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Conventional and molecular detection of *Vibrio cholerae* isolated from environmental water with the prevalence of antibiotic resistance mechanisms.

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ABSTRACT

Environmental water is an important source for *Vibrio cholerae*, which is autochthonous to the aquatic environment, monitoring this bacterium in water is important for control of cholera. *Vibrio cholerae* represents an enormous public health problem around the world, especially in developing countries. One hundred samples were collected and selected. All presumptive isolates were confirmed by using a series of biochemical tests including Oxidase test, Simmon Citrate test, DNase test, Indole test, Klingler Iron Agar (KIA) test, MacConkey agar test and motility. Confirmed *Vibrio cholera* strains were then screening for slide agglutination test by using commercially antisera polyvalent and monovalent O1 and O139 for determining strain serotype. The resistance to antibiotics by Vibrio cholerae was determining by using thirteen standardized disc diffusion including Amikacin, Ceftriaxone, Ceftazidime, Gentamycin, Tetracycline, Streptomycin, Tobramycin, Cephotaxime, Nalidixic Acid, Norfloxacin, Cephalothin, Rifampicin, Cefixime. From one hundred water samples were detected, fifty-six samples were motile and positive for biochemical tests. Fifteen isolates confirmed as *Vibrio cholera* by Polymerase Chain Reaction (PCR) assay with primers designed for *ctx*A and 241bp band was observed. They showed sensitive to all antibiotics except Amikacin, Streptomycin, Cefixime, Norfloxacin, Cephalothin. the aim of this study was determined the accurate method for detection of *Vibrio cholerae* in environmental water. In the current study, we found that the molecular method using Polymerase Chain Reaction performance using the *ctx*A gene-specific primers for detection of *Vibrio cholerae* was faster and accurate and specific.

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INTRODUCTION

Cholera remains a perpetual health problem in the world by causing significant morbidity and mortality throughout human history. This diarrheal disease results in large volumes of watery stool lead to rapid dehydration and deaths 50-70% of patients without medicine (Faruque *et al.*, 1998). The disease caused by mucosal pathogenic bacteria called *Vibrio cholerae* is a major public health problem in developing countries. Although *V. cholerae* is acquired from environmental sources, a small portion only of environmental bacteria can cause cholera (Lutz, 2013). *V. cholera* is classified into 206 serogroups based on variations in the heat-

stable somatic O antigens, only serogroups O1 and O139 are associated with cholera (Barzamini *et al.*, 2014). In the aquatic environment, several kinds of living organisms were infected with *V. cholera*, including plants, protozoa, bivalves, birds, the exoskeleton of chitin. These organisms regarded as an environmental reservoir for the bacterium (Vezzulli *et al.*, 2010). The virulence factor of *Vibrio cholerae* known as cholera toxin (CT), initiates a pathway in epithelial cells that leads to the severe diarrhea characteristic of cholera known as rice-water stool (RWS) (Morris, 2011). The first stage of infection, bacteria rapidly replicate and increase expression of key virulence factors. The middle stage of infection is characterized by upregulation of the chemotaxis and flagellar genes. This allows *V. cholerae* to exit the luminal fluid of the small intestine. Finally, in the late stage of infection, chemotaxis becomes repressed as *V. cholerae* is shed in the RWS (Nguyen *et al.*, 2017). Cholera enterotoxin was encoded by *ctx*AB gene, which responsible of severe diarrhea, consist of two subunits one A subunit (*ctx*A) that provides the activity of intracellular, and five B subunit (*ctx* B) that binds with the cell receptor (P, 2014). The *ctx* AB gene existing in all strains of *V. cholerae* and retain on the 6.9kb of CTX prophage that is combined within a chromosome (Maysa and Al-Shukri, 2017). The infection by *V. cholerae* dose, not a systemic infection, therefore antibiotics are not essential to determination cholera symptoms and cannot be used as a treatment for the disease; however, it is important to combine oral rehydration therapy with antibiotic treatment. Cholera treatment should be used as an oral solution containing glucose, sodium chloride, potassium chloride and trisodium citrate with antibiotics tetracycline and quinolones to reduce the symptoms of cholera (Kitaoka and Pukatzki, 2011). The problems of *V. cholerae* are become drug-resistant to antibiotics by having several mechanisms including efflux pumps, chromosomal mutations, developing genetic resistance via the exchange of conjugative plasmids, conjugative transposons, integrons or self-transmissible chromosomally integrating SXT elements (Garg *et al.*, 2001).

