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Genetic Diversity of *Vibrio cholerae* O1 in Argentina and Emergence of a New Variant

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The genetic diversity of *Vibrio cholerae* O1 strains from Argentina was estimated by random amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (PFGE). Twenty-nine isolates carrying the virulence genes *ctxA*, *zot*, *ace*, and *tcpA* appeared to represent a single clone by both typing methods; while 11 strains lacking these virulence genes exhibited several heterogeneous RAPD and PFGE patterns. Among the last group, a set of isolates from the province Tucumán showed a single RAPD pattern and four closely related PFGE profiles. These strains, isolated from patients with diarrhea, did not produce the major *V. cholerae* O1 virulence determinants, yet cell supernatants of these isolates caused a heat-labile cytotoxic effect on Vero and Y-1 cells and elicited significant variations on the water flux and short-circuit current in human small intestine mounted in an Ussing chamber. All these effects were completely abolished by incubation with a specific antiserum against El Tor hemolysin, suggesting that this virulence factor was responsible for the toxic activity on both the epithelial cells and the small intestine specimens and may hence be involved in the development of diarrhea. We propose "Tucumán variant" as the designation for this new cluster of cholera toxin-negative *V. cholerae* O1 strains.

Cholera reemerged in Latin America after being absent from the continent for around 100 years. The first case occurred in Peru in 1991 and was caused by a *Vibrio cholerae* O1 biotype El Tor strain that soon spread to the rest of Latin America at the rate of one country a month (4). The disease appeared in Argentina in February 1992 and has caused seven epidemic outbreaks since then. Several studies have demonstrated that a single clone was responsible for the Latin America epidemic (3, 26, 34). However, this strain appears to have undergone genetic changes in Peru throughout the years (9), and a second strain has been associated with cholera cases occurring in Mexico and Central America (10). Among the different typing techniques, pulsed-field gel electrophoresis (PFGE) has proved to be highly discriminatory for the analysis of *V. cholerae* O1 and O139 genetic diversity. This method has been successfully used for molecular epidemiology surveillance in monitoring the dissemination of this pathogen and the emergence of new epidemic clones (1, 3, 9, 10, 27). Therefore, in this study we applied PFGE to the study of human and environmental *V. cholerae* O1 isolates referred to our laboratory from different regions of Argentina during the seven epidemic outbreaks. Additionally, we employed random amplified polymorphic DNA (RAPD) analysis as a second typing technique. This methodology has been used in genetic diversity analysis of different bacterial species, including *Escherichia coli* (22, 23, 25) and *V. cholerae* (19, 28).

The major virulence determinants normally found in epidemic *V. cholerae* O1 strains are the potent enterotoxin cholera toxin (CT), the colonization factor toxin-coregulated pilus (TCP), and the regulatory protein ToxR (12). Over the last years, it has been demonstrated that these virulence factors are encoded within the genomes of two filamentous bacteriophages, i.e., CTXϕ phage (35), which contains the *ctxAB* operon coding for CT, and VPIϕ phage (17), which carries the TCP gene cluster. Since TCP acts as the receptor of CTXϕ, acquisition and expression of this virulence determinant should precede infection by CTXϕ; however, strains lacking TCP have been transfected in vitro with CTXϕ (11), providing a possible explanation for the emergence of new epidemic *V. cholerae* strains.

On the other hand, strains lacking these pathogenicity factors have been reported with increasing frequency to be associated with diarrheal disease, causing mild-to-severe cholera-like diarrhea (30, 31; S. I. Honda, K. Shimoiriasa, A. Adachi, K. Saito, N. Asano, T. Taniguchi, T. Honda, and T. Miwatani, Letter, Lancet **ii:**1486, 1988). These strains have been historically described as nontoxigenic (NT) *V. cholerae* O1 (30, 31), meaning that they do not produce the classical cholera-associated toxins. Although the exact mechanisms by which NT *V. cholerae* O1 strains induce diarrhea have not been elucidated, the importance of this pathogen as an etiologic agent of diarrhea has become evident over the last years. As a matter of fact, several countries where an active surveillance of cholera is under way have demonstrated the emergence of genetically related clusters of NT strains that could be called *V. cholerae* O1 "variants" (6, 24, 31). In Latin America, Coelho et al. identified one of these variants, called Amazonia, which was isolated in the northern region of Brazil (6). They also demonstrated that these strains exhibited cytotoxic activity, caused

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TABLE 1. Characteristics of strains studied

Origin(s) (*n*)	Yr of isolation	Place(s) of isolation (*n*)	Serotype(s) (*n*)	Presence of CT (as determined by ELISA)	Presence of:				RAPD type (*n*)	PFGE type (*n*)
					zot	*zot*/*ace*	*ctxA*	*tcpA* (El Tor)		
Human (1)	1992	Salta (1)	Inaba (1)	+	−	−	+	+	1 (1)	1 (1)
Human (20), environment (5)	1992–1997	Salta (12), Jujuy (7), Tucumán (3), Chaco (1), Santa Fe (1), Buenos Aires (1)	Inaba (6), Ogawa (19)	+	+	+	+	+	1 (25)	1 (25)
Human (1), environment (1)	1993–1994	Jujuy (1), Salta (1)	Ogawa (2)	+	+	+	+	+	1a (2)	1 (2)
Environment (1)	1993	Salta (1)	Ogawa (1)	+	+	+	+	+	1b (1)	1 (1)
Human (4)	1994	Tucumán (4)	Inaba (2), Ogawa (2)	−	−	−	−	−	2 (4)	2a (4)
Human (1), environment (1)	1994	Tucumán (2)	Inaba (1), Ogawa (1)	−	−	−	−	−	2 (2)	2b (2)
Human (1)	1998	Tucumán (1)	Ogawa (1)	−	−	−	−	−	2 (1)	2c (1)
Human (1)	1993	Tucumán (1)	Inaba (1)	−	−	−	−	−	2 (1)	2d (1)
Environment (1)	1992	Salta (1)	Inaba (1)	−	−	−	−	−	3 (1)	NT (1)
Environment (1)	1997	Jujuy (1)	Ogawa (1)	−	−	−	−	−	4 (1)	3 (1)
Human (1)	1998	Santiago del Estero (1)	Inaba (1)	−	−	−	−	−	5 (1)	4 (1)

by El Tor hemolysin of *V. cholerae*, which evoked a unique vacuolating effect on Vero cells (7). This effect of El Tor hemolysin was also observed in a group of isolates of *V. cholerae* non-O1 recovered in India (21), suggesting that this virulence factor may play an important role in the development of diarrhea, especially in strains lacking other well-defined toxins, e.g., CT.

