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DISTRIBUTION OF VIRULENCE MARKERS IN CLINICAL AND ENVIRONMENTAL *Vibrio cholerae* NON-O1/NON-O139 STRAINS ISOLATED IN BRAZIL FROM 1991 TO 2000

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SUMMARY

One hundred seventy nine *Vibrio cholerae* non-O1/non-O139 strains from clinical and different environmental sources isolated in Brazil from 1991 to 2000 were serogrouped and screened for the presence of four different virulence factors. The Random Amplification of Polymorphic DNA (RAPD) technique was used to evaluate the genetic relatedness among strains. Fifty-four different serogroups were identified and *V. cholerae* O26 was the most common (7.8%). PCR analysis for three genes (*ctx*A, *zot*, *ace*) located of the CTX genetic element and one gene (*tcp*A) located on the VPI pathogenicity island showed that 27 strains harbored one or more of these genes. Eight (4.5%) strains possessed the complete set of CTX element genes and all but one of these belonged to the O26 serogroup suggesting that *V. cholerae* O26 has the potential to be an epidemic strain. The RAPD profiles revealed a wide variability among strains and no genetic correlation was observed.

KEYWORDS: *Vibrio cholerae* O26; CTX; Virulence genes; Genotypic variation; Epidemic potential.

INTRODUCTION

V. cholerae, a Gram-negative bacterium is the causative agent of cholera. The conventional method used to classify *V. cholerae* strains is a serotyping scheme based on epitopic variation cell surface lipopolysaccharide (LPS). Up to now, there is near 200 serogroups[37] and two serotypes (Inaba e Ogawa) identified for serogroup O1. The specie has been divided into serogroups O1 and non-O1 which differ each other for their ability to cause cholera epidemics[7]. Prior to 1992, it was believed that only *V. cholerae* O1 strains were responsible for pandemics of cholera and all major epidemics[16], and the non-O1 strains were non virulent responsible for sporadic cases of gastroenteritis[26] and extra-intestinal infections[24]. In 1992, an outbreak of cholera in India and Bangladesh, which subsequently spread into several parts of the subcontinent, was caused by a novel non-O1 strain: *V. cholerae* O139 Bengal[1].

V. cholerae O1 produces cholera toxin (CT) which is responsible for secretory diarrhea and is encoded by the *ctx*A and *ctx*B genes. Although CT is responsible for disease, the search for additional enterotoxins produced by *V. cholerae,* which has included volunteer studies of genetically engineered *ctx*-deleted *V. cholerae,* has led to discovery of new toxins[15]. The zonula occludens toxin (ZOT) acts on intestinal tight junctions to increase intestinal permeability[3,18,20]. The accessory cholera enterotoxin (ACE) increases potential difference across intestinal epithelium and alters ion transport[20]. The *ctx*A, *ctx*B, *zot* and, *ace* genes are located on the CTX genetic element, which is composed of a 4.5-Kb central region termed the core region or virulence cassette[30]. The CTX genetic element, a filamentous bacteriophage that infects *V. cholerae* and its close relative *V. mimicus*[4,12] is present on the chromosome of toxigenic strains and absent in non-toxigenic strains[22], but *V. cholerae* pathogenicity depends on a combination of factors as ability to produce a cholera toxin (CT) and to adhere and colonize the small intestine through colonization factor known as toxin-coregulated pilus (TCP). It had been accepted that CT and TCP were exclusively associated with clinical strains of *V. cholerae*, notably those belonging to serogroups O1 and O139, whereas reports on the incidence of CT among environmental strains are rare[6]. Similarly, TCP had rarely been reported among environmental strains of *V. cholerae*, suggesting that TCP is associated only with virulent *V. cholerae* O1 or O139 strains. However, in the last years, the presence of *ctx*AB and *tcp*A genes in non-O1/non-O139 toxigenic and non-toxigenic strains has been observed[31].

In the present study, PCR was used to detect the presence of *ctx*A, *zot*, *ace,* and *tcp*A genes on 179 *V. cholerae* strains. We also used RAPD-PCR to evaluate the genetic relatedness among some clinical and environmental *V. cholerae* non-O1/non-O139 strains.