The study aimed at determining the accuracy method for detection of *V. cholerae* in water via the following steps:

1. Isolation of *V. cholerae* from Kufa River
2. The conventional method for identification of these bacteria by using selective media and biochemical tests
3. Molecular method for identification of these bacteria by using PCR

Determining the prevalence and antibiotic resistance pattern

MATERIALS AND METHODS

Traditional methods for isolating and characterization *V. cholerae* from water samples were presented by (Colwell *et al.*, 1977). who modified protocols originally intended for clinical samples (Colwell *et al.*, 1977).

Study of location: the study was carried out in Kufa River/ Al-Najaf Al-Ashraf/Iraq, different locations were randomly selected from four regions which are Albohadari, Hawatem/Albodheres, Hawatem/ Albohaijasim, Hawatem/ Albotrad.

From September 2017 to August 2018, one hundred samples were collected and selected comprising of 30 water master for drinking, 20 Sewage drainage pipes, 20 from the River containing aquatic plant, 15 River centers, 15 River ranges.

Samples collection: One hundred milliliter of water were taken from each of the 100-water samples in a sterile container containing Carry-Blair transport media, sealed, labelled and transported immediately to the Biology Department laboratory of the Science Faculty/ Kufa University for further analysis.

Sample processing: culturing with traditional methods for detection of *V. cholera* were continuing to be improved with the result. The process involves each water sample was filtered by 0.45 µm Millipore filter then the membranes were transferred to slants with a cup containing 2ml of alkaline peptone water and incubated for 5-8 hours at 37°C. After enrichment, aliquots are subcultured onto a selective medium- TCBS (Thiosulphate Citrate Bile Salt Sucrose) agar and incubated for 24h at 37°C. The colonies were taken characterized by yellow, smooth, slightly flattened with opaque centers and translucent peripheries considered as *Vibrio* species and were sub-cultured on enrichment media such as blood agar for biochemical tests.

Biochemical Tests: All presumptive isolates were confirmed by using a series of biochemical tests including Oxidase test, Simmon Citrate test, DNase test, Indole test, kligler Iron Agar (KIA) test, MacConkey agar test and motility.

Serological Tests: Confirmed *V. cholera* strains were then screening for slide agglutination test by using commercially antisera polyvalent and monovalent O1 and O139 for determine strain serotype.

Preservation of Bacterial Isolates: for long-time preservation, stab the organism into the trypticase soya agar and incubated at 35°C. Close tubes with screw-cup and dip the cap into molten paraffin wax to seal, store at room temperature and transfer after one year (Vandepitte *et al.*, 2003).

Antibiotic Susceptibility: Muller Hinton agar and broth support the growth of *V. cholera* are suitable for detection of antibiotic susceptibility. Thirteen standardized disc diffusion was used for determining the sensitivity of *V. cholera* isolates according to Clinical and Laboratory Standards Institute (CLSI) guidelines for the following antibiotics: Amikacin (Ak, 30mcg), Ceftriaxone (Ci, 30mcg), Ceftazidime (Ca, 30mcg), Gentamycin (G, 10mcg), Tetracycline (T, 30mcg), Streptomycin (S, 10mcg), Tobramycin (Tb, 10mcg), Cephotaxime (Ce, 30mcg), Nalidixic Acid (Na, 30mcg), Norfloxacin (Nx, 10mcg), Cephalothin (Ch, 30mcg), Rifampicin (R, 5mcg), Cefixime (Cfx, 5mcg).

Extraction of Genomic DNA: the genomic DNA was extracted from a pure culture of *V. cholerae* cells by the phenol/chloroform method as described previously (Kochl *et al.*, 2005).

