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Virulence factors of non-O1 non-O139 *Vibrio cholerae* isolated in Córdoba, Argentina

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SUMMARY

V. cholerae non-O1 non-O139 serogroups isolated from clinical and environmental sources in Córdoba, Argentina, were analyzed for the presence and expression of virulence genes. Most of the strains studied contained the genes *toxR* and *hlyA*, but lacked *ctxA*, *zot, ace, tcpA* and *stn*. The culture supernatants were tested for hemolytic and cytotoxic activity. The enterotoxic potential of the strains was studied in a rabbit ileal loop assay and their genetic profiles were compared by PFGE. The environmental strains varied in their virulence phenotype and showed no-clonal relationships. The clinical strains were highly enterotoxic, hemolytic, proteolytic and showed indistinguishable PFGE profiles, although they differed in their cytotoxic activity. This is the first description, using cell culture and "in vivo" studies, of the virulence properties of non-O1 non-O139 *V*. *cholerae* from Argentina.

Key words: non-O1 non-O139 *V. cholerae*, enterotoxin, cytotoxin, hemolysin, multiplex PCR

RESUMEN

Factores de virulencia de *Vibrio cholerae* no-O1 no-O139 aislados en Córdoba, Argentina. En este trabajo se analizó la presencia y expresión de genes de virulencia en *V. cholerae* no-O1 no-O139 de origen clínico y ambiental, aislados en Córdoba, Argentina. La mayoría de las cepas estudiadas contiene los genes *toxR* y *hlyA*, pero no *ctxA*, *zot*, *ace*, *tcpA* y *stn*. Se analizó la actividad hemolítica y citotóxica de estas cepas en los sobrenadantes de cultivo, así como su potencial enterotóxico en ensayos de asa ileal ligada de conejo. Además, los aislamientos fueron comparados por sus perfiles genéticos en PFGE. Las cepas del medio ambiente mostraron variación en su fenotipo de virulencia y no mostraron relación clonal. Las cepas clínicas fueron muy enterotóxicas, hemolíticas, proteolíticas y mostraron perfiles indistinguibles de PFGE, aunque mostraron diferencias en su actividad citotóxica. En este trabajo se describen por primera vez, utilizando ensayos de cultivo celular e "in vivo", propiedades de virulencia de *V. cholerae* no-O1 no-O139 aislados en Argentina.

Palabras clave: *V. cholerae* no-O1 no-O139, enterotoxina, citotoxina, hemolisina, multiplex PCR

INTRODUCTION

Cholera disease, caused by toxigenic *Vibrio cholerae,* is a major public health problem in developing countries. Epidemiological surveillance and comparative molecular analysis of isolates have demonstrated clonal diversity among epidemic strains and a continual emergence of new clones of toxigenic *V. cholerae* (13, 20). The non-O1 non-O139 serogroups are associated to the emergence of new pathogenic variants of *V. cholerae* (21) and are potential receptors of virulence factors from toxigenic *V. cholerae* O1 (6).

Non-O1 non-O139 *V. cholerae* has been isolated from patients with acute secretory diarrhea worldwide (23). Nevertheless, the factors involved in the pathogenesis of non-O1 non-O139 serogroups are not well understood. It has been reported that these strains encode some putative virulence factors such as NAG-ST enterotoxin (2), hemolysins (24), and proteases (22). The study of these factors in clinical as well as environmental strains of non-O1 non-O139 *V. cholerae* isolated around the world will be of great value to elucidate the pathogenesis of non toxigenic cholera disease.

The aim of the present study was to investigate and compare the virulence determinants in fifteen non-O1 non-O139 *V. cholerae* strains isolated in Córdoba, Argentina. The presence of several virulence genes, and the ability to produce hemolysin, cytotoxin, protease and fluid accumulation, were analyzed. Also, the clonal relationship among the strains was determined by pulsed field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Bacteriology and serogrouping

Thirteen *V. cholerae* isolates were obtained from environmental water sources of Córdoba during the period 1991-1996: strains 1383, 208 and 286 from Suquía River; 140, 150, 183, 289 from

La Cañada creek; 210 and 287 from Northern Main Canal, 211 and 288 from Southern Main Canal; 217 from Dolores River and 218 from Calabalunga River. During the same period, under a cholera surveillance program carried out at the "Centro de Enterobacterias-División Laboratorio Central de Salud Pública de la Provincia de Cordoba", two *V. cholerae* clinical isolates were obtained from hospitalized patients who suffered an acute diarrhea syndrome indistinguishable from cholera, in Córdoba city (strains175 and 588). All the *V. cholerae* isolates included in this study did not agglutinate with either O1 or O139 antiserum.

