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Diarrheagenicity evaluation of attenuated *Vibrio cholerae* O1 and O139 strains in the human intestine ex vivo

J.M. Burgos[a], J.L. Perez[b], L. Garcia[b], G.S. Gonzalez[b], J.A. Benitez[c], F. Galindo[d], C. Silberstein[a], C. Ibarra[a,*]

[a]*Laboratorio de Fisiopatogenia, Departamento de Fisiología, Facultad de Medicina, Universidad de Buenos Aires, Argentina*
[b]*Instituto Finlay, Sueros y Vacunas, Havana, Cuba*
[c]*Grupo de Genética Molecular del Centro Nacional de Investigaciones Científicas, Havana, Cuba*
[d]*Hospital Nacional de Gastroenterología "Dr B. Udaondo", Buenos Aires, Argentina*

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Abstract

The recent spread of El Tor cholera in Latin America highlights the need for a safe and economical vaccine. The main approach for developing live recombinant vaccines has been to disarm known pathogenic strains of cholera toxin leaving intact antigens involved in protection. These recombinant vaccine candidates do not cause severe diarrhea, but they are too reactogenic for wide scale usage. We describe here a test capable of determining the diarrheagenic potential of attenuated *V. cholerae* strains. The functional test consists in the simultaneous recording of net water movement, electrical potential difference and short-circuit current across the human intestine ex vivo. We found that human tissues incubated with supernatants from the attenuated 638, 413 and 251a *V. cholerae* strains caused no changes in the ion conductances and water absorption in ileal and colon tissues allowing them to be assayed in volunteers.

Keywords: Vibrio cholerae; Water permeability; Short circuit current; Human intestine; Vaccine development

1. Introduction

Cholera constitutes an emergent concern for human health in many regions of the world. The outbreak of cholera epidemics in Latin America [1,2] and Southeast Asia [3] show that this illness continues to be a serious problem. The severe watery diarrhea is caused mainly by a polypeptide enterotoxin, cholera toxin (CT), secreted by *V. cholerae* O1 [4]. According to antigen O serotyping, more than 155 serogroups of *V. cholerae* have been identified. However, before 1992, only the O1 serogroup had been associated with epidemic and pandemic cholera [5]. O1 strains can be divided in two biotypes, namely classical and El Tor, with three different antigenic serotypes, Inaba, Ogawa and, in a much minor proportion Hikojima [6]. In October of 1992, a non-01 toxigenic strain of *V. cholerae* that caused an illness indistinguishable from cholera produced by the serogroup O1 was isolated for first time. This strain, subsequently designated *V. cholerae* O139, has had a high incidence in both adults and children in areas where cholera is endemic [7], and was isolated from a 2-year-old child with cholera-like diarrhea in Argentina [8]. It has been suggested that there is little if any pre-existing immunity against O139 resulting from natural exposure to *V. cholerae* O1 and that these cases reflect the beginning of the eighth cholera pandemic [5,8].

In humans, with either V cholerae O1 or O139, infection results from ingestion of the organism usually in contaminated food and water. Depending on the size of the inoculum and the susceptibility of the person, the incubation period for *V. cholerae* O1 infection can be as short as 12 h, and as long as 72 h [9]. This may be due in part to the fact that *V. cholerae* is highly acid labile. Thus, most ingested vibrios are killed in the acidic environment of the stomach. To

* Corresponding author. Depto. de Fisiología, Fac. de Medicina, UBA, Paraguay 2155, 7 mo piso, cp 1121 Buenos Aires, Argentina. Tel.: +54-1-9640503; fax: +54-1-9636287; e-mail: ibarra@fmed.uba.ar

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reach the small intestine, the organisms must cross the layer of mucus separating it from the enterocyte, counteract the peristalsis, compete with the normal flora and adhere to the intestinal mucosa. Thus, the size of inoculum, motility, chemotasis, the expression of critical enzymes such as mucinases and proteases, and adherence and colonization factors, are all critical in the pathogenesis of cholera [10].

Despite numerous efforts to develop attenuated *V. cholerae* strains that provide protection against El Tor biotypes of *V. cholerae* O1, a satisfactory cholera vaccine has not been achieved yet.