In the present study we identified a new cluster of *V. cholerae* O1, isolated from patients with diarrhea in Argentina, that was genetically distinct from the Latin America epidemic clone and from the Amazonia variant. These isolates did not produce CT, but they exhibited cytotoxic and enterotoxic activity, which could be attributed to El Tor hemolysin.

MATERIALS AND METHODS

Strains and culture conditions. A total of 40 strains of *V. cholerae* O1 biotype El Tor isolated from stools of infected individuals ($n = 30$) and from environmental surface waters ($n = 10$) were analyzed in this study. This group included strains from the seven epidemic outbreaks and the different locations affected in Argentina. They were kept at −70°C in the culture collection maintained at the Instituto Nacional de Enfermedades Infecciosas, ANLIS "Dr. Carlos G. Malbrán." The strains were isolated in the northwestern provinces of Salta, Jujuy, Chaco, Santiago del Estero, and Tucumán and in the central provinces of Buenos Aires, San Juan, and Santa Fe. The characteristics of these isolates are detailed in Table 1. Strain 3439, belonging to the Amazonia variant, was kindly provided by João Andrade, Serviço de Microbiologia e Immunologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil.

Detection of virulence factors. (i) Production of CT. The expression of CT was detected in filtered culture supernatants by enzyme-linked immunosorbent assay (ELISA) using GM_1 ganglioside-coated plates as previously described (29). The toxin bound to this receptor was detected with polyclonal anti-CT serum, peroxidase-conjugated anti-rabbit IgG, and H_2O_2–*o*-phenylenediamine as a chromogenic substrate.

(ii) Virulence-associated genes. The genes coding for CT, Zot, and Ace (*ctxA*, *zot*, and *ace*) were detected by colony blotting using specific probes, labeled with dUTP-digoxigenin. The probes were kindly provided by James Kaper and James Nataro, Center for Vaccine Development, University of Maryland, Baltimore. They consisted of an *Eco*RI 540-bp fragment of pCVD27, corresponding to the *ctxA* gene; an *Xba*I-*Cla*I 500-bp fragment of pBB241 corresponding to *zot*; and a *Cla*I 300-bp segment of pCVD628 containing *ace* and *zot* sequences. The colony blot hybridization assay was performed under high-stringency conditions on nylon membranes (20).

The strains showing negative results for *ctxA* by colony blot hybridization were also tested by a highly sensitive heminested PCR to confirm the absence of the *ctxAB* operon. Aliquots of 200 μl of stationary-phase cultures were centrifuged, suspended in 900 μl of distilled water, boiled for 10 min, and used as the template source (approximately 8 to 10 μg of DNA/ml) for the amplification reaction. The assay was performed as previously described (33), using primers 5′ GTGGGAATGCTCCAAGATCAAATCG 3′ (external forward) and 5′ ATTG CGGCAATCGCATGAGGCGT 3′ (external reverse) for the first reaction and primer 5′ GATATGCAATCCTCAGGGTATCC 3′ (internal), along with the external forward primer, for the second-round PCR.

The *tcpA* gene, coding for the TCP was detected by a multiplex assay, using specific primers for El Tor and Classical sequences, as previously described (32). The oligonucleotides 5′ GAAGAAGTTTGTAAAAGAAGAACAC 3′ and 5′ G AAAGGACCTTCTTTCACGTTG 3′ were used as primers for the El Tor fragment of 471 bp; 5′ CACGATAAGAAAACCGGTCAAGAG 3′ and 5′ ACCAA ATGCAACGCCGAATGGAG 3′ were used as primers for the Classical fragment of 617 bp.

Genetic diversity. (i) RAPD analysis. DNA templates were prepared as described above. PCR was carried out as previously described using primer 258 (5′ AGCCAGTTTC 3′) (23). Reaction products were analyzed by electrophoresis on 1.2% agarose (Gibco-BRL, Grand Island, N.Y.). RAPD profiles were defined on the basis of those bands that were consistently detected in different amplification reactions.

(ii) PFGE. PFGE was performed as described by Mahaligam et al. (18), with some modifications. Briefly, DNA was prepared in a solid SeaKem agarose plug (BioWhittaker Molecular Applications, Rockland, Maine) for digestion with 25 U of restriction enzyme *Not*I. PFGE was carried out in a CHEF-DR III electrophoresis chamber (Bio-Rad, Richmond, Calif.) in 1% PFGE-certified agarose gels (Bio-Rad) at 6 V/cm with the following pulse times: 6 s (8 h), 15 s (10 h), and 25 s (11 h) in 0.5× Tris-borate-EDTA electrophoresis buffer.

(iii) Data analysis. The relatedness among RAPD or PFGE patterns was estimated by the proportion of shared bands by applying the Jaccard coefficient (16). Data recording and calculations were performed using RAPDistance programs (version 1.04; J. Armstrong, A. Gibbs, R. Peakall, and G. Weiller, Australian National University, Canberra). The resulting matrixes of pairwise distances were used to generate phenograms based on the unweighted pair-group method using arithmetic averages (UPGMA) included in the MEGA (Molecular Evolutionary Genetics Analysis) software (version 1.02; S. Kumar, K. Tamura, and M. Nei, The Pennsylvania State University, University Park).

Toxicity assays. The NT *V. cholerae* O1 strains isolated in Tucumán were further characterized by toxicity assays. Bacterial cultures grown in AKI medium (15) at 37°C for 18 h with shaking (120 rpm) were centrifuged. The supernatants were filtered through 0.22-μm-pore-size membranes and assayed for toxicity in the suckling mouse model, on cell cultures, and in human small intestine tissue. An autochthonous CT-producing strain (ST10568/95) was used as the control.