Bacterial growth conditions and supernatant preparation

V. cholerae was grown on dialyzed Brain Heart Infusion Broth (BHI, Oxoid, England) (15) under shaking conditions at 37 °C for 16 h. Cultures were centrifuged at 10.000 g and 4 °C for 30 min and the supernatants were used as "centrifuged supernatants" (containing about 1 x 10^5 – 1 x 10^6 cfu ml^{-1}) or were sterilized by filtration through a 0.22 µm low-binding protein filter (Millipore Corp.). Aliquots of the supernatants were heat-treated by incubation at 65 °C during 15 min. Trypsin treatment was done as follow: culture supernatants were precipitated with 50% ammonium sulfate at 4 °C, centrifuged, resuspended in 1/10 volume of phosphate-buffered saline (PBS) and dialyzed against Hepes 50 mM at 4 °C for 1 h. Aliquots of the concentrated supernatants were incubated with 0.2 volume of trypsin 25 µg ml^{-1} (Serva, Boehringer Ingelheim) at 37°C during 3 hours. The reaction was stopped with fetal bovine serum (Gibco-BRL Laboratories, N.Y.) in a final dilution of 1/100. Concentrated supernatants without trypsin-treatment were used as controls.

Antimicrobial susceptibility test

Bacterial strains were examined by the disk diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS) (17) to determine the susceptibility to the following antibiotics (Oxoid, England): chloramphenicol (30 µg), ampicillin (10 µg), cephalothin (30 µg), trimethoprim /sulfamethoxazole (1.25/23.75 µg), ciprofloxacin (5 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), streptomycin (10 µg), and tetracycline (30 µg). When no interpretive criteria for *V. cholerae* were available based on the NCCLS guidelines, breakpoints for *Enterobacteriaceae* were applied. ATCC strains *E. coli* 25922, *S. aureus* 25923, *P. aeruginosa* 27853 and *E. faecalis* 29212 were used as controls.

Polymerase chain reaction assay (PCR)

Three sets of multiplex and one simplex PCR were used to determine the presence of the genes: *ctxA* (encoding the enzymatic subunit of cholera toxin) and *tcpA* (encoding the major structural subunit of toxin co-regulated pilus, El Tor variant) (10); *zot* (encoding zonula occludens toxin) and *ace* (encoding accessory cholera enterotoxin) (21); *toxR* (encoding the transcriptional activator ToxR) (10) and *hlyA* (encoding the El Tor hemolysin) (1); and *stn* (encoding the termostable toxin NAG) (18). The primers used are shown in Table 1.

The following were added to obtain each 25 µl of PCR mixture: 2.5 µl of 10X PCR amplification buffer (500 mM KCl; 100 mM Tris-HCl pH 9.0; 1% Triton X-100, Promega Corporation), 2.5 µl of $MgCl_2$ 50 mM, 0.25 µl dNTPs (25 mM each), 0.4 µl (20 pmol) of each of the primers, 0.25 µl (1.25 U) of Taq DNA polymerase (Promega Corporation) and 1 µl of DNA template. DNA samples were prepared as follow: bacterial colonies were resuspended in 500 µl of water, centrifuged, washed twice with water and boiled for 10 minutes. The PCR conditions for all the reactions included a first step of 10 min at 94 °C and a final step of 10 min at 72 °C. The middle step consisted of 30 cycles of: a) *ctxA* - *tcpA*: 1.5 min at 94 °C, 1.5 min at 60 °C and 1.5 min at 72 °C; b) *zot* - *ace*: 1.5 min at 94 °C, 1.5 min at 55 °C and 1.5 min at 72 °C; c) *toxR* – *hlyA*: 1.0 min at 94 °C, 1.0 min at 62 °C and 4.0 min at 72 °C; d) *stn*: 1.5 min at 94 °C, 1.5 min at 53 °C and 1.5 min at 72 °C. *V. cholerae* O1 biotype El Tor and non-O1 non-O139 *V. cholerae stn*$^+$ were used as positive controls.