Construction of live attenuated *V. cholerae* oral vaccines, by recombinant technology that introduces deletions in the genes encoding cholera toxins, has elicited high antibody responses but also lead to the development of mild to moderate diarrhea during the human tests with volunteers [11, 12]. Although the pathophysiology of the residual diarrheagenic potential of these strains has yet to be elucidated, one possibility is that additional, as yet unidentified, enterotoxins specific for the human intestine may be present. An alternative explanation is the "colonization reactogenicity" hypothesis which suggests that bacterial colonization induces symptoms including diarrhea by a mechanism that does not involve a bacterial toxin per se [13].

The purpose of the present report was to design a functional test that allows the evaluation of attenuated *V. cholerae* strains with regard to their capacity to modify fluid absorption across human intestine ex vivo. Here we describe an experimental approach, previously employed in other epithelial tissues, that allows the simultaneous recording of the net water movement, electrical potential difference and short-circuit current across the tissue [14]. Our results indicate the diarrheagenic potential of new and attenuated candidate strains for cholera vaccines.

2. Materials and methods

2.1. *Vibrio cholerae* strains and media

Wild type and attenuated *V. cholerae* strains utilized are listed in Table 1. Strain 251a was derived from SG251 (kindly provided by Dr Richard A. Finkelstein University of Missouri School of Medicine, Columbia, M.O.) as described in refs [17] and [18]. Briefly, SG 251 was transformed with a suicide vector containing part of the core region of the virulence cassette. The antibiotic resistant co-integrate was allowed to segregate in antibiotic free medium to produce strain 251a, This strain lost the entire core region by recombination between RS elements.

Bacteria were routinely grown in LB medium and conserved in the same medium supplemented with 20% glycerol at −70°C. Culture supernatants were prepared by centrifugation of overnight cultures (37°C, LB broth) followed by filtration through a 0.45-μm filter (Millipore) and checked for toxin production using an ELISA. Then, they were concentrated 20 times using an ultra filtration system (Amicon) and a filter PM10 (M_r 10,000 cutoff). The concentrate was washed two times with 20 volumes of an standard Ringer solution (NaCl 114 mM, KCl 4.5 mM, $NaHCO_3$ 25 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.2 mM, glucose 25 mM; pH = 7.4) and stored at −20°C until needed. The concentrated supernatants were reconstituted to the original volume when they were added to Ussing chambers.

2.2. Determination of LD_{50}

LD_{50} was determined using the infant mouse model. Dilutions from 10^3 to 10^7 vibrios in 20 μl phosphate-buffered saline were orally inoculated into groups of six to ten 2- to 3-day-old mice, and mortality was determined after 72 h.

Table 1
Bacterial strains

V. cholerae strain	Relevant genotype	Reference
C7258	Wild type, 01, El Tor, Ogawa, Perú 1991	15
C6706	Wild type, 01, El Tor, Inaba, Perú 1991	15
E7946	Wild type, 01, El Tor, Ogawa,	16
SG251	Wild type, 0139, India	—
638	ctx A^- ctxB^- zot^- ace^- $orfU^-$ cep^- HA^- $mucinase^-$ mutant from C7258	17
413	ctx A^- ctxB^- zot^- ace^- $orfU^-$ cep^- mutant from C6706	18
251a	ctx A^- ctxB^- zot^- ace^- mutant from SG 251	This paper
CVD 109	ctx A^- ctxB^- zot^- ace^- $orfU^-$ cep^- mutant from E7946	19
CVD 103-HgR	ctx A^- hlyA^-, Hg^{2+} resistant mutant from 569B (Classical Inaba)	20

2.3. Ligated rabbit ileal loop assay

Ileal loop assays were performed as described previously [18]. Briefly, New Zealand adult rabbits were fasted for 24 h, the small intestine withdrawn and ligated approximately 10 cm from the appendix. The intestine was divided into 5–6 cm segments by ligatures and injected with 10^8 cfu of live vibrios in 0.5 ml of phosphate buffered saline. After 16–18 h the animals were sacrificed and loop length and fluid volume obtained. Results are expressed as FA = fluid accumulated (ml)/length(cm). Results are given as the mean ± standard error (mean ± SE).