(i) Suckling mouse assay. A 100-μl volume of filtered supernatants was inoculated intragastrically into 2- to 3-day-old BALB/c mice. After 3 h at 28°C, the

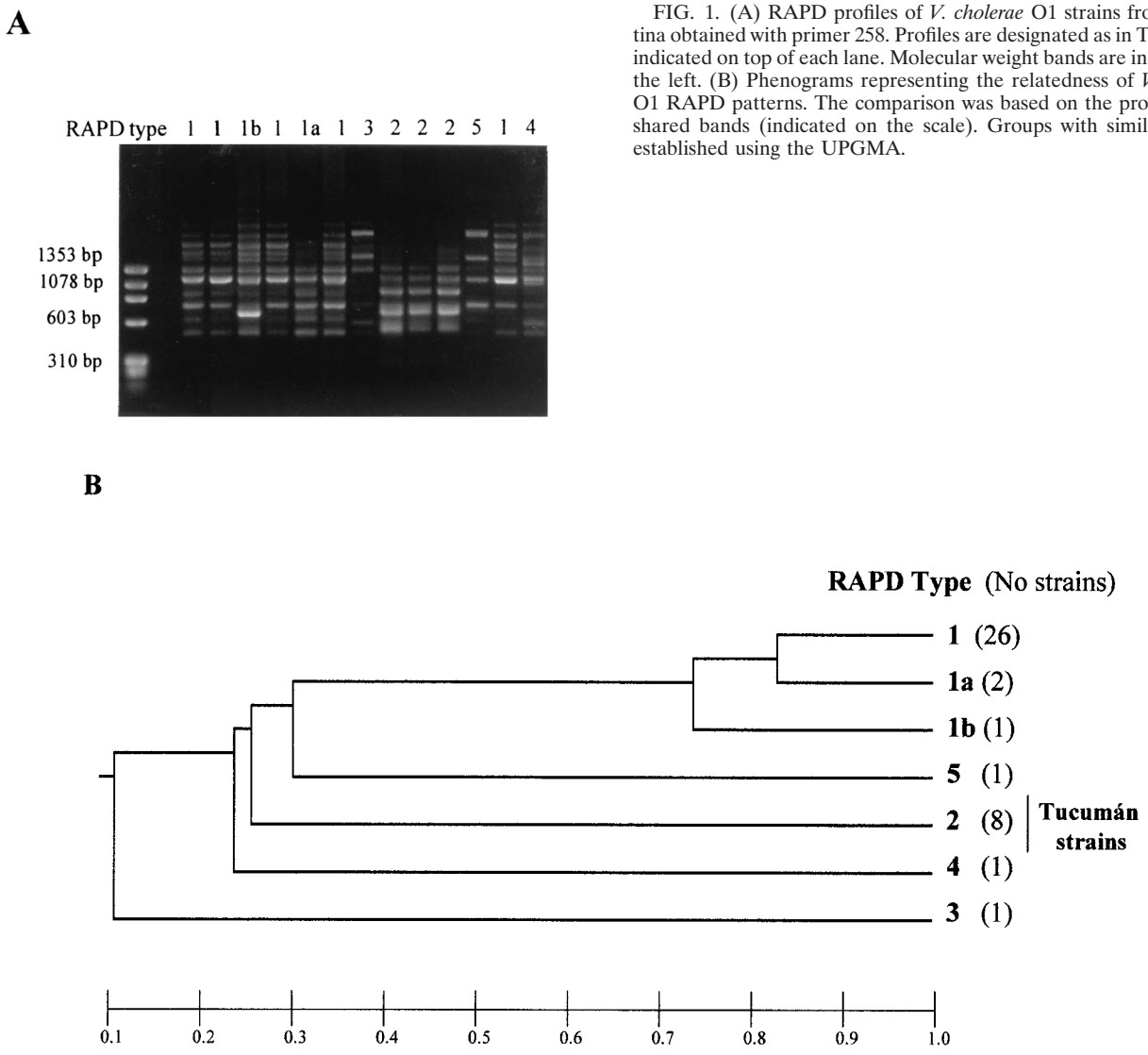

FIG. 1. (A) RAPD profiles of *V. cholerae* O1 strains from Argentina obtained with primer 258. Profiles are designated as in Table 1 and indicated on top of each lane. Molecular weight bands are indicated on the left. (B) Phenograms representing the relatedness of *V. cholerae* O1 RAPD patterns. The comparison was based on the proportion of shared bands (indicated on the scale). Groups with similarity were established using the UPGMA.

animals were killed and the fluid accumulation was calculated as the ratio of the weight of the whole intestine to that of the rest of the body (13). The assay was considered positive if this ratio was ≥0.08.

(ii) Cytotoxicity assay and inhibition with anti-El Tor hemolysin antiserum. Supernatants were tested on Vero cells and Y-1 mouse adrenal cells grown in 96-well flat-bottom multiplates, following the conditions recommended by the American Type Culture Collection (R. Hay, J. Caputo, T. R. Chen, M. Marvin, P. McClintock, and Y. Reid [ed.], American Type Culture Collection cell lines and hybridomas, 8th ed., p. 46-48, American Type Culture Collection, Rockville, Md., 1994). A total of 200 μl of the supernatants was applied in each well, and morphological changes in the cells were recorded after 3, 5, and 20 h of incubation at 37°C in a 5% CO_2 atmosphere. The assay was repeated after heat treatment of the supernatants for 5 min at 100°C. For the toxicity inhibition assay, culture supernatants were mixed with equal volumes of serially diluted anti-El Tor hemolysin antiserum (5) and incubated for 30 min at room temperature prior to the cytotoxicity assay on Vero cells.

(iii) Functional tests using human small intestine. Fragments of human jejunum were obtained from adult patients (informed consent was given) suffering from stomach cancer. After ablation, the mucosa and submucosa layers were dissected from macroscopically unaffected regions of the small intestine and mounted as a diaphragm in a modified Ussing chamber (0.94 cm^2). The tissue was immediately bathed with Ringer solution (concentrations are millimolar: 114 NaCl, 4.5 KCl, 25 NaHCO3, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 1.2 K_2HPO_4, 0.2 KH_2PO_4, 25 glucose), maintained at 37°C, and bubbled with carbogen. Transepithelial net water movement (Jw) was monitored automatically in a modified Ussing chamber connected to a special device (2). In the other chamber, the spontaneous potential difference and short circuit current (Isc) were recorded by an automatic voltage clamp system. Once the tissue reached steady values, 250 μl of filtered supernatants of *V. cholerae* O1 strains (CT^+ and CT^-) or noninoculated medium (control) were added to the mucosal bath (time zero), whereas Jw and Isc were continuously measured for 30 min. Because of tissue variability, data are presented as ΔJw (Jw at time − Jw at time zero), and ΔIsc (Isc at time − Isc at time zero). For the inhibition test, 200 μl of each culture supernatant was mixed with an equal volume of antihemolysin antiserum (5) and incubated for 30 min at room temperature prior to the enterotoxicity assay in the Ussing chamber.

