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Genetic diversity and antibiotic resistance of clinical and environmental *Vibrio cholerae* suggests that many serogroups are reservoirs of resistance

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SUMMARY

Vibrio cholerae is an important human pathogen and the cause of cholera. Since genetic variation and antibiotic resistance of strains have implications for effective treatment of the disease, we examined the genetic diversity and antibiotic resistance profile in 92 clinical strains (serogroup O1) and 56 environmental strains (O1 antigen, 42 strains; non-O1 antigen, 14 strains) isolated in Brazil between 1991 and 1999. Clinical and environmental O1 strains showed greater drug resistance compared to environmental non-O1 strains. Nearly all clinical O1 strains were resistant to one or more antibiotics while half of the environmental O1 and non-O1 strains were resistant to one or more antibiotics. No plasmids or class 1 integrons were detected in the strains by PCR analysis. Multilocus enzyme electrophoresis analysis (MLEE) suggests most of the O1 strains belong to a single (South American) clone that is related but different to seventh-pandemic strains isolated from other parts of the world. Our results show that there is a close genetic relationship between clinical and environmental O1 strains and that many serogroups and the environment can be a reservoir for antibiotic resistance.

INTRODUCTION

Vibrio cholerae is the aetiological agent of the human diarrhoeal disease cholera. Since 1817, seven cholera pandemics have been recorded. The current seventh pandemic caused by an El Tor biotype strain began in 1961 on the island of Sulawesi and soon spread worldwide [1]. In January 1991, cholera appeared in many Latin American countries after 100 years of absence [2], and in Brazil, 167 718 cholera cases were documented and 2009 deaths recorded between 1991 and 1999. The disease began mainly in the northeast and accounted for 92·1% of the countries total cases [3–5]. As in many other regions of the developing world, poor sanitation and contaminated environmental conditions promoted cholera epidemics in several Brazilian states such as Pernambuco and Alagoas [4]. As such, studying the genetic relatedness and molecular nature of these *V. cholerae* strains has implications for many geographical regions of the world.

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Table 1. V. cholerae *strains used in this study*

Region (states)*	Source†	Year of isolation									Total
		1991	1992	1993	1994	1995	1996	1997	1998	1999	
North	CO1	5	6			1					12
(AM, PA, AP)	EO1/non-O1		7								7
North West	CO1			5	9	8	10	3	3	7	45
(CE, PB, PE, SE, BA)	EO1/non-O1	2	9	5			2	4	3	4	29
South East	CO1			16	10	1					27
(ES, RJ, SP)	EO1/non-O1	3	2	3	4	3					15
South	CO1			2						5	7
(PR, SC, RS)	EO1/non-O1		1				3				4
Total		10	25	31	23	13	15	7	6	16	146

* AM, Amazonas; PA, Pará; AP, Amapá; CE, Ceará; PB, Paraíba; PE, Pernambuco; SE, Sergipe; BA, Bahia; ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul.
† CO1, Clinical strains of the serogroup O1; EO1/non-O1, environmental strains of the serogroup O1 and/or non-O1.

In order to better understand the molecular epidemiology and origin of pathogenic strains, various molecular-based techniques have been used to compare the relationships among clinical and environmental isolates including multilocus enzyme electrophoresis (MLEE) [6], pulsed-field gel electrophoresis (PFGE) [7], ribotyping [8], amplified fragment length polymorphisms (AFLPs) [9], randomly amplified polymorphic DNA (RAPD) [10, 11], and DNA sequencing [12]. These techniques have shown that *V. cholerae* is predominantly clonal but that several clonal variants exist within the population.

It has been proposed that epidemic *V. cholerae* strains are derived from non-toxigenic environmental strains by the acquisition of virulence genes [12, 13]. MLEE analysis of clinical isolates from the 'Latin American epidemic' shows that these strains are related to seventh-pandemic isolates from other parts of the world but differed from the seventh-pandemic isolates at a single locus (leucine aminopeptidase) [6, 14, 15]. In a previous study, we described the prevalence of virulence genes in clinical and environmental *V. cholerae* strains isolated in Brazil over a 9-year period (1991–1999) and found that 'environmental' strains can be reservoirs of virulence genes [11]. These findings highlight the need to monitor genes in *V. cholerae* that have roles in virulence or that promote the survival and persistence as this relates to epidemic potential.

