve as a reservoir of antibiotic-resistant genes in aquatic systems. The MARI is often used to determine the antibiotic resistance-associated health risk (Fri et al., 2018).

Non-O1 *V. cholerae* strains resistant to oxytetracycline, streptomycin, sulphadiazine and tetracycline were isolated from environmental and seafood samples from southeast India (Sathiyamurthy et al., 1997). Kumar & Lalitha (2013) demonstrated resistance in non-O1/non-O139 *V. cholerae* strains isolated from seafood to cefpodoxime, ticarcillin, augmentin and colistin. Sulca et al. (2018) reported resistance in *V. cholerae* isolates from Lima (Peru) seawater to 12 antimicrobial drugs such as ampicillin, penicillin, amoxicillin, nitrofurantoin, kanamycin, amikacin, azthreonam, ciprofloxacin, gentamycin, co-trimoxazole, ceftazidime and nalidixic acid. In Egypt, Ahamed et al. (2018) reported resistance to nalidixic acid, erythromycin, sulfamethoxazole and

chloramphenicol, in *V. cholerae* isolates from fish and shrimp retail markets.

Mechanisms of drug resistance in *Vibrio cholerae*

It is well established that the environment itself plays an important role in the acquisition of antibiotic resistance by *V. cholerae* and the four stages envisaged in this process are the emergence of novel resistance genes, mobilization, transfer to pathogens and dissemination (Bengtsson-Palme et al., 2018). Among these, the emergence and mobilization events likely occur all the time while environmental factors, such as selective pressure, fitness cost, and dispersal determine whether these events actually result in establishing novel genes in populations.

It is also reported that antibiotic exposure results in increased rates of mutations and recombination as well as an increase in integrase activity, thus compounding the multiple effects that excessive usage of antibiotics can have on the emergence and enrichment of antibiotic resistance in bacterial populations. ICEs integrate and replicate with the host chromosome and can excise themselves and transfer between bacteria by conjugation. Many of the antibiotic resistance genes in *V. cholerae* are physically linked with mobile genetic elements (MGEs) and disseminate to closely or distantly related bacterial species by lateral and vertical gene transfer. It has been reported that a single isolate of *V. cholerae* may harbour as many as 40 different AMR encoding genes that can confer resistance against 22 antibiotics representing nine different classes of antimicrobial drugs (Verma et al., 2019). They found that irrespective of their geographical origins, both pathogenic and non-pathogenic *V. cholerae* can acquire AMR to promote their subsistence. The organism is capable of acquiring DNA from the environment by transformation, conjugation and transduction. It is known that the spread of antibiotic-resistant *V. cholerae* is facilitated by horizontal gene transfer via self-transmissible mobile genetic elements, including SXT elements (Table 1). Previous studies demonstrated that antimicrobial resistance genes in *V. cholerae* contribute antibiotic resistance by reducing membrane permeability or active efflux of the antibiotics (Table 1), modifying the drug targets by post-transcriptional, translational modifications and hydrolysis or chemical modification of antibiotics (De Pascale et al., 2010; Morar et al., 2012; Olaitan et al., 2014; Verma et al., 2019).

Methods of determining antibiotic resistance *V. cholerae*

The antibiotic resistance profile of *V. cholerae* to available antibiotics can be determined by using phenotypic and genotypic methods (Table 2). The culture-based/ phenotypic methods are routinely employed in the laboratories. In this method, the isolated organism is evaluated for their growth in response to specific antibiotic concentrations by susceptibility tests, and the resistance of the bacterium can be detected directly from the susceptibility testing. Kirby–Bauer disc diffusion technique that implements the guidelines adopted from the

Table 1. Mechanisms of drug resistance in *Vibrio cholerae*

	Mechanisms	Description	Reference
1	Bacterial efflux pumps	*VcaM* (ATP driven pump) confers resistance to tetracycline, norfloxacin, ciprofloxacin and doxorubicin	Kitaoka et al., 2011; Kumar & Varela, 2012; Varela et al., 2013; Das et al., 2020
		MATE-family efflux systems confer resistance to hydrophilic fluoroquinolones, aminoglycosides and norfloxacin.	
		MFS transporters confer resistance to chloramphenicol and nalidixic acid.	
		MFS efflux protein EmrD-3 confers resistance to linezolid, rifampicin, erythromycin and chloramphenicol	
		RND efflux pumps confers resistance to polymyxin B, erythromycin and penicillin.	
2	Spontaneous mutations	Chromosomal mutations confers resistance to tetracycline, erythromycin, chloramphenicol and quinolones	Kitaoka et al., 2011, Das et al., 2020