RESULTS

Virulence factors of *V. cholerae* O1 strains. A total of 2,042 isolates of *V. cholerae* O1 have been submitted to our institute, the National Reference Laboratory, since 1992; of these, 1,947 were of human origin and 95 were from environmental waters. The strains were phenotypically characterized by both serotyping and biochemical tests. A total of 40 isolates was selected for this study, including human and environmental strains of both

FIG. 2. Map showing the province of Tucumán (1), where the new variant of NT *V. cholerae* O1 isolates was recovered, and the rest of the provinces mentioned in Table 1: Jujuy (2), Salta (3), Chaco (4), Santiago del Estero (5), Santa Fe (6), and Buenos Aires (7).

serotypes Inaba and Ogawa, isolated in different regions during the seven outbreaks. Twenty-eight (70.0%) strains carried the virulence-associated genes *ctxA*, *zot*, *ace*, and *tcpA* (Table 1), while the remaining isolates were negative for all the virulence factors analyzed. Only one *V. cholerae* O1 Inaba strain (ST19/92) of human origin, recovered in Salta during the first outbreak in 1992, harbored the genes *ctxA* and *tcpA* but lacked those coding for Zot and Ace. All the *ctxA*-positive strains carried the *tcpA* gene corresponding to El Tor sequence, and all but one expressed CT as shown by ELISA, with the exception of one *V. cholerae* Inaba strain (CH3/94), isolated from a patient with cholera during the outbreak in 1994, that did not produce the toxin although it had the corresponding gene (Table 1).

Genetic diversity. Primer 258 was selected from a total of six oligonucleotides evaluated for RAPD analysis-PCR, because it showed the highest discriminatory power. Using this primer, the 40 *V. cholerae* O1 strains studied gave rise to seven different electrophoretic patterns that had a total of 30 bands ranging from 400 to 1,500 bp (Fig. 1A). All the *ctxA*-positive strains appeared to be closely related, exhibiting only three band profiles. RAPD type l was the most prevalent, including 26 isolates of human and environmental origin, isolated during the seven outbreaks and belonging to both serotypes Inaba and Ogawa. Type 1a (two strains) and 1b (one strain) also included toxigenic isolates that differed from type 1 in two bands and one band, respectively. The NT strains ($n = 11$) were more heterogeneous, giving rise to four distinct band patterns. However, it was noteworthy that eight of the NT strains, isolated in the province of Tucumán (Fig. 2) between 1993 and 1998, presented a single band profile (type 2) which shared only 27% of the bands with RAPD type 1 (Fig. 1B). Type 4 comprised one Ogawa strain of environmental origin, while RAPD types 3 and 5 consisted of one isolate from the environment and one of human origin, respectively, that exhibited unique band patterns, sharing less than 32% of the bands with the rest of the electrophoretic types (Fig. 1B).

When analyzed by *Not*I PFGE, all the *ctxA*-positive strains exhibited a single band pattern, PFGE type 1 (Fig. 3A), in accord with the results obtained by RAPD analysis. On the other hand, the eight NT *V. cholerae* O1 strains isolated in the province of Tucumán that showed a distinct RAPD type (type 2) also appeared to be closely related by PFGE, giving rise to four similar band profiles (types 2a, 2b, 2c, and 2d). All these

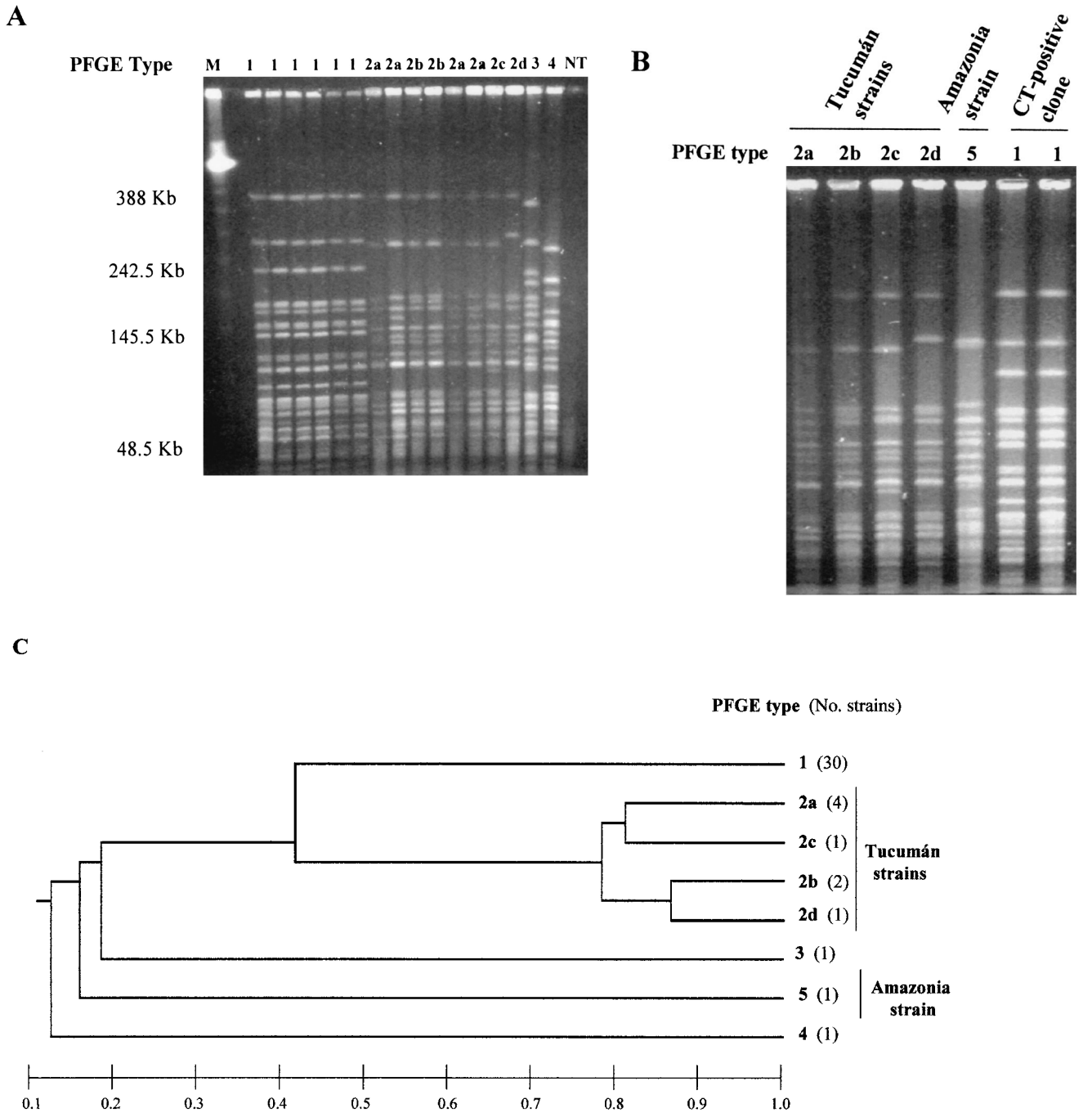

FIG. 3. (A) PFGE profiles of *V. cholerae* O1 strains from Argentina obtained by digestion of chromosomal DNA with *Not*I. Profiles are designated according to Table 1 and are indicated on top of each lane. Molecular weight markers are indicated on the left. (B) PFGE profiles of *V. cholerae* O1 strains from Argentina and from the Amazonia variant strain 3439. (C) Phenograms representing the relatedness of *V. cholerae* O1 PFGE patterns. The comparison was based on the proportion of shared bands (indicated on the scale). Groups of similarity were established using the UPGMA.