MATERIALS AND METHODS

Bacterial strains: A total of 179 *V. cholerae* non-O1/non-O139 strains isolated from faeces of suspected cholera patients (139 strains) and from environmental (40 strains from water of different ecosystems)

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Table 1
Primers sequences used for detection of virulence genes by PCR

Gene	Primer sequence(5' - 3')	Size (bp)	Reference
*ctx*A	CTCAGACGGGATTTGTTAGGCACGTCTATCTCTGTAGCCCCTATTACG	301	19
zot	GCTATCGATATGCTGTCTCCTCAAAAAGCCGACCAATACAAAAACCAA	900	21
ace	AGAGCGCTGCATTTATCCTTATTGAACTCGGTCTCGGCCTCTCGTATC	600	21
*tcp*A-$_{ET}$	GAAGAAGTTTGTAAAAGAAGAACACGAAAGGACCTTCTTTCACGTTG	471	19

were selected for this study. These strains were isolated during 1991 to 2000 and belonged to the collection of National Reference Laboratory for Cholera and Enteric Diseases at Oswaldo Cruz Institute (Fiocruz/RJ). Clinical strain V. *cholerae* O1, classical biotype (569B) and one environment strain *V. cholerae* non-O1/non-O139 (E8498, provided by R. R. Colwell, University of Maryland, USA); the *ctx*A, *zot, ace,* and *tcp*A genes and absent and present on these two strains, respectively, and were used as controls.

Isolation and identification of *V. cholerae* strains were performed according to the guidelines from the Brazilian Health Ministry[23].

The strains that did not agglutinate with either O1 or O139 antisera were considered non-O1/non-O139. They were sent to the National Institute of Health, Tokyo, Japan for serogroup identification.

PCR assay: A PCR - based assay was used to determine the presence of *ctx*A, *zot*, *ace*, and *tcp*A genes in individual strains[29]. The primers used for this assay and the expected amplicon sizes for each gene are listed in Table 1. The following were added to each 25 µL of PCR mixture: 2.5 µL of 10x PCR buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl [pH 9.0]); 2 µL of the 160 µM dNTP mix (dATP, dTTP, dGTP, dCTP); 1 µL (20 pmol) each of the primer; 0.2 µL (1U) of *Taq* DNA polymerase (Amersham Pharmacia) and 17.3 µL of double-distilled water. PCR was carried out in 0.5 mL microcentrifuge tubes with 24 µL of the PCR mixture described above and 1 µL of template. Whole-cell suspensions were used as template for all PCRs. Cells were harvested from overnight growth on nutrient agar (Oxoid), transferred into 500 µL of water and vortexed to homogeneity. Suspensions were boiled at 100 ºC for 20 min and then stored at -20 ºC. The solution was overlaid with a drop of sterile mineral oil (Sigma), and PCR was performed in an automated thermocycler (Perkin Elmer). The PCR reaction was performed on 30 cycles: 95 ºC for one min, 60 ºC for one min, 72 ºC for 1.5 min, and then kept at 72 ºC for seven min. A non-template control and the strains 569B and E8498 were included in each run as negative and positive controls for the absence/presence of *ctx*A, *zot*, *ace*, and *tcp*A genes, respectively. Presence of PCR product was visualized on 1% agarose gels after stained with ethidium bromide. A 100 base-pair molecular size ladder (Amersham Pharmacia) was included in each gel.

RAPD-PCR: In order to evaluate the genomic relatedness among clinical and environmental *V. cholerae* non-O1/non-O139 strains, 26 *V. cholerae* non-O1/non-O139 strains that hosted any virulence-associated gene and 12 non-virulent strains were selected for RAPD-PCR (Table 3).

Genomic DNA was extracted following the procedure described by AUSUBEL *et al*.[2]. Preliminary assays were performed with *V. cholerae* and sixteen 10-nucleotide random primers drawn for different purposes (**784**: 5'-GCGGAAATAG-3'; **785**: 5'-CCGCAGCCAA-3'; **786**: 5'-GCGATCCCCA-3'; **787**: 5'-AACGCGCAAG-3'; **788**: 5'-GTGGATGCGA-3'; **789**: 5'-AGCCAGTTTA-3'; **790**: 5'-GTCAACGAAG-3'; **791**: 5'-GAGGACAAAG-3'; **792**: 5'-GGTACTCCCA-3'; **793**: 5'-GACCGACCCA-3'; **794**: 5'-ACTGAACGCA-3'; **795**: 5'-GAGACGCACA-3'; **796**: 5'-ACCTCAGCTG-3'; **797**: 5'-AGCGTCACTG-3'; **798**: 5'-TGACCCGCCG-3'; **799**: 5'-GGCTTGGCCG-3') to select the primers that generated clear and reproducible bands. The primers 784, 785 and 791 were found to be the most appropriate. Amplification reactions