Determination of protease and hemolytic activities

The protease activity was estimated inoculating approximately 10^3 cfu of each strain with a Steers multireplicator onto LB agar plates supplemented with 3% of casein. To achieve the desired density of the bacterial suspensions, 0.5 Mc Farland turbidity inoculum obtained from overnight colonies emulsified in sterile saline (0.85% NaCl) were properly diluted. Regular colony counts were performed to verify the final inoculum density in terms of cfu ml^{-1}. The plates were incubated 18 h at 37 °C and triplicates of the diameter of the clear halo around colonies were measured.

Table 1. Primers used in this study

Gene	Primer	Sequence	Product size	Reference
ctxA	FctxA	5´-CTCAGACGGGATTTGTTAGGCACG-3´	301-bp	11
	RctxA	5´-TCTATCTCTGTAGCCCCTATTACG-3´		
tcpA	FtcpA	5´-GAAGAAGTTTGTAAAAGAAGAACAC-3´	471-bp	11
	RtcpA	5´-GAAAGGACCTTCTTTCACGTTG-3´		
zot	Fzot	5´-CACTGTTGGTGATGAGCGTTATCG-3´	243-bp	21
	Rzot	5´-TTTCACTTCTACCCACAGCGCTTG-3´		
ace	Face	5´-GCTTATGATGGACACCCTTTA-3´	284-bp	21
	Race	5´-GTTTAACGCTCGCAGGGCAAA-3´		
toxR	FtoxR	5´-GCCGTCGACCAATGGAATTACCTTGATGTGCAA-3´	931-bp	this work (1)
	RtoxR	5´-CGGCTTAAGGGCTTGAGTCCACCAGTATGTTTT-3´		
hlyA	FhlyA	5´-CCAAGTGGTGAAGCGGCGGA-3´	1915-bp	this work (1)
	RhlyA	5´-TTTTGGCATCCGGTGTCGCG-3´		
stn	Fstn	5´-GGTGCAACATAATAAACAGTCAACAA-3´	375-bp	this work (2)
	Rstn	5´-TAGTGGTATGCGTTGCCAGC-3´		

(1) Primers designed from *V. cholerae* El Tor N16961 sequence (Reference 11).
(2) Primers designed from *stn* gene sequence (Accession Number M85198

Protease activity was classified in low (+), intermediate (++) and high (+++) when the halo ranged from 7-9 mm, 10-12 mm and 13-15 mm, respectively.

The hemolytic activity was determined by the production of a clear halo around bacterial colonies grown on LB-agar plates supplemented with 5% (v/v) sheep red blood cells, overnight at 37 °C. To quantify the hemolytic activity, sterile culture supernatants were diluted 1/10 in PBS pH 7.3 and 0.8 ml of dilution was mixed with 0.2 ml of a 5% (v/v) sheep red blood cells suspension equilibrated in PBS. The mixture was incubated at 37° C during 30 min, centrifuged at 2,000 rpm during 5 min and the absorbance was measured spectrophotometrically at 540 nm. A volume of 0.2 ml of 5% (v/v) red blood cells suspension was mixed with 0.8 ml of PBS or 0.8 ml of water and used as negative and 100% of hemolysis controls, respectively. Values were expressed as the percentage of hemolysis.