PFGE types differed from the prevalent PFGE type 1; they lacked six DNA fragments and had six extra bands of different sizes (Fig. 3A).

In order to determine whether the group of strains from Tucumán were genetically related to the isolates belonging to the Amazonia variant, recently identified in Brazil, we compared representative strains from both locations by PFGE. As shown in Fig. 3B, Amazonia strain 3439 exhibited a distinct electrophoretic pattern, different from those of the strains from Tucumán and from the CT-positive isolate pattern 1.

The strains from Tucumán appeared in a separate branch of the phenogram, with more than 78% of bands in common (Fig. 3C). This group of isolates shared less than 42% of the bands with the rest of the PFGE profiles and only

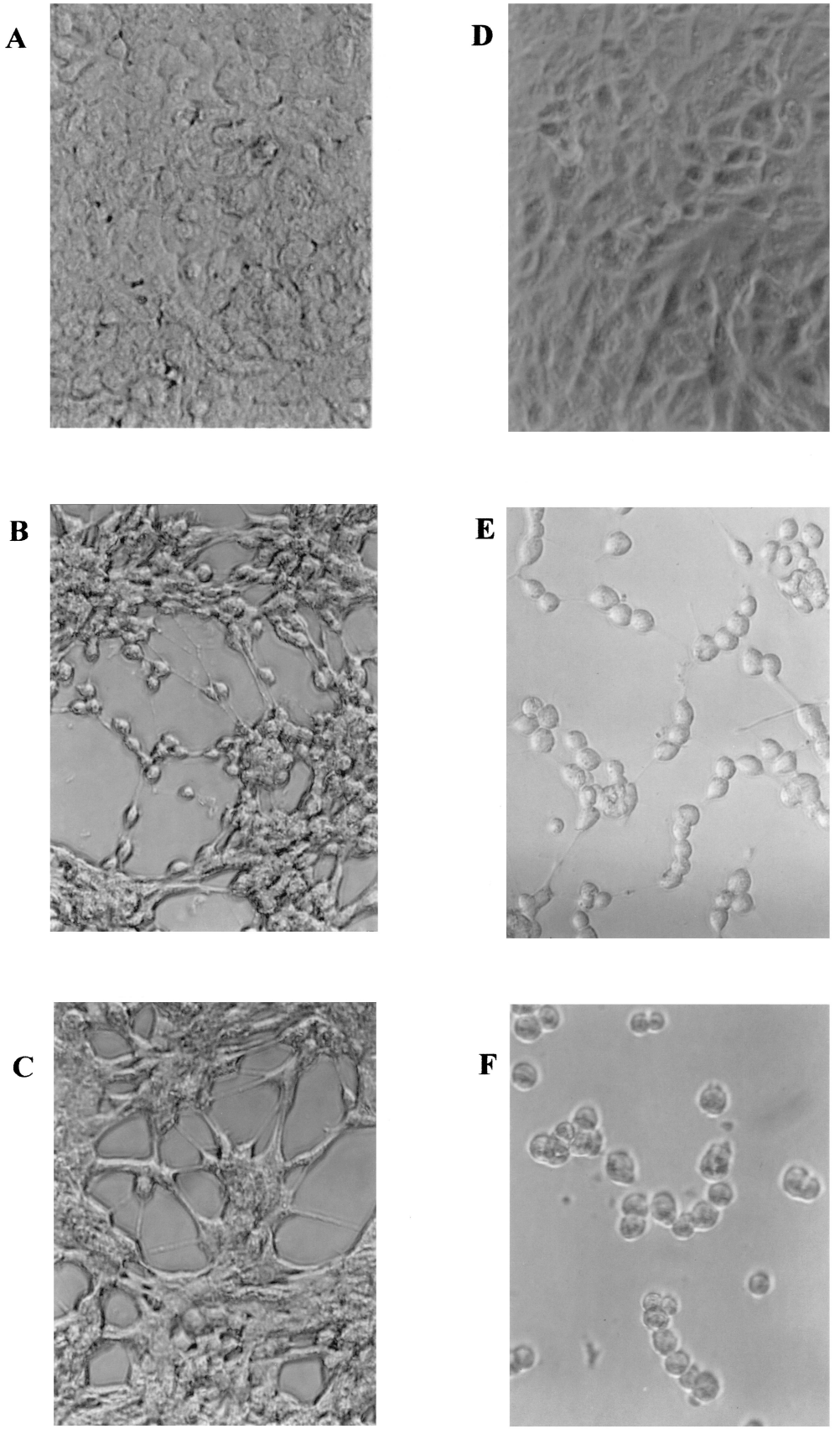

FIG. 4. Normal Y-1 adrenal cells (A) and cytotoxic effect evoked by culture supernatant of the CT producing strain of *V. cholerae* O1 ST10568/95 (B) and the CT-negative strain T12550/94 (C). Also shown are normal Vero cells (D), cytotoxicity elicited by culture supernatant of the CT-producing strain of *V. cholerae* O1 ST10568/95 (E), and the CT-negative strain T12550/94 (F). Cells are unstained, and light microscopy was used with no phase contrast (approximate magnification, ×84).