Polymerase Chain Reaction (PCR) Assay: in this study, we used specific primer F: 5-CAAATGATGATAAGTTATATCGG- 3´and R: 5´-GACCAGACAATATAGTTTGACC- 3´(CUSABIO/China), which designed according to *ctx*A gene sequences. This primer was detection and amplifying 241 base pairs of *the ctx*A gene (Barzamini *et al.*, 2014). The primer was provided in a lyophilized form by dissolved in TE buffer to give a final concentration of 100 pmol/µL, ninety microliters of stock solution were mixed with 100µl TE buffer to an obtained working solution. PCR mixture was set up in a total volume of 30µl included 15µl of PCR premix (Solgent/Korea), 2µl of primer and 5µl of extracted DNA, the rest volume was completed 6µl of sterile deionized distilled water. PCR reaction tubes were centrifuged briefly to mix and bring the contents to the bottom of the tubes and placed into thermocycler PCR instrument where DNA was amplified as indicating in below Table 1.

Table 1: Program used to amplify the ctxA gene.

Stage	Temperature (Time)	
Initial denaturation	94°C for 5min	
Denaturation	94°C for 1min	30cycle
Annealing	51°C for 1min	
Extension	72°C for 30sec	
Final extension	72°C for 7min	

Then 5µl of PCR product was analyzed by 1.7% agarose gel electrophoresis.

RESULTS AND DISCUSSION

We take 100 ml of water sample because a small amount of water sample is not enough for contain enough bacterial cells for detection. Thus, it is important to examined appropriate volumes of water by using several methods to determine the presence of *V. cholerae* in a given sample (Huq *et al.*, 2012). All isolates were tested, and the positive ones were selected. From one hundred water samples were detected, fifty-six samples with colonies characterized by yellow, smooth, flattened with two to four mm in diameter, opaque centers and translucent peripheries on TCBS agar medium which had pH above of 7 and motile and positive for Simmon citrate, Indole, Oxidase, DNase and Alkaline/Acid for KIA and growth on MacConkey agar characteristics as *Vibrio* species (Table 2), but only 15 isolates confirmed as *V. cholera*. These fifteen isolate showed agglutination with polyvalent and monovalent O1 antisera, and two strains represented Ogawa from other strains that showed Inaba (Table 3).

The susceptibility of *V. cholerae* isolates to antibiotics. Each *V. cholerae* strain was subjected to antibiotic susceptibility test to a battery of antibiotics (Table 4). The fifteen isolates exhibited an identical response to each antibiotic examined. They were sensitive to Ceftriaxone (Ci), Ceftazidime (Ca), Tobramycin (Tb), Cephotaxime (Ce), Gentamycin (G), Nalidixic Acid (Na), Rifampicin (R) and Tetracycline (T). On the other hand, ten strains included polyvalent Inaba were resistant to Amikacin (Ak), four strains to Streptomycin (S), one strain numbered 91 was resistant to Cefixime (Cfx), one strain to Norfloxacin (Nx), one strain to Cephalothin (Ch).

The bacterial DNA were isolated from the pure *V. cholerae*, then electrophoresed on 1.7% gel agarose, and one band was observed.

The assay of polymerase chain reaction (PCR) was carried out on the DNA extracted from the *V. cholerae*, and the negative control sample with primers designed for ctxA and 241bp band was observed. The results of PCR between strains of *V. cholerae* isolated from different regions of Al-Najaf Al-Ashraf listed in the previous table shown in (Figure 1), only the genome of *V. cholerae* is positive.

Table 2: Biochemical tests for one hundred samples isolated from water, fifty-six samples were positive to biochemical tests, but others were negative.