It is important from the view of public health to determine the genetic relatedness among clinical and environmental *V. cholerae* isolates and to study the evolution of the strains over time and across geographic areas. Such information would allow public health laboratories to monitor the movement and identify the origins of pathogenic *V. cholerae* strains. Such information might also be useful in designing and implementing prevention and intervention strategies. In the work reported here, clinical and environmental *V. cholerae* isolates were studied for their genetic relatedness, antibiotic resistance profile and the presence of plasmids and class 1 integrons.

MATERIALS AND METHODS

Bacterial strains

The *V. cholerae* strains used in this study were isolated between 1991 and 1999 from several different Brazilian states (Table 1) and belong to the culture collection of the Centro de Referência Nacional para Cólera e outras Enteroinfecções (CRNCE), Departamento de Bacteriologia, IOC, Fiocruz, Rio de Janeiro, Brazil. We studied 92 clinical isolates with serogroup O1 and 56 environmental isolates having either O1 antigen (42 strains) or non-O1 antigen (14 strains). Bacterial strains were incubated for 3–6 h in alkaline peptone water (pH 9·0) and were then streaked onto thiosulphate citrate bile salts sucrose agar (TCBS; Oxoid Ltd, Basingstoke, UK) to verify the purity of *V. cholerae* strains. All strains were confirmed by serogrouping as described previously [11].

Antimicrobial susceptibility

Clinical and environmental strains of *V. cholerae* were examined for resistance to amikacin (30 μg), ampicillin (10 μg), nalidixic acid (30 μg), ceftriaxone

(30 μg), cefamandole (30 μg), ofloxacin (5 μg), streptomycin (10 μg), sulphonamides (300 μg), tetracycline (30 μg), trimethoprim–sulphamethoxazole (25 μg), chloramphenicol (30 μg), cephalothin (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), and furazolidone (100 μg) with commercial antibiotic disks (Oxoid). A 6-h culture of *V. cholerae* in tryptic soy broth (TSB; Difco, Becton, Dickinson & Co., Sparks, MD, USA) was spread plated on Mueller–Hinton agar (Difco) and the disks were applied. Plates were incubated for 24 h at 37 °C. The National Committee for Clinical Laboratory Standards has established interpretative criteria for *V. cholerae* for the following drugs: ampicillin, chloramphenicol, sulphonamides, tetracycline, and trimethoprim–sulphamethoxazole [16]. Tentative zone-size criteria have been proposed for furazolidone and nalidixic acid [17]. Zone-size criteria standardized for the Enterobacteriaceae were used to interpret results of other susceptibility tests [16]. Strains were recorded as susceptible, intermediately resistant or resistant based on inhibition zone sizes. A control strain of *Escherichia coli* (ATCC 25922) was used in these studies.

Isolation of plasmid DNA

Plasmid extraction was carried out by using the QIAprep Spin Miniprep kit, according to the recommendations of the manufacturer (Qiagen Inc., valencia, CA, USA). Electrophoresis and visualization of plasmids were carried out as previously described [18]. *E. coli* 39R861 and V517 were used as reference plasmid control strains [19, 20].

Detection of class 1 integrons by PCR analysis

All strains were tested for the presence of class 1 integrons by PCR analysis using the primers qacEΔ1 (ATCGCAATAGTTGGCGAAGT; accession no. X15370) and sul1 (GCAAGGCGGAAACCCGCGCC; accession no. X12869), specific for 3′-CS [21] and the primers in-F (GGCATCCAAGCAGCAAGC; accession no. U12338) and in-B (AAGCAGACTTGACCTGAT; accession no. U12338), which amplify the region between 5′-CS and 3′-CS [21]. PCR were performed as described previously [21]. The annealing temperatures of 58 °C (qacEΔ1/sul1 primers) and 55 °C (in-F/in-B primers) were used. Integron 5′-CS- and 3′-CS-positive *E. coli* O118:[H16] strains CB6175 (1·6-kb amplified fragment), and CB6365 (1·0-kb amplified fragment) and the strain CB8220 (integron 5′-CS and 3′-CS negative strain) [22] were used as controls. A 100-bp DNA ladder (Gibco–BRL Life Technologies Inc., Gaithersburg, MD, USA) was used as a size marker during electrophoresis of PCR products.