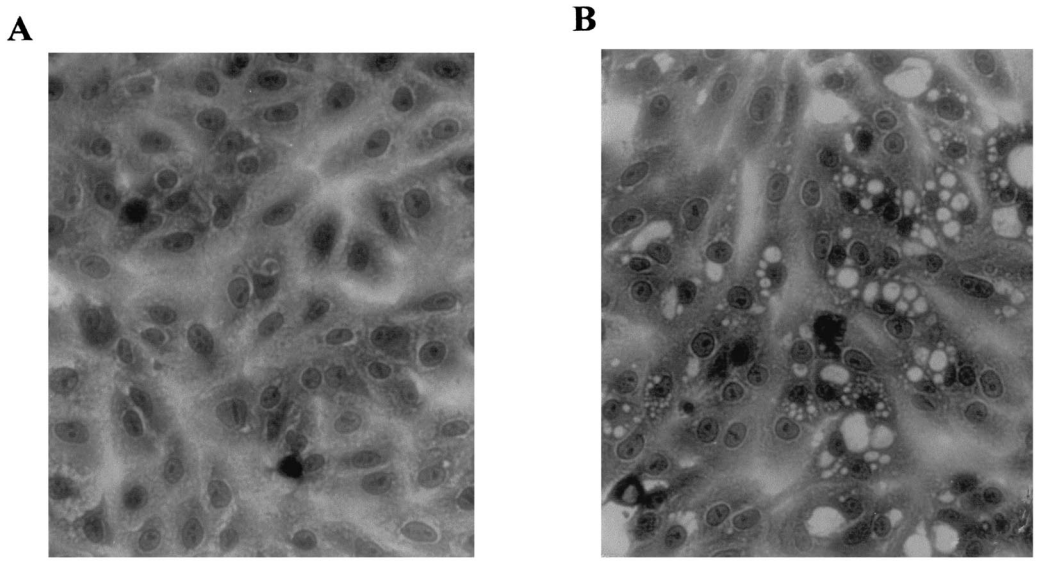

FIG. 5. Normal Vero cells (A) and vacuolization effect evoked by the culture supernatant of Tucumán T12550/94 strain (B). Cells were fixed with methanol and stained with Giemsa (approximate magnification, ×170).

17.2% with the PFGE pattern of the Amazonia strain (Fig. 3C). Thus, PFGE could discriminate among the Tucumán isolates, but it confirmed the clonal relatedness revealed by RAPD analysis.

Strain S1W/92, of RAPD type 3, could not be typed by PFGE due to endonuclease activity (Fig. 3A). In agreement with the results obtained by RAPD analysis, the NT strain SE717/98, isolated from a child with severe diarrhea, exhibited a unique PFGE band pattern (pattern 4), as did the environmental strain J254W/97 (pattern 3).

New variant of pathogenic *V. cholerae* O1. The group of eight *V. cholerae* O1 strains isolated in the province of Tucumán (Fig. 2), showing fingerprints different from those of the *ctx*-positive strains isolated in Argentina, comprised seven clinical and one environmental isolates. *V. cholerae* O1 was the only pathogen isolated from six of the diarrhea patients, while in the remaining case, strain T522/98 was recovered along with *Shigella* spp. As was observed among the epidemic isolates, the new variant strains reacted as typical *V. cholerae* representatives in biochemical tests, and most of them were susceptible to antimicrobial agents, except for T13074/94 (resistant to streptomycin) and T5957/93 (resistant to trimethoprim-sulfamethoxazole, sulfisoxazole, and vibriostatic O129). Biotyping, hemolysis, and hemagglutination tests showed that these strains belong to the El Tor biotype; i.e., they were Voges-Proskauer positive, were hemolytic for sheep and horse erythrocytes, and agglutinated chicken erythrocytes. However, unlike the epidemic strains and other typical El Tor strains, the isolates from Tucumán were sensitive to polymyxin.

None of these strains harbored the genes coding for the cholera-associated factors CT, Zot, Ace, and TCP, yet they were able to cause diarrhea, suggesting they might produce other virulence factors involved in the development of the disease. To evaluate whether the NT *V. cholerae* O1 strains from Tucumán secreted any extracellular product showing toxic effect on epithelial cells, filtered supernatants of these strains were tested on confluent Vero and Y-1 cell monolayers. All the supernatants evoked morphological changes and cell death on both cell lines when grown in AKI medium. After 5 h of incubation, Y-1 cells were irregularly shaped, with bulging projections, and the cell monolayer was disrupted, although the cells did not appear rounded as did those treated with a CT-producing strain supernatant (Fig. 4A to C). Vero cells were damaged and completely detached by the effect of the Tucumán strains, while the CT-positive strain supernatant evoked disruption of the monolayer, with cells remaining attached to the plastic wells (Fig. 4D to F). The cytotoxic effects of all strain supernatants on Vero and Y-1 cells were abolished after heat treatment at 100°C for 10 min. When tested in the suckling mouse model, none of the filtered cultures caused fluid accumulation, exhibiting ratios of ≤ 0.06.

El Tor hemolysin is responsible for the cytotoxic effects of the Tucumán strains. Cytotoxic effects similar to those evoked by the Tucumán strains had been recently described in the Amazonia variant by Coehlo et al. (6, 7), who showed that such cytotoxicity was very likely related to a vacuolating activity of the El Tor hemolysin. In light of these findings, we decided to repeat the toxicity assay on Vero cells using dilutions of the supernatants, which allowed us to observe the same kind of vacuolization effect (Fig. 5). Furthermore, we performed an inhibition assay, using a polyclonal antiserum specific for El Tor hemolysin (5). Incubation with this antiserum completely abolished the toxic effects (both vacuolization and cell death) caused by the NT strains from Tucumán on Vero cells. Moreover, as determined by the titers of antihemolysin antiserum required for neutralization, some of the strains appeared to produce a higher amount of El Tor hemolysin (for instance, T777/94, 1:100; T12550/94, 1:1,000). These results were in agreement with differences observed in the cytotoxic effects, which were more severe in the strains producing more hemolysin.