Biochemical tests Sample number	pH	Simmon citrate	Indole	Oxida	DNase	KIA	Motility	MacConkey	Result
1	7.5	-	+	+	-	A/A			Negative
2	7.2	-	+	+	+	K/A	Motile		Negative
3	6.9	-	+	+	+	K/A	Motile		Negative
4	6.7	-	+	+	+	K/A	Motile		Negative
5	7.8	+	+	-	+	K/A		G	Negative
6	7.4	+	+	-	-	K/A	Motile	G	Negative
7	7.9	+	+	+	+	K/A	Motile	G	Positive
8	7.7	+	+	-	+	K/A		G	Negative
9	7.5	-	+	+	+	K/A		G	Negative
10	7.5	+	+	+	+	K/A	Motile	G	Positive
11	6.7	-	-	+	+	K/A	Motile		Negative
12	6.7	-	-	+	-	K/A			Negative
13	7.2	+	+	+	+	K/A	Motile	G	Positive
14	7.5	+	+	+	+	K/A	Motile	G	Positive
15	7.5	+	+	+	+	K/A	Motile	G	Positive
16	7.46	-	+	-	-	A/A			Negative
17	8.19	+	+	+	+	K/A	Motile	G	Positive
18	8.13	+	+	+	+	K/A	Motile	G	Positive
19	7.95	+	+	+	+	K/A	Motile	G	Positive
20	8.26	+	-	-	-	A/A			Negative
21	8.3	+	+	+	+	K/A	Motile	G	Positive
22	7.33	+	+	-	-	A/A			Negative
23	7.4	-	+	-	-	A/A			Negative
24	8.2	+	+	+	+	K/A	Motile	G	Positive
25	8.19	+	+	+	+	K/A	Motile	G	Positive
26	7.86	+	+	+	+	K/A	Motile	G	Positive
27	8.33	+	+	+	+	K/A	Motile	G	Positive
28	7.53	+	+	+	+	K/A	Motile	G	Positive
29	8	+	+	+	+	K/A	Motile	G	Positive
30	8.3	+	+	+	+	K/A	Motile	G	Positive
31	7.81	+	+	+	+	K/A	Motile	G	Positive
32	7.75	+	+	+	+	K/A	Motile	G	Positive
33	8.5	+	+	+	+	K/A	Motile	G	Positive
34	7.93	+	+	+	+	K/A	Motile	G	Positive
35	7.99	+	+	+	-	K/A			Negative
36	8.05	+	+	+	-	K/A			Negative
37	8.11	+	+	+	-	K/A			Negative
38	7.9	+	+	+	+	K/A	Motile	G	Positive
39	7.3	-	-	+	-	K/A			Negative
40	7.45	+	+	+	+	K/A	Motile	G	Positive
41	8	+	+	+	+	K/A	Motile	G	Positive
42	8.3	+	+	+	+	K/A	Motile	G	Positive
43	8.44	+	+	+	+	K/A	Motile	G	Positive
44	7.5	-	+	+	-	K/A			Negative
45	8.31	-	-	-	-	A/A			Negative
46	7.68	+	+	+	+	K/A	Motile	G	Positive
47	7.51	+	+	+	+	K/A	Motile	G	Positive

Continued on next page

Table 2 continued

48	7.65	+	+	+	+	K/A	Motile	G	Positive
49	7.68	+	+	+	+	K/A	Motile	G	Positive
50	7.6	+	+	+	+	K/A	Motile	G	Positive
51	7.46	+	+	+	+	K/A	Motile	G	Positive
52	7.67	+	+	+	+	K/A	Motile	G	Positive
53	7.5	+	+	+	+	K/A	Motile	G	Positive
54	7.43	+	+	+	+	K/A	Motile	G	Positive
55	7.66	+	+	+	+	K/A	Motile	G	Positive
56	7.65	+	+	+	+	K/A	Motile	G	Positive
57	8.04	+	-	+	-	K/A			Negative
58	7.87	-	+	+	-	A/A			Negative
59	7.76	-	-	+	-	A/A			Negative
60	8.07	+	+	+	+	K/A	Motile	G	Positive
61	8.07	-	-	+	-	A/A			Negative
62	7.96	+	+	+	+	K/A	Motile	G	Positive
63	7.96	+	+	+	+	K/A	Motile	G	Positive
64	8.04	-	+	+	+	A/A			Negative
65	8	-	-	-	-	A/A			Negative
66	7.9	+	+	+	+	K/A	Motile	G	Positive
67	7.68	+	+	+	+	K/A	Motile	G	Positive
68	7.51	+	-	+	+	K/A			Negative
69	7.65	-	+	+	-	A/A			Negative
70	7.68	-	-	-	-	A/A			Negative
71	7.46	-	+	+	-	A/A			Negative
72	7.6	+	+	+	+	K/A	Motile	G	Positive
73	7.88	+	+	+	+	K/A	Motile	G	Positive
74	7.5	-	+	-	-	A/A			Negative
75	7.75	-	+	+	-	A/A			Negative
76	8.12	-	+	+	-	A/A			Negative
77	7.93	+	+	+	+	K/A	Motile	G	Positive
78	7.9	+	+	+	+	K/A	Motile	G	Positive
79	8.05	+	+	+	+	K/A	Motile	G	Positive
80	8.04	+	+	+	+	K/A	Motile	G	Positive
81	7.86	+	+	+	+	K/A	Motile	G	Positive
82	8.33	+	+	+	+	K/A	Motile	G	Positive
83	7.53	+	+	+	+	K/A	Motile	G	Positive
84	8	+	-	+	+	K/A	Motile		Negative
85	8.3	+	+	+	+	K/A	Motile	G	Positive
86	7.46	+	-	+	+	K/A			Negative
87	8.19	+	+	+	+	K/A	Motile	G	Positive
88	8.13	-	-	-	-	A/A			Negative
89	7.95	-	+	+	-	A/A			Negative
90	8.26	-	+	+	-	A/A			Negative
91	7.87	+	+	+	+	K/A	Motile	G	Positive
92	7.57	+	+	+	+	K/A	Motile	G	Positive
93	7.84	-	+	+	-	A/A			Negative
94	7.93	-	+	+	-	A/A			Negative
95	7.8	-	+	+	-	A/A			Negative
96	7.8	-	+	+	-	A/A			Negative
97	7.4	-	+	+	-	A/A			Negative
98	7.96	+	+	+	+	K/A	Motile	G	Positive