Table 2. Methods of determining antibiotic resistance in *V. cholerae*

	Types of method	Examples	Reference
1.	Phenotypic methods	Disk diffusion or Kirby-Bauer method: Placing a test strain of bacteria, on an agar plate, and observing bacterial growth near antibiotic impregnated discs.	Omulo et al., 2015; Khan et al., 2019
		Epsilometer testing (E-test): It is a type of diffusion test which uses an antibiotic impregnated plastic test strip placed on agar.	
		Dilution: It allows the growth and identification of bacterial populations in suspension. The two basic types of dilution are microdilution and macrodilution, wherein broth and agar are the most commonly used mediums.	
		VITEK-2 (semi-automated) test: detect growing bacteria on the basis of turbidity.	
		Sensititre (automated) test: Based on fluorescence emission of the growing bacteria.	
		MALDI-TOF mass spectrometry: It is a form of time-of-flight mass spectrometry in which bacterium is subject to matrix-assisted laser desorption.	
		Automated systems: it can be used to replicate the manual processes	
2.	Genotypic methods	Polymerase chain reaction (PCR) is rapid and efficient molecular tool for targeting antibiotic resistant genes such as *floR* (chloramphenicol), *sul*II (sulfamethoxazole); *strA* and *strB* (streptomycin); *dfr18* (trimethoprim), *tet* (tetracycline) etc.	Fluit et al., 2001; Khan et al., 2019
		DNA Microarray and DNA chips: Multiple genes can detect simultaneously by binding the complementary DNA to a target gene. DNA arrays employ cDNA fragment probes on nylon membrane, where each DNA chip has a glass or silicon platform for probe binding.	
3.	Emerging methods	Microfluidics-based diagnostics: It use a small amount of fluid and a variety of testing methods, such as optical, electrochemical, and magnetic.	Khan et al., 2019
		Fluorescence proteins and dyes are commonly used for tagging resistant biomarkers.	
		Bioluminescence or ATP bioluminescence assay (ATP-BLA): enzyme based method mediated by luciferase enzyme.	

Clinical Laboratory Standards Institute (CLSI) is employed (Bauer, 1966; CLSI, 2006). This disc diffusion test is regarded as a qualitative test to classify an organism as being susceptible or resistant. For quantitative estimate of susceptibility, the minimum inhibitory concentration (MIC) of an antibiotic against a bacterial isolate is determined by micro broth dilution in microplates and by agar dilution. PCR assays are also developed for targeting antibiotic resistance genes (Table 2).

Continuous monitoring of AMR *V. cholerae* is crucial for early detection of increasing resistance patterns since the organism is vulnerable to develop unpredictable resistance patterns, based on their genetic plasticity. In addition, the resistance profile changes with the prevailing environmental conditions, time, geographical location and country. These data serve as guidelines in the selection of antibiotics for treatment. It also helps to reduce the use of broad-spectrum antibiotics and to slow down resistance development.

Conclusion

Cholera continues to be one of the leading causes of morbidity and mortality worldwide. In the last few decades, AMR *V. cholerae* has developed rapidly and spread throughout the globe. Due to the increase of drug resistance in *V. cholerae* strains, the treatment of infection became more and more challenging. Antimicrobial resistance is a global health problem, with resistance to common antibiotics found in all regions of the world. The clinical isolates showed similar resistance patterns as the environmental isolates. Multidrug resistant *V. cholerae* O1/O139 strains were associated with cholera outbreaks in the country. The difference in drug resistance pattern was reported in *V. cholerae* strains from various geographical regions. More information on the antibiotic resistance patterns of *V. cholerae* O1/O139/ non-O1, nonO139 prevalent in the aquatic environment is needed to give more insight into the environmental reservoirs of toxigenic resistant strains, resistance mechanisms and their distribution which would help in better management and control of antimicrobial resistant *V. cholerae* strains.

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