Multilocus enzyme electrophoresis (MLEE)

Methods of cell lysate preparation, protein electrophoresis, and selective enzyme staining have been described elsewhere [6, 23]. Twelve enzymes encoded by chromosomal genes were assayed in all isolates: ADH (alanine dehydrogenase, EC 1.4.1.1); IDH (isocitrate dehydrogenase, EC 1.1.1.42); ME (malic enzyme, EC 1.1.1.40); EST (esterase, EC 3.1.1.1); PGD (6-phosphogluconate dehydrogenase, EC 1.1.1.44); MDH (malate dehydrogenase, EC 1.1.1.37); PGM (phosphoglucomutase, EC 2.7.5.1); GPI (glucose phosphate isomerase, EC 5.3.1.9); G6P (glucose-6-phosphate dehydrogenase, EC 1.1.1.49); P1 (peptidase leucyl-leucyl leucine, EC 3.4.3); P2 (peptidase leucyl-glycyl glycine, EC 3.4.3), and LAP (leucyl-leucyl aminopeptidase, EC 3.4.11.1). Each band that appeared on the gel was considered a distinctive electromorph (mobility variants). The bands were numbered in order of increasing rate of anodal migration and were equated with alleles at corresponding structural gene loci. The bands were collected into matrices indicating the presence or absence (scored as 1 or 0 respectively) and strains having identical allelic profiles for all the loci studied were designated as electrophoretic type. No null reactions were included in the analysis. Simple matrices were obtained by comparing pairs of strains using the Jaccard coefficient and dendrograms were constructed using the unweighted-pair group method with arithmetic mean (UPGMA). For these analyses, the NTSYS software package (version 2.02, Exeter Software, Setauket, NY, USA) was used.

RESULTS AND DISCUSSION

Analysis of antibiotic resistance

Antibiotic-resistant *V. cholerae* strains are increasingly being found worldwide [24–28]. Antimicrobial susceptibility tests showed that all clinical and environmental *V. cholerae* O1 and non-O1 strains were susceptible to nalidixic acid, trimethoprim–sulphamethoxazole, chloramphenicol and gentamicin (Table 2). Of the 92 clinical *V. cholerae* O1 strains tested, 98% showed resistance to one or more drugs

Table 2. *Percentage of antimicrobial resistance of clinical and environmental* V. cholerae *strains*

	% of clinical O1 strains ($n=92$)			% of environmental O1 strains ($n=42$)			% of environmental non-O1 strains ($n=14$)		
Antimicrobial drugs*	S	I	R	S	I	R	S	I	R
Amikacin (30 μg)†	69	30	1	62	31	7	100	0	0
Ampicillin (10 μg)	7	10	83	52	0	48	36	0	64
Nalidixic acid (30 μg)	100	0	0	100	0	0	100	0	0
Ceftriaxone (30 μg)	76	17	7	72	14	14	100	0	0
Cefamandole (30 μg)	85	10	5	76	12	12	100	0	0
Ofloxacin (5 μg)	98	2	0	100	0	0	100	0	0
Streptomycin (10 μg)	5	18	77	29	0	71	0	29	71
Sulphonamides (300 μg)	64	4	32	26	3	71	71	0	29
Tetracycline (30 μg)	21	76	3	83	0	17	86	7	7
Trimethoprim–sulphamethoxazole (25 μg)	100	0	0	100	0	0	100	0	0
Chloramphenicol (30 μg)	100	0	0	100	0	0	100	0	0
Cephalothin (30 μg)	86	12	2	67	10	23	100	0	0
Ciprofloxacin (5 μg)	98	2	0	100	0	0	100	0	0
Gentamicin (10 μg)	100	0	0	100	0	0	100	0	0
Furazolidone (100 μg)	60	—‡	40	76	—	24	64	—	36

* S, susceptible; I, intermediate; R, resistant.
† Concentration in parentheses.
‡ Intermediate resistance is not considered for furazolidone [17].