Table 3: Agglutination test for fifteen isolates of *V. cholerae*, isolates 7 and 13 showed Ogawa, but other isolates represented Inaba.

Sample number	Polyvalent	Ogawa	Inaba
7	+	-	+
10	+	+	-
13	+	-	+
18	+	+	-
24	+	+	-
40	+	+	-
48	+	+	-
55	+	+	-
62	+	+	-
63	+	+	-
72	+	+	-
73	+	+	-
82	+	+	-
91	+	+	-
92	+	+	-

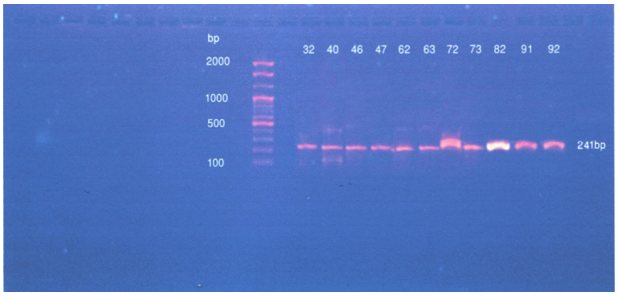

Figure 1: Detection of the *ctx*A(241bp) gene by PCR amplification in *V. cholerae* species: first line from left L-molecular DNA ladder 100bp, lanes (32, 40, 46, 47, 62, 63, 72, 73, 82, 91,92) positive isolates.

A thiosulfate citrate bile salts sucrose (TCBS) agar, highly selective medium, removes most nontarget bacteria in experimental samples but is not enough for isolation of *V. cholerae* from environmental samples because many bacteria existing in natural water sources can growth and produce yellow colonies on TCBS agar whose appearance is like that colony of *V. cholerae*. Additionally, the series of biochemical tests commonly used to identify *V. cholerae* (Choopun, 2002). The conventional methods including culturing, serotyping and biochemical testing which used to identify *V. cholerae* are consumed the time and laborious, while molecular techniques such as PCR are suitable for detection of bacterial culture because of rapid, specificity, and high accuracy (Barzamini *et al.*, 2014). PCR was effectively for detecting of toxigenic *V. cholerae* directly in seawater and samples. This is providing evidence for an environmental reservoir for pathogenic bacteria (Lipp *et al.*, 2003). The detection of *V. cholerae* O1 using PCR method was described by Koch *et al.* (1993) in food samples, they amplification specific sequences *ctx*AB gene within the cholera toxin by seeded oysters, crabmeat, shrimp, and lettuce with *V. cholerae* and washed with alkaline peptone water (6-8h), they resulted only Vibrio stains contain *ctx*AB gene (Koch, 1993). Shirai *et al.* were used specific primers for detection of *ctx* operon of *V. cholerae* by PCR isolated from rice water stool samples of patients with cholerae. They give rise to that the strains of *V. cholerae* were identical with those obtained (Shirai, 1991). Vibrio that confirmed and identified as Vibrio spp. By using PCR from Vibrio strains isolated from seawater was demonstrated by (Kim, 2015). Other researchers used cultural and PCR methods for the presence of *V. cholerae* in a total of 245 samples, including 35 samples from water. The results showed 80 samples (19 water) were positive by the PCR method, while the samples were positive by cultural method, only 59 samples (12 water). The study proved the excellent tool for detection of *V. cholerae* in the water environment is PCR (Maheshwari *et al.*, 2011).