Analysis of cytotoxic activity

Sterile culture supernatants were examined for their cytotoxic activity on Cos-7 cells monolayers. Eukaryotic cells were grown in Dulbecco modified Eagle's medium (DMEM) (Sigma Chemical Co. Ltd., USA) supplemented with 5% (v/v) of fetal bovine serum (FBS) (Gibco-BRL Laboratories, N.Y.) at 37°C in a humidified 5% CO_2 atmosphere.

a) *Microscopic examinations.* Roughly 4 x 10^5 Cos-7 cells were seeded on 3-cm culture dishes and incubated during 24 h under standard conditions as described above. Then the culture media was replaced with DMEM supplemented with 1% (v/v) FBS and inoculated with 1/10 volume of sterile culture supernatant. As negative control, the Cos-7 cells were inoculated with BHI alone. Morphological changes of the cells were examined by phase-contrast microscopy in a Zeiss Axiovert 135IM microscope.

b) *Quantification of cytotoxic activity.* About 2 x 10^4 Cos-7 cells were added in each well of the 96-well plates and incubated 24 h under standard conditions. At this time the number of cells reached 4 x 10^4 cells/well. After fresh medium replacement with 1% (v/v) FBS, the cells were inoculated with 1/10 volume of culture supernatant and incubated for 2 h. The cell viability was measured using a CellViability AQ 96 system (Promega Corporation) according to the manufacturer conditions. Cos-7 cells treated with 1/10 volume of BHI were used as viability control. A mix of DMEM containing 1/10 volume of BHI was used as a blank. The absorbance at 490 nm was obtained with an ELISA Reader Plate (BioRad, Richmond Calif.). The values were expressed as the reciprocal of the absorbance at 490 nm in order to indicate cytotoxicity. Each determination was done by duplicate and the results are the media from two individual experiments.

Rabbit ileal loop assay

The ligated ileal loop test was performed as described by De and Chatterjee (5). Briefly, New Zeeland rabbits weighing 2.0 – 2.5 kg were starved for 48 h before the experiment. Rabbits were anesthetized by subcutaneous injection of ketamine (40.0 mg kg^{-1}) and acepromazine (5.0 mg kg^{-1}). The small intestine was withdrawn and ligated at a distance of 10 cm from the ileocaecal region. Ten to twelve intestinal loops of 6-8 cm, separated by uninoculated segments of 2 cm, were ligated in each animal. The loops were inoculated with 1 ml of bacterial

Table 2. Putative virulence factors in *V. cholerae* non-O1 non-O139 strains

Strain	Origin[1]	Virulence genes[2]			Proteolysis	Hemolysis[3]	Cytotoxicity[4]	FA[5]
		toxR	*hlyA*	*ctxA, ace, zot, tcpA, stn*		%	OD^{-1} ± SD	V/L ± SD
V. cholerae non-O1 non-O139								
1383	E	+	+	-	+++	2.1	11.20 ± 5.59	0.52 ± 0.26
140	E	+	+	-	+++	61.2	1.70 ± 0.16	1.01 ± 0.22
150	E	+	+	-	+	70.5	12.80 ± 3.82	0.42 ± 0.01
175	C	+	+	-	+++	61.1	14.37 ± 4.94	0.97 ± 0.09
183	E	+	+	-	+++	63.4	1.83 ± 0.03	nd
208	E	+	+	-	++	2.4	2.34 ± 0.53	0.23 ± 0.05
210	E	+	+	-	++	0.8	1.52 ± 0.23	0.19 ± 0.04
211	E	+	-	-	++	0.2	1.93 ± 0.21	0.07 ± 0.05
217	E	+	+	-	+++	6.9	4.27 ± 0.82	0.85 ± 0.39
218	E	+	+	-	+++	62.1	5.48 ± 1.07	1.01 ± 0.16
286	E	+	+	-	+++	4.8	8.88 ± 1.05	0.80 ± 0.08
287	E	+	+	-	+++	0.8	2.80 ± 0.18	0.46 ± 0.19
288	E	+	+	-	+++	5.7	2.29 ± 0.04	0.14 ± 0.05
289	E	+	+	-	+++	2.7	2.61 ± 0.75	0.60 ± 0.31
588	C	+	+	-	+++	71.4	1.64 ± 0.21	0.99 ± 0.06
V. cholerae O1 El Tor biotype								
C	+	+	+	nd	nd	1.73 ± 0.52	1.77 ± 0.12	
E. coli DH5α								
L	nd	nd	nd	nd	nd	nd	0.03 ± 0.01	