and the majority of strains were resistant to ampicillin (83%) and streptomycin (77%). Half of the environmental *V. cholerae* O1 strains tested were resistant to 1–3 drugs and most isolates were resistant to streptomycin (71%) and sulphonamides (71%). Of the 14 environmental non-O1 strains, the majority of strains (65%) showed resistance to one or two drugs, particularly to streptomycin (71%) and ampicillin (64%). Only clinical and environmental *V. cholerae* O1 strains showed varying levels of resistance to amikacin, ceftriaxone, cefamandole and cephalothin, whereas none of the environmental *V. cholerae* non-O1 strains were resistant to amikacin, ceftriaxone, cefamandole, ofloxacin, cephalothin and ciprofloxacin. Varying degrees of resistance to ampicillin, streptomycin, sulphonamides, tetracycline and furazolidone were observed among clinical and environmental *V. cholerae* O1 and non-O1 strains. Interestingly, one strain of clinical origin (no. 281) and one environmental O1 strain (no. 23) showed resistance to seven antibiotics. No correlation was found among antibiotic susceptibility patterns and the year and place of isolation of the strains.

In the present work, antimicrobial susceptibility tests performed on clinical and environmental O1 and non-O1 strains showed that both clinical and environmental O1 strains exhibited higher frequencies of resistance to certain antibiotics compared to environmental non-O1 strains. Differences in the frequency of resistant strains observed in our study may reflect the existence of different R-types of *V. cholerae* in different geographical locations in Brazil. As indicated by Chakraborty et al. [24], higher frequencies of resistance detected among clinical isolates may reflect the fact that under more selective pressures of antimicrobial agents, clinical strains showing multidrug resistance are selected. Furthermore, in this study, it is plausible to assume that some of the environmental O1 strains with antibiotic resistance profiles (and MLEE patterns, see below) similar to clinical strains represent strains that have been shed into the environment from clinical cases. Alternatively, while speculative, it is possible that all these strains are derived from the seventh-pandemic strain by the absence or loss of virulence genes. The increased frequency of O1 environmentally resistant strains may reflect their selection after treatment and/or the widespread use of antibiotics by the population, for the treatment of cholera and other purposes.

Our results for the environmental non-O1 strains are in contrast with other studies showing non-O1

isolates exhibit high levels of resistance to antimicrobial agents [25, 26]. It is possible that these variations are associated with regional differences or may reflect differences on antibiotics usage in different parts of the world. As we found that O1 and non-O1 strains have increased resistance to ampicillin, streptomycin, sulphonamides and furazolidone, it may be that these strains share a common mechanism for the development of antibiotic resistance. We found that a high percentage of clinical O1 strains (76%) showed intermediate susceptibility to tetracycline. This is of great concern because tetracycline is the antibiotic of choice for the treatment of cholera [29]. Tetracycline-resistant *V. cholerae* O1 strains have been responsible for major epidemics around the world [10, 24, 30] and in Brazil, two *V. cholerae* strains showing multiple drug resistance (including to tetracycline) were isolated from cholera patients in the state of Ceará between 1991 and 1993 [27].

Almost all O1 and non-O1 strains displayed susceptibility to the broad-spectrum fluoroquinolones, such as ciprofloxacin and ofloxacin. It is important to note, however, that two clinical O1 strains showed intermediate resistance to these drugs while clinical studies have shown that these drugs are effective in the treatment of cholera [31, 32], the emergence of fluoroquinolone-resistant strains of *V. cholerae* O1 has been reported [28]. The results from this study suggest that the treatment of diarrhoeal patients with alternative drugs such as fluoroquinolones will probably become problematic in the future due to the emergence of resistance.

Analysis of plasmids and integrons by PCR

None of the strains included in this study harboured any plasmid or class 1 integrons as detected by the PCR analysis used here. Since plasmids and plasmid-encoded resistance are rarely found among *V. cholerae* O1 strains [25, 32, 33], and class 1 integrons could account for only few markers, our data suggest that other determinants of antibiotic resistance are present in these strains. The presence of the trimethoprim–sulphamethoxazole element has been described in *V. cholerae* strains conferring resistance to trimethoprim, streptomycin, sulphamethoxazole, and chloramphenicol [34] and while its prevalence can be determined in future studies, its occurrence in some of these isolates could explain the high percentage of strains showing resistance to streptomycin and sulphonamides.