(Alishahi, 2013) were isolate *V. cholerae* from patients by using multiplex PCR assay with three separate primers for *ctx*A, *tox*R, and *omp*U genes, which is important in pathogenicity and toxigenicity. They found 72 samples identified as *V. cholerae* carried *tox*R, and *omp*U genes but only 61 isolates carried *ctx*A gene. They indicate that pathogenicity of *V. cholerae* does not depend on the existence of the cholerae toxin encoded by *ctx* gene (Alishahi, 2013).

In this study, specific primers were designed for *ctx*A gene screening of *V. cholerae* by using PCR, similar to the study by (Barzamini *et al.*, 2014), who using specific primers for *ctx*A gene of cholera toxin-producing *V. cholerae* in water and wastewater by PCR assay.

Contaminated water in developing countries plays an important role for transmission of pathogenic bacteria to the human and causes cholera, that regarded as serious problems in healthy countries. Therefore, the rapid detection of *V. cholerae* in water its important for early diagnostic and prevent the transmission of these bacteria.

Most strains of *V. cholerae* in this study were resistant to at least two antibiotics of a different group, but there are several antibiotics were still more potent against *V. cholerae*.

Resistance to antibiotics usually varies from one place to another, *V. cholerae* becomes to antibi-

Table 4: Inhibition zone (mm) of antimicrobial susceptibility tests of *V. Cholerae* toward different commonly used of antibiotics.

sample	Ca	Ci	Ak	S	Cfx	Tb	Ce	R	G	Na	Nx	Ch	T
7	20	30	R	28	28	20	30	25	22	30	25	20	22
10	30	33	R	R	22	22	33	15	18	18	25	22	25
13	20	35	R	R	25	25	30	22	20	25	25	20	21
18	25	30	15	12	25	25	30	18	25	25	25	18	23
24	20	30	7	R	30	25	30	17	18	30	25	20	20
40	20	30	18	12	30	30	30	18	18	30	25	25	21
48	30	30	R	R	30	30	35	15	25	30	30	25	20
55	30	30	R	15	30	25	30	25	25	20	25	R	26
62	25	30	R	12	25	25	30	15	30	25	25	20	22
63	30	30	30	30	30	30	30	30	30	30	R	18	23
72	30	35	30	30	30	30	30	30	30	30	30	25	23
73	30	33	R	12	30	25	30	20	25	25	25	20	20
82	25	33	R	15	25	25	20	25	25	25	25	25	24
91	25	30	R	12	R	25	25	30	25	30	30	20	25
92	25	30	R	15	25	25	20	25	25	25	25	22	20

otics by several mechanisms including efflux pumps, chromosomal mutations and genetic resistance. The resistance genes can be able to transfer to commensals bacteria or enteric pathogens in the human gut by sharing traits (Dengo-Baloi, 2017).

Antimicrobial treatment is important for a treat of patients with cholera, although rehydration therapy is the support of therapy. The antibiotics doxycycline and tetracycline were the drugs of choice for the treatment of cholera for long of a time, but the resistance by *V. cholerae* against antibiotics was demonstrated by many studies (Chillarge, 2015). (Gupta, 2016) demonstrated *V. cholerae* resistant to ampicillin and cotrimoxazole, but all isolates were sensitive to tetracycline, doxycycline, levofloxacin and azithromycin (Gupta, 2016).

CONCLUSIONS

Water is the main source of *V. cholerae* can transfer to the human, which causes fatal disease. Therefore we should examine the water by isolation these bacteria and detection by several methods to know the accuracy and faster method for identification. Tow methods used in this study for detection of *V. cholerae*, conventional and molecular method, the PCR technique is faster and accuracy.

Antibiotics are used in combination with rehydration therapy for the treatment of cholerae because antibiotics are widely used for the treatment of cholerae. Therefore, the number of pathogenic *V. cholerae* strains becomes resistant to one or more of antibiotics. This study used several antibiotics to be determining the sensitivity of *V. cholerae* to these antibiotics.

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