2.4. Functional test using human intestine

Fragments of fresh human intestine were obtained from surgical operations on adult patients with cancer (informed consent was given). Tissue was taken from a macroscopically normal area within the "security zone" of the extirpated intestine section and placed in ice-cold high K^+-Ringer solution (in mM: 120 KCl, 10 $NaHCO_3$, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 1.2 K_2HPO_4, 0.2 KH_2PO_4, 25 glucose) to preserve the transport functions [14]. Before the experiments the mucosa and submucosa layers were dissected from the underlying tissue (always at 4°C) and mounted as a diaphragm in a modified Ussing chamber (1.76 cm^2). Immediately, both sides of the tissue were bathed with the standard Ringer solution and bubbled with 95% O_2–5% CO_2. The bathing solution was maintained at 37°C with water-jacketed reservoirs connected to a constant temperature circulating pump. Our model of the Ussing chamber has in fact two chambers. Each one has a mucosal and serosal compartment divided by the mounted tissue.

Transepithelial net water movement (J_w) was followed automatically by a modified Ussing chamber connected to a special device [21]. Briefly, the tissue was placed against a nylon mesh by a hydrostatic pressure of 10 cm of H_2O. Water movement across the tissue was followed by displacement of a photo-opaque solution inside a glass capillary tube connected to the mucosal side of the chamber via an intermediate chamber. The liquid meniscus movement in the glass capillary was detected using an electro-optical device which was connected to a computer. The sensitivity of this instrument is in the order of 50 nl.

In the other chamber, the spontaneous potential difference (PD) was recorded across the calomel electrodes, via agar bridges placed adjacent to the epithelium under open-circuit condition. Short circuit current (I_{sc}) was recorded by an automatic voltage clamp system that maintained the PD at zero mV. Two mV pulses made each 10 minutes across the intestinal tissue under short-circuit conditions allowed calculation of the tissue conductance (G_t) according to Ohm's law: $I_{sc} = PD \times G_t$.

Once the tissue reached steady values, 200 μl of culture supernatant from either wild-type or attenuated strains were added to the mucosal bath whereas Jw and Isc were continuously measured during at least 60 min. Because of tissue variability, data are presented as ΔJ_w, ΔI_{sc} and ΔG_t, where $\Delta J_w = (J_w$ at time) − (J_w at time 0), $\Delta I_{sc} = (I_{sc}$ at time) − (I_{sc} at time 0) and $\Delta G_t = (G_t$ at time) − (G_t at time 0).

3. Results

3.1. Effect of different *V. cholerae* strains on animal models

Determination of LD_{50} in the infant mouse model inoculated with different *V. cholerae* strains is shown in Table 2. Deletion of the virulence cassette genes in attenuated strains by genetic manipulation (Table 1) caused a significant increase in LD_{50} with respect to those obtained with the corresponding wild type strains (Table 2). The attenuated strains were also atoxigenic in the ligated rabbit ileal loop assays as shown in Fig. 1. However, these results do not exclude potential reactogenicity in humans due to the expression of unknown secretagogues specific for human intestine. Culture supernatant from attenuated and their parental wild-type *V. cholerae* strains were also studied ex vivo with human intestine.

3.2. Effect of *V. cholerae* strains on human intestine

Under basal conditions, a net absorptive J_w was observed when the human intestine was placed between two identical Ringer solutions in the Ussing chamber. Fig. 2 shows the typical results. The height of each vertical stroke indicates the amount of water moving from the mucosal to the serosal bath in 1 min (absorptive J_w).

Table 2
Determination of LD_{50} in the Infant Mouse cholera model

V. cholerae strain	Properties	LD_{50}	*n*
Virulent strains			
C7258	El Tor, Ogawa	$(1.50 \pm 0.02) \times 10^3$	6
C6706	El Tor, Inaba	$(1.30 \pm 0.03) \times 10^3$	8
E7946	El Tor, Ogawa	$(2.10 \pm 0.03) \times 10^3$	6
SG 251	O139	$(4.00 \pm 0.03) \times 10^3$	8
Attenuated strains			
638	El Tor, Ogawa	$(1.00 \pm 0.05) \times 10^7$	8
413	El Tor, Inaba	$(2.30 \pm 0.02) \times 10^7$	8
251a	O139	$(1.10 \pm 0.03) \times 10^7$	8
CVD 109	El Tor, Ogawa	$(3.00 \pm 0.02) \times 10^7$	6