MLEE analysis of genetic relatedness

In order to better understand the genetic relatedness of the strains in this study we used MLEE. Table 3 shows the electromorphs of 18 electrophoretic types (ETs) obtained from 92 clinical O1 and 56 environmental *V. cholerae* O1 and non-O1 isolates. For comparison purposes, the alleles profiles of *V. cholerae* N16961 (El Tor, seventh pandemic) and *V. cholerae* 395 (sixth-pandemic classical biotype) strains were also included. These data indicate that most of the clinical O1 isolates (97%) and all of the 42 environmental O1 strains belonged to one genetically indistinguishable ET (ET1). On the other hand, the environmental *V. cholerae* non-O1 isolates showed a variety of ETs (ET3–ET17). One clinical O1 strain (no. 238), recently (1999) isolated in Pará, showed the same alleles profile of N16961, corresponding to ET2. A distinct ET (ET3) was shown in clinical O1 strain (no. 152), isolated in Ceará (1999), suggesting it has an independent origin. On the other hand, a clinical O1 strain (no. 189), isolated in the state of Rio de Janeiro, in 1993, gave the same allelle profile of the strain 395 (ET18). The dendrogram in the Figure shows that all the strains of ET1 are closely related to strains of the seventh pandemic (ET2), although they differed at the LAP locus (Table 3).

The data obtained from our MLEE analysis of Brazilian *V. cholerae* strains suggests that most clinical O1 strains (97%) belong to a single clone and are closely related to environmental O1 strains, regardless of their year and place of isolation. These findings are in agreement with those obtained by RAPD analysis in a previous study [11]. Furthermore, in support of other studies, our MLEE results showed that clinical and environmental O1 strains are closely related to seventh-pandemic strains from other parts of the world, but differ from those strains at the LAP locus [6, 14, 15]. Our earlier study [11] showed that some clinical strains lacked specific virulence genes (CTX and VPI genes) but this was more common in the environmental isolates with none having a complete set of virulence genes tested. Together, these data indicate that these strains represent the South American variant of the seventh-pandemic strain with the allele 3 LAP, or are derivatives of that variant. The data could be further interpreted to suggest that the environmental O1 strains are derived from the clinical form and appear to have lost some virulence properties and genes. This possibility was suggested previously by us [11] and is reinforced here by our current data.

Table 3. *Allele profiles of 92 clinical O1 and 56 environmental* V. cholerae *O1 and non-O1 isolates*

ET	Origin (no. of isolates)	Serogroup (no. of the strain)	Alleles present in each enzymatic locus*											
			ADH	IDH	ME	EST	PGD	MDH	PGM	GPI	G6P	P1	P2	LAP
1	Clinical (89) Environmental (42)	O1	1	1	2	4	3	3	3	2	5	2	1	3
2	Clinical (1)	O1 (238)	1	1	2	4	3	3	3	2	5	2	1	1
3	Clinical (1)	O1 (152)	1	1	2	6	3	3	3	3	4	2	1	3
4	Environmental (1)	Non-O1 (16)	1	1	2	5	3	4	3	4	4	2	1	3
5	Environmental (1)	Non-O1 (41)	1	1	2	5	3	3	3	3	4	2	1	1
6	Environmental (1)	Non-O1 (47)	1	2	2	5	3	3	3	3	4	2	1	1
7	Environmental (1)	Non-O1 (44)	1	3	2	5	3	3	3	3	4	2	1	1
8	Environmental (1)	Non-O1 (45)	1	1	2	5	3	4	3	3	4	2	1	1
9	Environmental (1)	Non-O1 (48)	1	3	2	5	3	3	3	3	4	2	1	2
10	Environmental (1)	Non-O1 (52)	1	2	2	5	3	3	3	4	5	2	1	1
11	Environmental (1)	Non-O1 (51)	1	1	2	5	4	3	4	4	4	2	1	1
12	Environmental (1)	Non-O1 (61)	1	3	2	5	4	3	4	4	4	2	1	2
13	Environmental (1)	Non-O1 (62)	1	2	2	5	3	3	3	3	5	2	1	1
14	Environmental (1)	Non-O1 (63)	1	1	2	5	3	3	3	3	4	2	1	2
15	Environmental (1)	Non-O1 (49)	1	2	2	5	3	3	3	3	5	2	1	2
16	Environmental (1)	Non-O1 (65)	1	2	2	5	3	3	3	3	4	2	1	2
17	Environmental (1)	Non-O1 (66)	1	1	2	4	3	3	3	3	5	2	1	3
18	Clinical (1)	O1 (189)	1	1	2	4	2	3	2	3	5	2	1	2
	Control strain (El Tor)	O1 (N16961)	1	1	2	4	3	3	3	2	5	2	1	1
	Control strain (Classical)	O1 (395)	1	1	2	4	2	3	2	3	5	2	1	2