[1] E= environmental, C= clinical, L= laboratory. [2] Determined by PCR assay (see Table 1). [3] Relative activity referred to 100% hemolysis control. [4] Results expressed as the reciprocal of the optical density at 490 nm. The mean of the control non-treated cells was 1.24 ± 0.02 OD^{-1}. [5] Fluid accumulation, expressed as the volume to length ratio (ml cm^{-1}). The results are the mean of triplicate individual experiments. nd= not determined.

suspension adjusted to 5 x 10^4 - 5 x 10^5 cfu ml^{-1} (14). Negative control loops were injected with PBS alone or with *E. coli* DH5a, while positive control loops were injected with *V. cholerae* O1 biotype El Tor. After 18-20 h the animals were killed, the loops were excised and the fluid accumulation (FA) was expressed as the ratio between volume (ml) and length (cm) of each loop. Each strain was assayed at least in three rabbits.

Pulsed-field gel electrophoresis (PFGE)

The genomic DNA was prepared in agarose plugs as previously described (3) and digested twice with 14 U of Not I per 100 µl block. The chromosomal DNA fragments were analyzed by PFGE on a CHEF DR-II apparatus (BioRad, Richmond Calif.). The electrophoresis was carried out during 24 h at 200 V with pulse times of 0.1 to 35.0 sec. *V. cholerae* O1 biotype classical and El Tor, and *V. cholerae* O139 were used as controls.

RESULTS

Identification of virulence genes by PCR

All the strains belonging to clinical or environmental sources yielded the *toxR* and *hlyA* specific amplicons, with the exception of strain 211 that was negative for *hlyA* (Table 2). As expected, none of the strains amplified the virulence genes *ctxA*, ace and zot, contained in the CTXF prophage, nor tcpA whose product is a colonization factor and acts as a receptor for the CTXF bacteriophage (Table 2) or *stn*, encoding the toxin NAG-ST (data not shown). The absence of the CTXF prophage was confirmed by Southern blot of genomic DNA using the *ctxA* PCR fragment as a probe (data not shown).

Antimicrobial susceptibility and production of protease and hemolysin

Most of the non-O1 non-O139 *V. cholerae* isolates were susceptible to all antibiotics tested, but three environmental strains were resistant to ampicillin (strains 150, 183 and 211), and one showed intermediate resistance level to streptomycin (strain 140) (data not shown).

To characterize the secreted components potentially involved in epithelial cell damage, we investigated the production of proteases and hemolysins. All the strains were positive for protease activity in 3% casein plates. For easier comparison, this activity was classified in low, intermediate and high according to the size of the halo as described in Materials and Methods (Table 2). All the strains but strain 211 produced β-hemolysis when grown on sheep blood-agar plates. However, the quantification of the hemolytic activity in the sterile supernatants showed differences between isolates. Strains were considered markedly or weakly hemolytic according to activities higher than 60% (strains 140, 150, 175, 183, 218 and 588) or lower than 10% (strains 1383, 208, 210, 211, 217, 286, 287, 288 and 289), respectively (Table 2). All heat-inactivated supernatants lost their hemolytic activity (data not shown).

Cytotoxic and enterotoxic activities

The production of cytotoxic compounds was evaluated on eukaryotic Cos-7 cells. Most of the supernatants from non-O1 non-O139 *V. cholerae* produced cell rounding and decolling of the monolayer after 2-6 h of treatment (Fig 1). This effect was also seen using other cell lines, e.g. CHO cells (data not shown). However, after 2 h of incubation some strains induced a drastic reduction in cell viability (strains 1383, 150, 175, and 286) while the others have a slightly detectable activity (Table 2). All the supernatants showed more than five fold reductions in cytotoxicity when they were filter-sterilized. This effect was probably due to protein adsorption since it was reverted using albumin-saturated filters (data not shown). To study the putative polypeptide nature of the cytotoxic compound, supernatants were concentrated and treated with trypsin, which largely diminished the cytotoxic activity (Fig 2). Also, a heat treatment of supernatants (65°C, 15 min) completely abolished this activity (Fig 2).