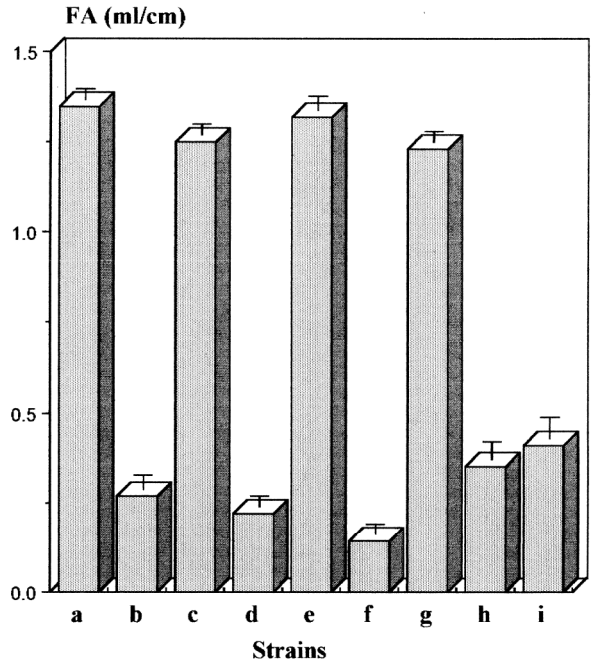

Fig. 1. Toxin production in vivo measured in ligated rabbit ileal loops. a: C7258; b: 638; c: C6706; d: 413; e: SG251; f: 251a; g: E7946; h: CVD 109; i: CVD 103-HgR.

The mean values obtained for J_w and the electric parameters measured simultaneously in the ileum and colon under basal conditions are shown in Table 3.

Addition of culture supernatant from wild-type *V. cholerae* strains to the mucosal side of the human intestine resulted in an increase of I_{sc} (Fig. 3A) and G_t (Fig. 3B) with a decrease in the net absorptive J_w (Fig. 2A and 4). In contrast, tissues exposed to supernatants from attenuated *V. cholerae* strains derived from C7258, C6706 and SG251, showed no variations in either J_w (Fig. 4), I_{sc} (Fig. 3A) or G_t (Fig. 3B) in the first 60 min of incubation. Similar effects were observed in both ileum and colon. The absence of the effect could not attributed to a cell death, since the J_w inhibition was found when a wild-type strain in the same tissue was used (Fig. 2B).

In our analysis we have included the known attenuated vaccine strains CVD 103-HgR and CVD 109. Both failed to demonstrate reactogenicity in ligated rabbit ileal loop (Fig. 1), suckling mouse assays (Table 2) or Ussing chambers assays using rabbit tissues [19]. Although attenuated in much the same manner, CVD 103-HgR is non-reactogenic in human studies [22, 23] and CVD 110 derived from CVD 109 caused mild to moderate diarrhea in 70% of volunteers [24].

In agreement with these results, we have observed a significant inhibition of the absorptive J_w when culture supernatant from CVD 109 but not from CVD 103-HgR was used (Fig. 5). In both cases, PD, I_{sc} and G_t remained unchanged for almost 60 min of incubation (Table 4).

Table 4 summarizes the mean differences in J_w, I_{sc} and G_t obtained from 60 min of incubation with culture supernatants from either wild-type or attenuated *V. cholerae* strains.

Table 3
Net absorptive water flux and electric parameters in human intestine under basal conditions

Human tissue	Ileum	Colon
PD (mV)	1.5 ± 0.4 (9)	5.8 ± 1.1 (22)*
I_{sc} (μA/cm^2)	12.2 ± 3.5 (9)	21.0 ± 3.4 (22)
G_t m/cm^2)	6.9 ± 0.9 (9)	5.0 ± 0.7 (21)
J_w (μl/min.cm^2)	0.16 ± 0.03 (8)	0.20 ± 0.02 (20)