Table 2
Distribution of *Vibrio cholerae* non-O1/non-O139 serogroups isolated from different sources

Serogroup (Sources)*	Number of strains belonging to each serogroup
O2(H), O12(H), O21(H), O22(H), O27(H), O31(H), O33(E), O41(E), O43(H), O48(H), O49(H), O52(H),O53(E), O99(E), O100(E), O103(H), O141(H), O151(E),O195(E), O203(E)	1
O5(H), O14(H), O18(H), O19(H/E), O23(H), O32(H), O40(H/E), O56(H), O97(H), O101(E), O119(H)	2
O7(H), O13(H/E), O39(H/E), O51(H/E), O64(H), O69(H), O74(H), O94(H), O110(H/E)	3
O17(H), O28(H/E), O45(H), O68(H/E), O112(E)	4
O62(H/E)	5
O11(H)	6
O8(H), O36(H/E)	7
O6(H/E)	8
O10(H), O34(H/E)	12
O37(H/E)	13
O26(H/E)	14
O UK (H)	6
TOTAL	179

Sources*: (H) = Human, (E) = Environment, (H/E) = Human and environment, O UK = Unknown serogroup - not typable.

Table 3
Characteristics of *Vibrio cholerae* non-O1/non-O139 serogroups analyzed by RAPD-PCR

Isolate No. IOC	Serogroup	Source	Presence of analyzed genes
157, 3874, 3905	O6	Human	*zot*
13153	O7	Human	*zot, ace*
1614	O7	Human	——-
14721	O8	Human	——-
4005	O10	Human	*zot*
15251, 15380	O10	Human	——-
13165	O14	Human	——-
2556	O17	Human	*zot, ace*
3647, 16352	O26	Human	*zot*
1717, 10626	O26	Human	*zot, ace*
11043, 13663, 15677	O26	Human	*zot, ace tcp*A
3340, 4756, 6958, 11159, 13151	O26	Human	*ctx*A, *zot, ace*
4010	O26	Human	*ctx*A, *zot, ace, tcp*A
2494	O26	Environment	*ctx*A, *zot, ace, tcp*A
13653	O27	Human	——-
13488	O34	Human	——-
4482	O37	Human	*zot, ace*
13154, 16580	O37	Human	——-
14831	O45	Human	*tcp*A
14813	O45	Human	——-
13662	O51	Human	*zot*
3833	O51	Environment	*zot, ace, tcp*A
14833	O56	Human	——-
7098	O94	Human	*zot*
15381	Rough strain	Human	——-
17155	Not typable	Human	*ctx*A, *zot, ace*, *tcp*A

were performed in 25 µL volume containing 2.5 µL of 10x reaction buffer, 200 µM each of dNTP, 20 pmol of primer, 3 mM of $MgCl_2$, 2 U of Taq DNA polymerase and 20 ng of genomic DNA under a drop of mineral oil.

The PCR reaction was performed on 30 cycles: 94 ºC for one min, 36 ºC for one min, 72 ºC for 1.5 min, and than kept at 72 ºC for 10 min. A non-template control was included in each RAPD-PCR run. The PCR amplification products were separated by electrophoresis on 1.5% agarose gels and visualized after staining with ethidium bromide.

RESULTS

The serogrouping results are shown in Table 2. None of the 179 *V. cholerae* isolates agglutinated with antisera to O1 or O139, but 173 of them (96.6%) could be divided in 54 different serogroups, with O26 (7.8%) and O37 (7.3%) being the most prevalent.

Of the 179 isolates, 27 strains harbored one or more of the virulence-associated genes (Table 4). Eight (4.5%) strains were *ctx*A gene positive being observed only in *V. cholerae* O26. All the *V. cholerae* O26 strains, without exception, were positive for one or more virulence-associated encoding genes. The *ctx*A gene was not present independent of the *zot* and *ace* genes, but *zot* gene was found to be distributed in 19 strains of *V. cholerae* non-O1/non-O139 (17 clinical and two environmental) in the absence of the *ctx*A and/or *ace* genes. The *tcp*A gene was present in eight strains of the *V. cholerae* non-O1/non-O139, which contained one or more gene of the CTX genetic element. However, this gene was also present in one isolate (strain 14831) negative for all genes of the CTX element studied. Furthermore, five clinical strains that were positive for *ctx*A, were negative for *tcp*A.