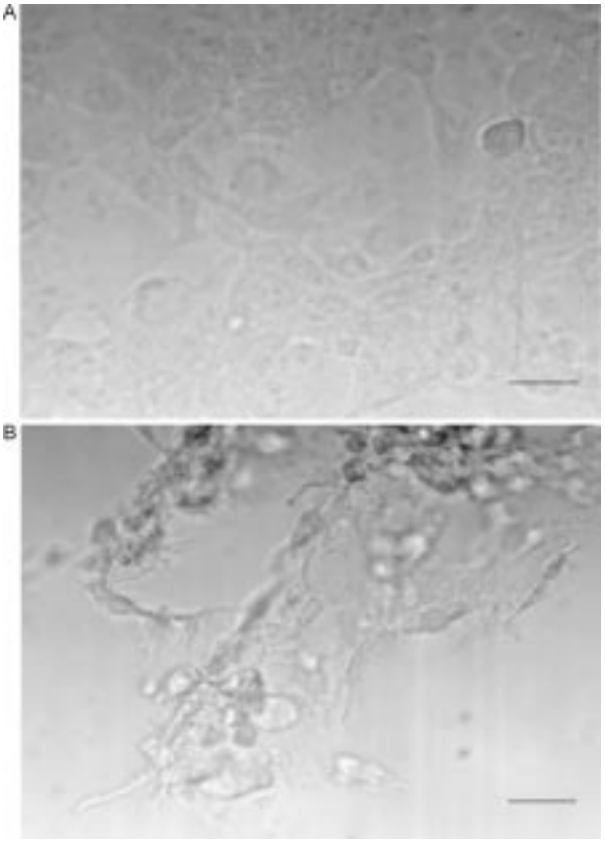

Figure 1. Cytotoxicity of non-O1 non-O139 *V. cholerae* supernatant on Cos-7 cells. Cultured cells were treated with 1/10 volume of BHI (A) or sterile supernatant from *V. cholerae* 175 (B) for 2 h and photographed under phase-contrast microscopy. Bar, 20 µm.

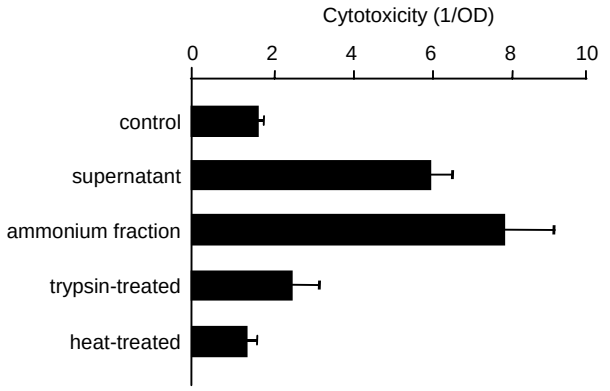

Figure 2. Effect of trypsin or heat on the cytotoxic activity. Cultured Cos-7 cells were treated with BHI (control), *V. cholerae* 175 supernatant, or different *V. cholerae* 175 supernatant fractions prepared according to Materials and Methods, and cytotoxicity was determined.

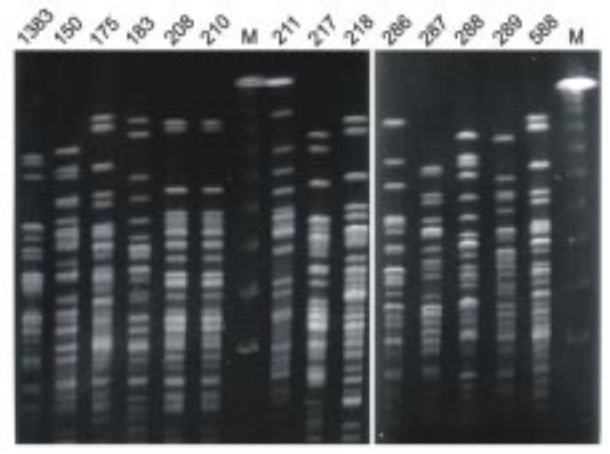

Figure 3. PFGE of non-O1 non-O139 *V. cholerae*. Genomic DNA was digested with *Not I* as described in Materials and Methods. Numbers of strains are indicated. M: Lambda DNA Ladder (BioRad).