In contrast, when the CT-positive strain ST10568/95 was pre-

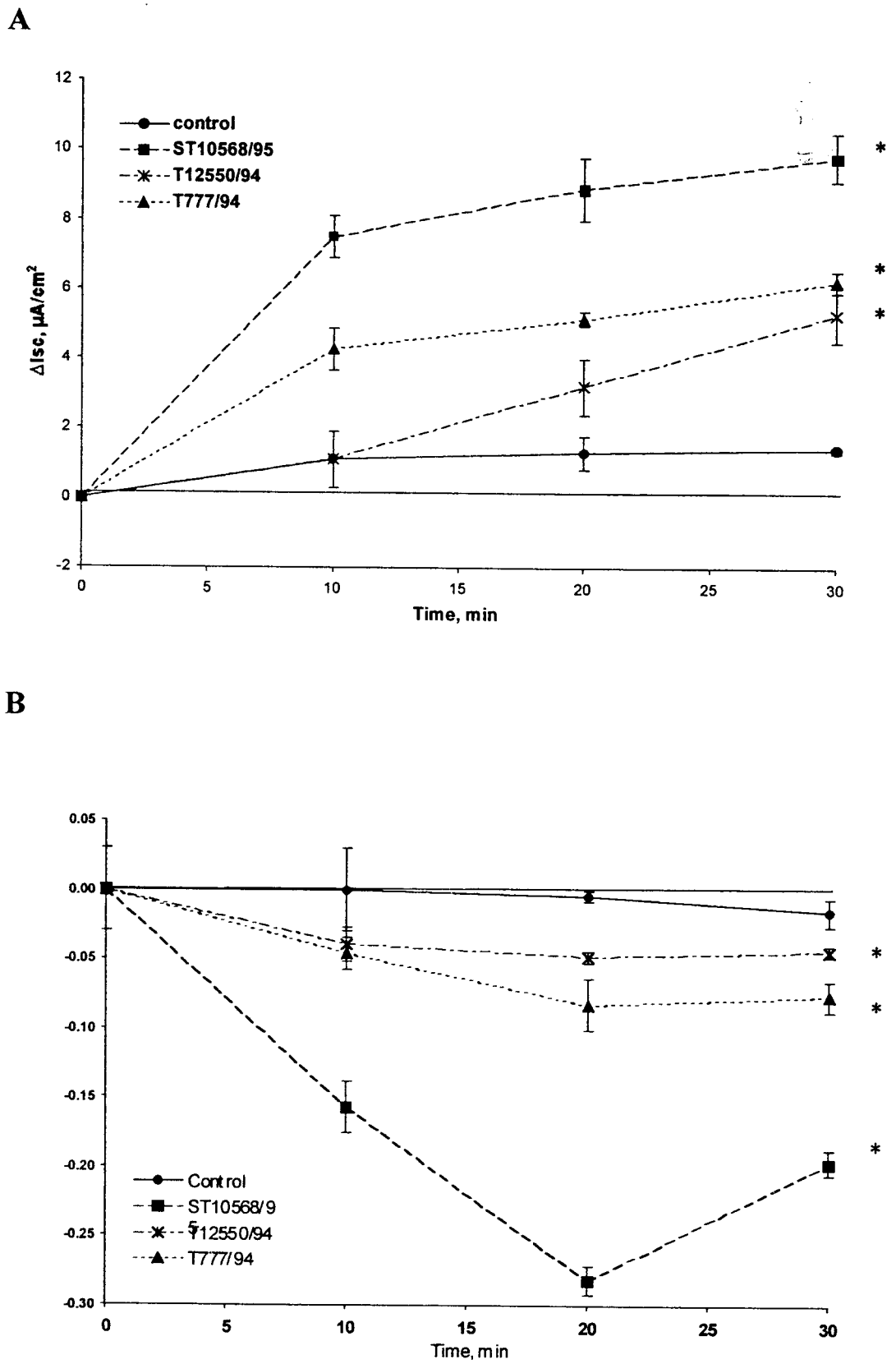

FIG. 6. Effect of CT-positive (ST10568/95) and CT-negative (T12550/94 and T777/94) *V. cholerae* culture supernatants on Isc (A) and Jw (B) measured in human small intestine. Values are means for at least two experiments at each time point. Error bars show ±1 standard error. Time zero is the time at which culture supernatant was added to the mucosal bath (*, $P < 0.05$ compared with the control).

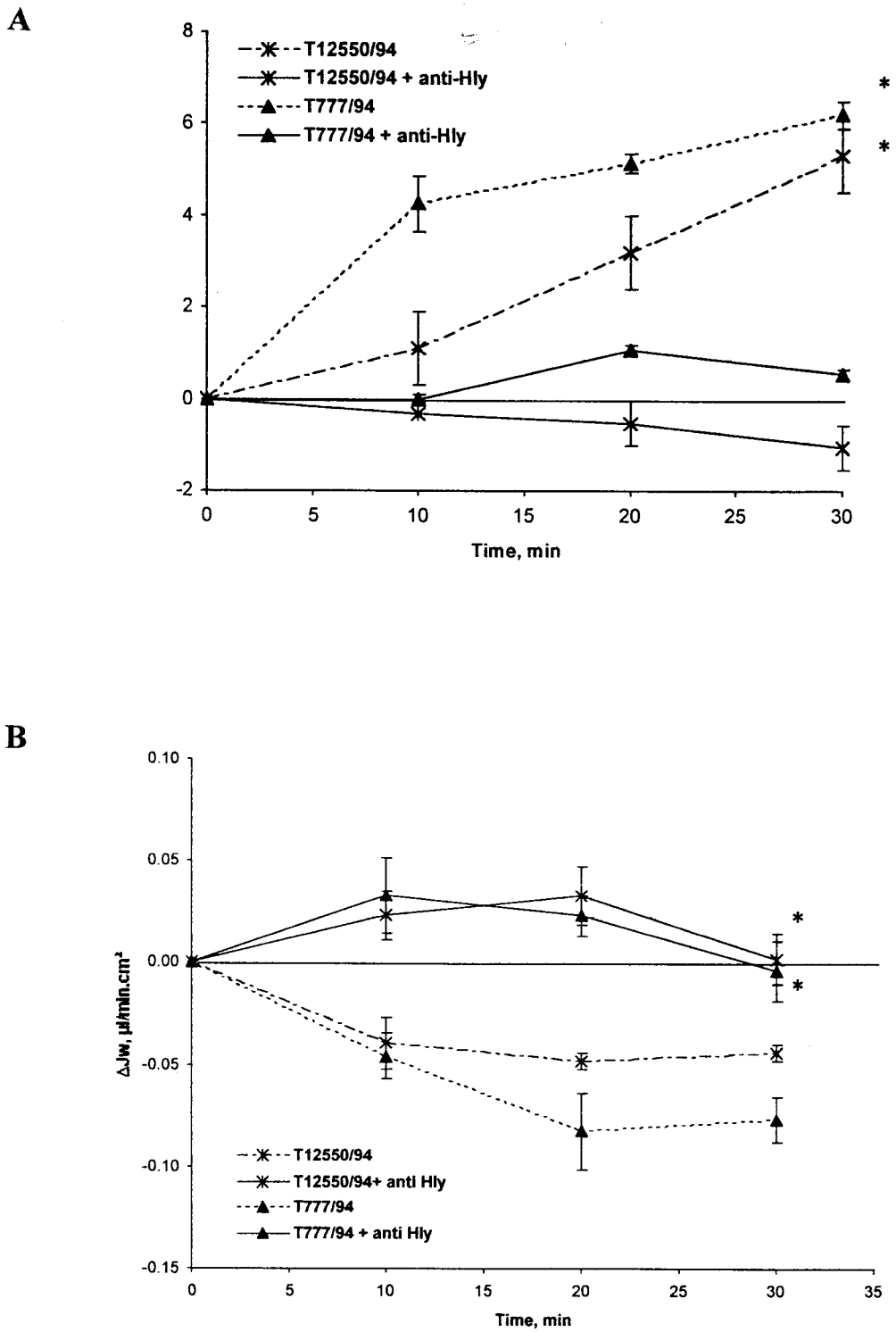

FIG. 7. Inhibition of Isc (A) and Jw (B) effects by anti-El Tor hemolysin antiserum. Tucumán *V. cholerae* strain supernatants were preincubated with equal volumes of antiserum diluted 1:100 for 30 min at 37°C. Values are means for at least two experiments. Error bars show results ± 1 standard error (*, $P < 0.05$, comparing each strain antigen-antibody complex with the corresponding supernatant effect).

incubated with the same antihemolysin antiserum, cytotoxic effects were still observed, probably due to the action of CT.