* $P < 0.01$, *t*-test

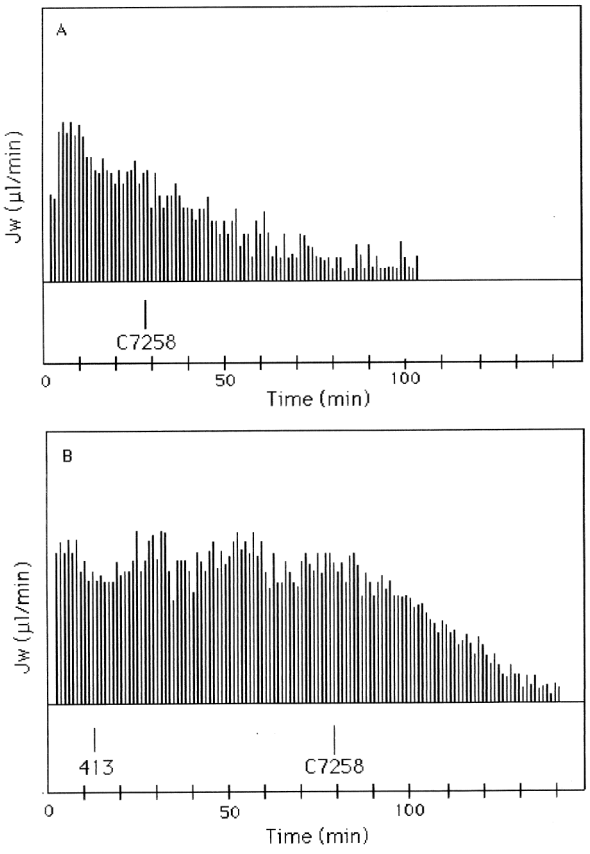

Fig. 2. Net absorptive J_w across human intestine. Screen captured from a typical experiment obtained when the tissue was mounted between two identical Ringer solutions under a hydrostatic pressure gradient of 10 cm of H_2O (mucosal side positive). The height of each line is proportional to amount of water moving in 1 min.