Most of the isolates showed enterotoxic activity although the FA values indicated variations between the strains (Table 2). The difference in FA ratios between highly and lower hemolytic strains was not statistically significant ($p>0.05$). Nevertheless, highly hemolytic strains produced higher FA ratios respect to the negative control (E. coli DH5α) and lower hemolytic strains produced lower FA ratios respect to the positive control (*V. cholerae* O1), both with statistically significant differences ($p<0.05$) according to a non-parametric test (ANOVA, Kruskal-Wallis).

Genetic relationship between non-O1 non-O139 *V. cholerae* isolates

Two pairs of indistinguishable genetic patterns were observed, one for the two clinical strains (175 and 588) and the other for two environmental strains isolated in Córdoba city (208 and 210). Ten different patterns were obtained for the others isolates (Fig. 3) and one was nontypeable (strain 140, not shown). All the patterns were different from that of toxigenic *V. cholerae* O1 biotype classical and El Tor, and O139 serogroup (data not shown).

DISCUSSION

In this study we analyzed fifteen strains of non-O1 non-O139 *V. cholerae* isolated in Córdoba, Argentina, during the period 1991-1996. All the isolates lacked the *ctxA* gene encoding the A subunit of the cholera toxin. Also, the genes *ace, zot, tcpA* and *stn*, associated to pathogenicity in *V. cholerae*, were absent in these isolates. On the contrary, all the strains contained the *toxR* gene, encoding the transcriptional regulator ToxR, present in most O1 and non-O1 non-O139 *V. cholerae* strains.

All the strains analyzed were proteolytic. It has been reported that *V. cholerae* proteases interfere with the intercellular tight-junctions of cultured epithelial cells (16). Although most strains analyzed in this work were cytotoxic, some did not affect the integrity of the Cos-7 cell monolayer. All the strains except for *V. cholerae* 211 harbored the *hlyA* gene encoding the El Tor hemolysin and showed hemolytic phenotype. Nevertheless, two groups of strains were determined, with hemolytic activities higher than 60% and lower than 10%, respectively. The two clinical isolates are in the former group, suggesting a putative role for the hemolytic activity in the pathogenesis of the cholera-like disease. Hemolysins from non-O1 non-O139 *V. cholerae* have been described to have cytotoxic and cell vacuolating activity on cultured HeLa and Vero cells (4, 8). The supernatants of some non-O1 non-O139 *V. cholerae* studied in this work produced dramatic cytotoxic effect on Cos-7 cell monolayers (Table 2) although they did not produce vacuolization on the same cell line. The results suggest that a proteic compound produce the cytotoxic activity. The molecular mechanisms responsible for this activity remain to be further characterized.

Most of the isolates, including environmental strains, showed enterotoxic activity. The highly hemolytic strains are also highly enterotoxic, suggesting a putative relation between the hemolytic activity and the enterotoxic phenotype as previously described (12). Both clinical isolates were highly enterotoxic and hemolytic although they showed a significant difference in cytotoxicity. At present, the pathogenesis of non-O1 non-O139 *V. cholerae* gastroenteritis is not well understood. It is possible that a concerted action of several secreted enzymes, as demonstrated for the accessory toxins in *V. cholerae* O1 (9), may be essential for the pathogenesis of non-O1 non-O139 *V. cholerae* to cause disease in the absence of classical cholera toxin. Also, as recently suggested, non-O1 non-O139 *V. cholerae* may produce previously undiscovered colonization factors that induce fluid accumulation by unknown mechanisms (7).

The DNA profiles of non-O1 non-O139 *V. cholerae* strains indicate that both clinical isolates are closely related, suggesting a probably common origin. The isolates from water sources showed a variety of genetic profiles coexisting in this environment.

Other authors recently described the genetic diversity of *V. cholerae* O1 in Argentina (19). This is the first study documenting the potential virulent factors, as determined from cell culture and "in vivo" studies, of non-O1 non-O139 *V. cholerae* isolates from clinical and environmental sources in Argentina.

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