El Tor hemolysin effects in Ussing chamber. In order to evaluate the effects on water flux and ion transport in the human intestine, we examined the filtered cultures of CT-negative *V. cholerae* strains T12550/94 and T777/94 from Tucumán (showing moderate and severe effects on cell lines, respectively) on small intestine mucosa in an Ussing chamber.

The effects caused by these strain supernatants and by that of CT-positive strain ST10568/95 are shown in Fig. 6. Under basal conditions, a net absorptive Jw (0.21 ± 0.03 μl min^{-1} cm^{-2}) and potential difference (3.0 ± 1.3 mV) were observed when the human small intestine was placed between two identical Ringer solutions in the Ussing chamber. Addition of culture supernatant from the CT-positive strain to the mucosal side resulted in a significant increase of Isc (Fig. 6A) and concomitant decrease in the Jw (Fig. 6B). Tissues exposed to the NT strains T12550/94 and T777/94 showed significant inhibition variations of Isc and Jw ($P < 0.05$) (Fig. 6A and 6B, respectively) at 30 min of incubation compared with the control. As observed on Vero cells, preincubation with anti-El Tor hemolysin-specific serum completely abolished the toxic effects caused by strains T12550/94 and T777/94 on the human small intestine mounted in the Ussing chamber (Fig. 7). Further, we have observed that purified *V. cholerae* O1 El Tor hemolysin is able to produce a significant Jw inhibition ($\Delta Jw = -0.062 \pm 0.002$, $n = 4$, $P < 0.001$) and Isc stimulation ($\Delta Isc = +4.3 \pm 0.2$, $n = 4$, $P < 0.001$) when human small intestine was incubated for 30 min with a toxin concentration of 4.6 μg/ml (data not shown).

DISCUSSION

The present study constitutes the first report on the genetic diversity of *V. cholerae* in Argentina. The use of molecular epidemiology techniques allowed us to identify a new clonal cluster of *V. cholerae* O1, isolated in the northern province of Tucumán. As has been reported in several studies (3, 9, 18), we found that PFGE performed with restriction enzyme *Not*I was highly discriminating and reproducible for the genetic diversity analysis of *V. cholerae* O1. Additionally, we used RAPD analysis, which also turned out to be a valuable tool for this analysis. Although RAPD analysis was not as reproducible as PFGE (some of the bands did not consistently appear), the results obtained by both techniques were in agreement. Furthermore, RAPD analysis allowed the characterization of one strain (RAPD type 3 [Fig. 1A]) that could not be studied by PFGE due to endonuclease activity (Fig. 3A, nontypeable). The results of PFGE and RAPD analysis showed that all the *ctxA*-positive strains of *V. cholerae* O1 belong to a single clonal cluster, which includes Inaba and Ogawa isolates of human and environmental origin, recovered during the seven cholera outbreaks in Argentina. In agreement with the observation of Dalgsgaard et al., who studied the *V. cholerae* O1 strains isolated in Peru (9), our results support the hypothesis that there is a single epidemic strain in Latin America that reemerges from an environmental source after interepidemic periods. This strain probably remains in the environment in the state of viable but nonculturable forms (8). Thus, the *ctxA*-positive strains exhibited only three closely related RAPD subtypes (subtypes 1, 1a, and 1b), and a single PFGE pattern (pattern 1) which appeared to be identical to PFGE type P1 described by Dalgsgaard et al. (9) and PFGE type 38 described by Evins et al. (10).

In contrast, the NT *V. cholerae* O1 strains were more heterogeneous, giving rise to several distinct PFGE and RAPD types. Among these NT strains, the identification of a cluster of isolates from the province of Tucumán (Fig. 2) that exhibited a distinct RAPD type and four highly related PFGE patterns was noteworthy. Except for one isolate from the environment, all the isolates belonging to this cluster were recovered from patients suffering moderate-to-severe diarrhea, who lived in different villages near the Sali river, in the province of Tucumán. None of these isolates harbored the genes coding for the CT, yet they caused deleterious effects on both Vero and Y-1 cells. As deduced from the results of the cytotoxicity inhibition assay on Vero cells, El Tor hemolysin was responsible for the cytotoxic activity. Furthermore, the diluted culture supernatants of the Tucumán strains caused a vacuolization effect similar to that described recently in association with El Tor hemolysin (7, 21). This effect was also abolished by incubation with the corresponding antiserum. Like other hemolysins, El Tor hemolysin has been historically described as a pore-forming toxin (14, 37), and its effect has been demonstrated on erythrocytes and epithelial cells (38, 40). Although its role in diarrhea has been repeatedly suggested (7, 21, 36, 40), there is only one report demonstrating cytolethal activity of El Tor hemolysin on human intestinal cells up to now (39). Here, by means of the experiments conducted in the Ussing chamber, we went on to prove that the deleterious effects introduced in the cells actually induce an inhibition of water absorption concomitant with an increase in Isc in the human small intestine. These effects were inhibited by incubation with anti-El Tor hemolysin serum. Thus, the results observed both in Vero cells and in the human small intestine, as well as the effects showed by the purified El Tor hemolysin, support the idea that this virulence factor might be responsible for the development of diarrhea due to the strains from Tucumán.

Taking into account the fact that the NT *V. cholerae* O1 strains from the province of Tucumán that we identified in the present study were genetically different from the CT-positive clone and from the Amazonia variant identified in Brazil, we propose "Tucumán variant" as the designation for this newly identified group of *V. cholerae* O1 isolates.

Our findings stress the need to monitor the emergence and prevalence of NT strains, which not only are a source of morbidity as agents of sporadic disease but also represent a threat, since they might acquire the prophages VPIϕ and CTXϕ (11) and become potentially epidemic. Also, our results provide further evidence that El Tor hemolysin might be implicated in the disease caused by NT *V. cholerae* O1.

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