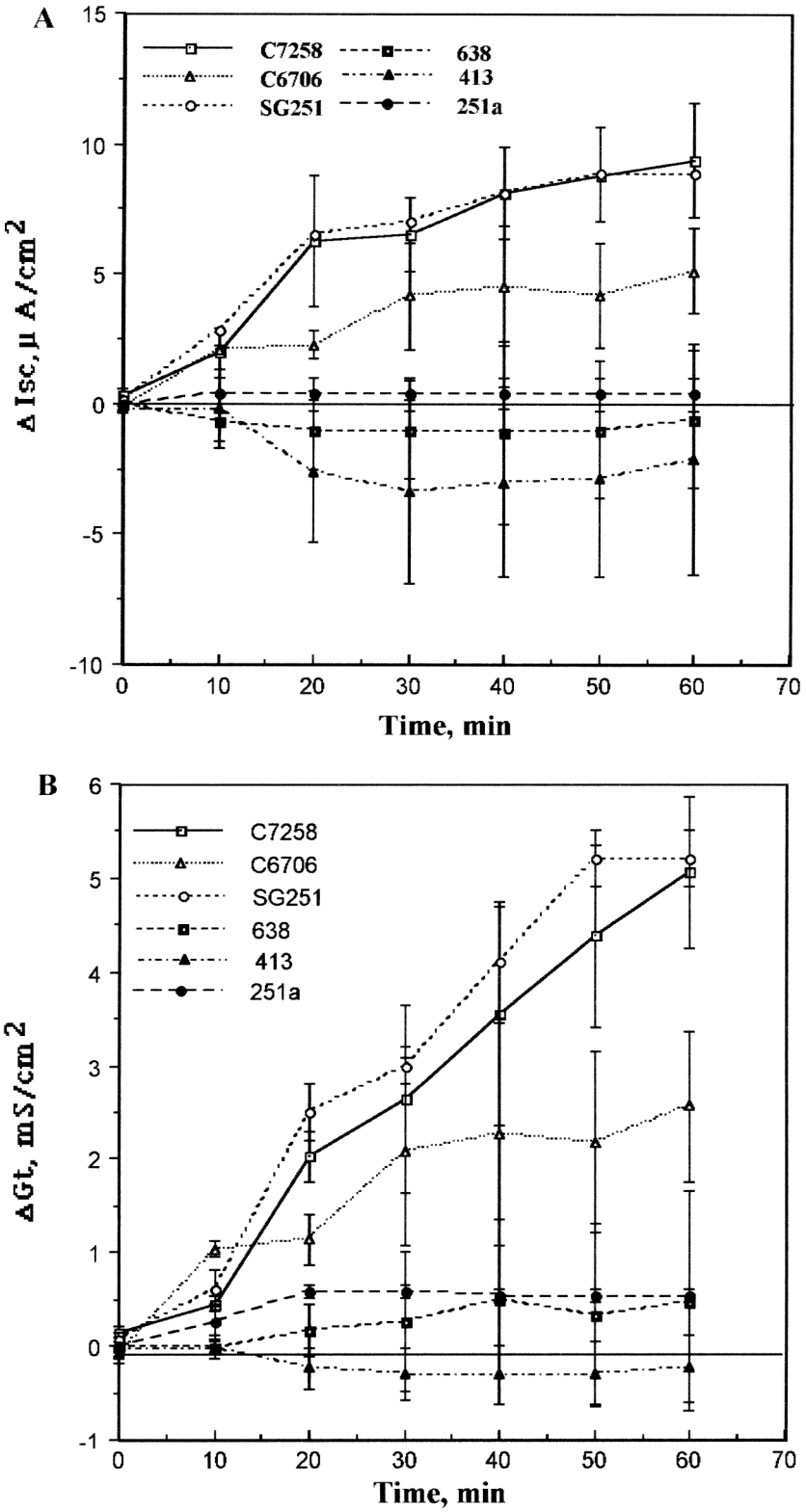

Fig. 3. Effect of wild-type and attenuated *V. cholerae* culture supernatants on I_{sc} (A) and G_t (B) measured in human intestine. Values are means for at least three experiments at each time point. Error bars show ±1 SE. Time 0 is the time at which the supernatant was added to the mucosal bath.

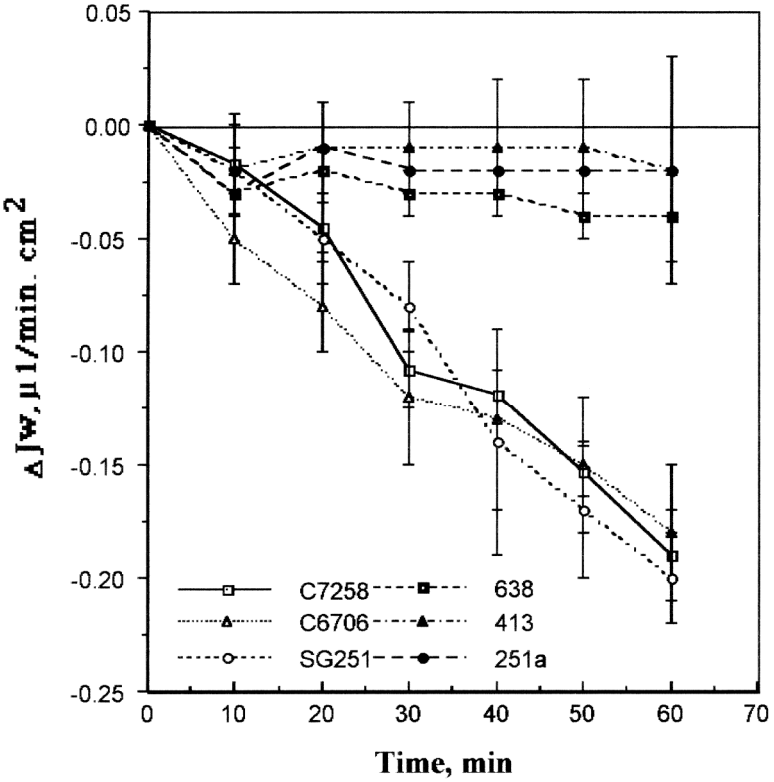

Fig. 4. Time course of ΔJ_w in human intestine after the supernatant addition of wild-type and attenuated *V. cholerae* strains. Curves represent means $\pm$ 1 SE of five experiments for C7258 and three experiments for the others.