The genetic relatedness of the clinical and environmental *V. cholerae* non-O1/non-O139 strains was investigated by RAPD-PCR. Three primers were selected in this study and the primer 785 generated the best amplification patterns for differentiation of *V. cholerae* strains belonging to the serogroups O6, O7, O8, O10, O14, O17, O27, O28, O34, O37, O45, O51, O56 and O94. A second profile was built with all 13 clinical and one environmental isolates of *V. cholerae* O26.

The RAPD-PCR with primer 785 with *V. cholerae* non-O1/non-O139 strains produced different fingerprints into the same serogroup: O6 (IOC 3874, 3905); O10 (IOC15251, 15380); O37 (IOC 4482, 13154, 16580) and most of the *V. cholerae* O26 strains. However, identical RAPD profiles were observed in the *V. cholerae* O6 (IOC 157, 3874); O26 (IOC 13151, 2494) and O45 (IOC 14813, 14831).

DISCUSSION

To date, ca. 200 serogroups of *V. cholerae* have been recorded, and two (O1 and O139) have been associated with major cholera epidemics. The other serogroups, referred as non-O1/non-O139, have not been associated with epidemics rather can cause sporadic diarrhea and occasionally outbreaks[30]. This outline distinction between serogroups is related to the observation that more than 95% of the strains belonging to serogroups O1 and O139 produce cholera toxin (CT)[15]. In contrast, more than 95% of the strains belonging to non-O1/non-O139 serogroups do not produce CT or TCP[25]. The detection of *ctx*A gene in only 4.5% of the strains in this study, is in agreement with the toxigenic profile of the *V. cholerae*. However, our results contradict the assumption that most cholera toxin-producing strains are also positive for TCP, since TCP is known to be the receptor for CTXϕ infection of *V. cholerae*. In fact, our results appear to be consistent with data from recent studies that report the presence of the two virulence genes found in epidemic strains, *ctx*AB and *tcp*A, in environmental and clinical strain of serogroups other than O1 and O139[5,10,27,28,31,32].

It has been suggested that most *V. cholerae* strains, especially those from the environment, lack the genes required to produce CT and the possibility of genetic exchange in the environment allows the potential emergence of new toxigenic clones. This mechanism of horizontal gene transfer plays an important role increasing the genetic variability of a bacterial species and also confers new phenotypes, such as virulence, to the recipient. The emergence of new toxigenic strains of *V. cholerae* and their selective enrichment during cholera outbreaks constitute essential mechanisms for the survival and evolution of *V. cholerae* and the genetic elements that mediate the transfer of virulence genes[11].

The *ctx*AB genes are carried in the genome of a lysogenic filamentous bacteriophage designated CTXϕ[36] and their dissemination

Table 4
Distribution of virulence genes in clinical and environmental of different serogroups of *Vibrio cholerae* non-O1/non-O139

Isolate No. IOC/Year	Origin	Source	Serogroup	Presence of virulence genes			
				ctxA	*zot*	*ace*	*tcp*A
157/91	Amazonas	Human	O6		+		
3874/92	Paraíba	Human	O6		+		
3905/92	Pernambuco	Human	O6		+		
13153/94	Pernambuco	Human	O7		+	+	
4005/92	Pernambuco	Human	O10		+		
2556/92	Paraíba	Human	O17		+	+	
1717/92	Pernambuco	Human	O26		+	+	
3340/92	Pernambuco	Human	O26	+	+	+	
3647/92	Pernambuco	Human	O26		+		
4010/92	Pernambuco	Human	O26	+	+	+	+
4756/92	Pernambuco	Human	O26	+	+	+	
6958/92	Ceará	Human	O26	+	+	+	
10626/93	Bahia	Human	O26		+	+	
11043/93	Pernambuco	Human	O26		+	+	+
11159/93	Pernambuco	Human	O26	+	+	+	
13151/94	Pernambuco	Human	O26	+	+	+	
13663/94	Pernambuco	Human	O26		+	+	+
15677/98	Ceará	Human	O26		+	+	+
16352/99	Ceará	Human	O26		+		
2494/92	Bahia	Environment	O26	+	+	+	+
16360/99	Ceará	Environment	O28		+	+	+
4482/92	Pernambuco	Human	O37		+	+	
14831/95	Ceará	Human	O45				+
13662/94	Pernambuco	Human	O51		+		
3833/92	Paraíba	Environment	O51		+	+	+
7098/92	Amazonas	Human	O94		+		
17155/00	Pernambuco	Human	NT	+	+	+	+