* ADH, alanine dehydrogenase; IDH, isocitrate dehydrogenase; ME, malic enzyme; EST, esterase; PGD, 6-phosphogluconate dehydrogenase; MDH, malate dehydrogenase; PGM, phosphoglucomutase; GPI, glucose phosphate isomerase; G6P, glucose-6-phosphate dehydrogenase; P1, peptidase leucyl-leucyl leucine; P2, peptidase leucyl-glycyl glycine; LAP, leucyl-leucyl aminopeptidase.
ET, Electrophoretic type.

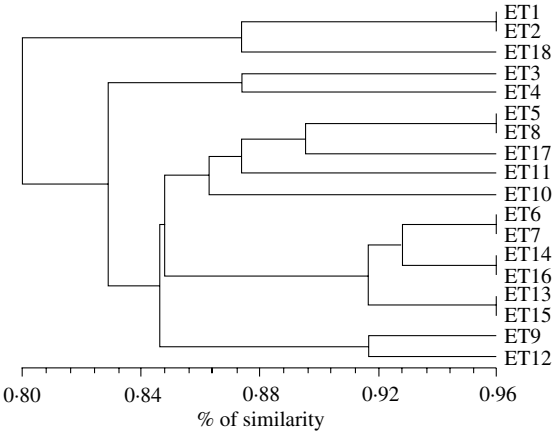

Fig. Dendrogram showing the degree of similarity of 18 electrophoretic types (ETs) of 92 clinical O1 and 56 environmental *V. cholerae* O1 and non-O1 strains. See Table 3 for ETs description.

Our findings that *V. cholerae* non-O1 strains are very variable in RAPD [11] and produce a variety of ETs (this study) are consistent with other studies and also shows the heterogeneous nature of *V. cholerae* non-O1 strains [35, 36]. Furthermore, the diverse nature of non-O1 environmental strains might represent the normal diverse flora endemic to the area.

Interestingly, we found a clinical O1 strain (strain 189) with the same allelic profile as strain 395 (a sixth-pandemic classical biotype) (ET18), suggesting they are closely related. One clinical O1 strain (strain 238; ET2) had the LAP 1 allele that is similar to strain N16961 (a seventh-pandemic El Tor strain). A distinct ET (ET3) was found in the clinical O1 strain 152. These findings are somewhat unexpected since the current seventh-pandemic strain in Latin America differs from the seventh-pandemic isolates from other parts of the world at the LAP locus [6, 15]. Strains 189 and 238 could be related to imported cases of cholera but we do not have additional information about the clinical cases from which these isolates were isolated to confirm this hypothesis. Strain 189, shown previously to have the sixth-pandemic *tcpA* gene [11] and same RAPD profile, is now shown to be identical to

the representative sixth-pandemic strain by MLEE. Therefore, based on a range of tests, strain 189 is not indistinguishable from sixth-pandemic strains and must have come from that clone. It appears that strain 152 evolved recently and may represent a transition state in the evolution of the seventh-pandemic clone and is consistent with evidence showing the rapid evolution of clones of *V. cholerae* in the last few years [36].

CONCLUSION

It appears that the continual emergence of new clones of *V. cholerae* strains may arise from multiple independent sources, possibly through natural selection involving unidentified environmental factors and immunity of the host population [9, 37]. Our results suggest that many serogroups of *V. cholerae* can be reservoirs of virulence and antibiotic resistance genes. As such, we strongly recommend continuous epidemiological surveillance focusing on the emergence of new clones of *V. cholerae* in both environmental and clinical sources. In addition, the high prevalence and reservoir of multiple antibiotic-resistant *V. cholerae* strains indicates the necessity of performing an epidemiological surveillance to periodically monitor the patterns of antibiotic susceptibility of *V. cholerae*.

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