IOC = Instituto Oswaldo Cruz, NT = Not typable strain.

to non-pathogenic strains may occur via phage-mediated horizontal gene transfer. The pilus colonization factor (TCP) is also known to act as a receptor for CTXϕ and is itself encoded by a recently described novel lysogenic bacteriophage, VPIϕ[17]. Hence, we presumed that only non-toxigenic *V. cholerae* strains that already contain TCP can acquire *ctx*AB via lysogenic conversion by CTXϕ infection. However, in our findings five human strains were *ctx*A$^+$/*tcp*A$^-$ (Table 4). Some authors have suggested that such isolates arose by TCP-mediated CTXϕ infection, with subsequent loss of the TCP island (VPIϕ)[11]. Alternatively, such strains may possess an as-yet-unidentified receptor and/or modes of CTXϕ acquisition that account for the presence of a CTXϕ prophage in these strains[12]. In our study the non-toxigenic strain positive for *tcp*A (IOC 14831-Table 3) may be a potential precursor of toxigenic strains because of its dual capacity to serve as efficient recipient of CTX phage and to colonize the human intestine by TCP-dependent mechanisms. Moreover, were identified eighteen *V. cholerae* non-O1/non-O139 strains (10%) that contained *zot* and/or *ace* but lacked *ctx*A. This finding is somewhat unexpected since the three genes are part of the CTX genetic element. However, similar results were obtained by VITAL BRAZIL *et al*.[35], which analyzed the prevalence of virulence-associated genes in *V. cholerae* strains isolated in Brazil and showed that 31 strains (8.3%) did not contain *ctx*A. Thus, we hypothesized that there was lost *ctx*A later integration of CTXϕ DNA into the *V. cholerae* chromosome.

In previous study was observed that when the RAPD reactions are performed under rigorous reaction conditions (same preparation of genomic DNA, brand and lot of enzyme), the pattern of bands is reproducible[21]. In this study, standardized RAPD method showed to be reproductive and ascertained the diversity of the serogroups of the *V. cholerae* analyzed, which was demonstrated by the presence of different subtypes into the same serogroup.

Identification of the non-O1/non-O139 serogroups of *V. cholerae* carrying virulence genes is very important since these new toxigenic strains with epidemic potential may emerge in the future. This finding is based first on the emergence of serogroup O139 (Bengal) in Bangladesh (CT positive) and Argentina (CT negative), each of which clearly evolved independently[33]. Second, the isolates from the sixth and seventh pandemic, and U.S. Gulf Coast belong to three different clones, each independently, evolved from environmental non-O1 *V. cholerae* isolates[16]. Third, the high relatedness of different serogroups of *V. cholerae* strains isolated in Brazil[34], and other epidemic serogroups have emerged, including *V. cholerae* O10[13], O12[9], O22[14], O31[26], O37[8],

and O141[10]. These findings stress the need for continued surveillance of non-O1/non-O139 *V. cholerae.*

RESUMO

Distribuição dos marcadores de virulência em cepas clínicas e ambientais de *Vibrio cholerae* não O1/não O139, isoladas no Brasil no período de 1991 a 2000

Cento e setenta e nove amostras de *V. cholerae* não O1/não O139, isoladas de casos clínicos (139) e de meio ambiente (40), no período de 1991 a 2000 no Brasil, foram caracterizadas antigenicamente pelo National Institute of Health (Japão) e investigadas quanto ao seu potencial genético de virulência, representado pelos genes *ctx*A, *zot*, *ace* e *tcp*A. As análises fenotípicas revelaram extraordinária diversidade antigênica, com a ocorrência de 54 diferentes sorogrupos, com prevalência para O26 (7,8%). A técnica de PCR, empregada na detecção dos genes localizados no elemento genético CTX (*ctx*A, *zot*, *ace*) e na Ilha de Patogenicidade de Vibrio-VPI (*tcp*A), possibilitou a identificação de 27 cepas contendo qualquer um desses genes. O gene *ctx*A (codificador da sub-unidade A de CT), só foi evidenciado no sorogrupo O26, sendo também o único capaz de se apresentar com o cassete de virulência de forma intacta. Com base nos resultados obtidos deste estudo preliminar, admite-se a hipótese da potencialidade destas cepas, evoluir para raças epidêmicas.

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