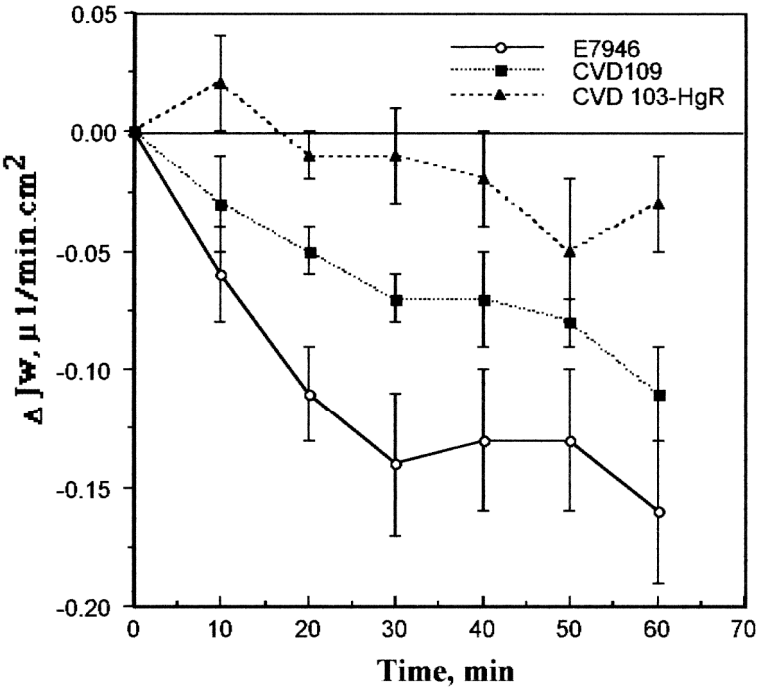

Fig. 5. Effect of *V. cholerae* culture supernatants from wild type E7946 and attenuated CVD 103-HgR and CVD 109 strains on J_w. Values are means for 3–4 experiments at each time point. Error bars show $\pm$1 SE.

Table 4
Effects of *V. cholerae* strains on transepithelial J_w, I_{sc} and G_t in the human intestine

V. cholerae	ΔJ_w (μl/min.cm^2)	ΔI_{sc} (μA/cm^2)	ΔG_t (m/cm^2)	*n*
Wild-type strains				
C7258	-0.19 ± 0.02 $P < 0.05$	$+9.38 \pm 2.16$ $P < 0.005$	$+5.06 \pm 1.93$ $P < 0.05$	5
C6706	-0.18 ± 0.03 $P < 0.025$	$+5.11 \pm 1.63$ $P < 0.05$	$+2.56 \pm 0.81$ $P <$ O.O5	3
SG251	-0.20 ± 0.02 $P < 0.025$	$+8.90 \pm 0.60$ $P < 0.05$	$+5.20 \pm 0.30$ $P < 0.01$	3
E7946	-0.16 ± 0.03 $P < 0.025$	$+7.41 \pm 2.50$ $P < 0.05$	$+3.84 \pm 0.29$ $P < 0.005$	3
Attenuated strains				
638	-0.04 ± 0.02 NS	-0.57 ± 2.61 NS	-0.48 ± 1.17 NS	4
413	-0.02 ± 0.05 NS	-2.10 ± 4.43 NS	-0.24 ± 0.37 NS	3
251a	-0.02 ± 0.02 NS	$+0.38 \pm 0.62$ NS	$+0.55 \pm 0.07$ NS	3
CVD 109	-0.11 ± 0.01 $P < 0.01$	$+0.47 \pm 1.09$ NS	$+0.08 \pm 0.61$ NS	4
CVD 103-HgR	-0.03 ± 0.02 NS	-0.56 ± 0.63 NS	-0.01 ± 0.52 NS	3

NS: not significant

4. Discussion

Diarrheagenicity of genetically attenuated *V. cholerae* vaccines in volunteers has been correlated with either different levels of intestinal colonization [19, 25] and/or unknown toxins [26, 27]. These reactogenic effects were largely unpredictable by current animal models [24, 27].

This is the first report on the use of a functional test to evaluate the potential reactogenicity of *V. cholerae* strains by assessing their capacity to modify net water and ion movement across the human intestine ex vivo.

Despite of heterogeneity in the water and salt transport mechanisms along the length of the gut, a similar reduction in the absorptive J_w associated with an increase in I_{sc} was observed in both small and large human intestine incubated with wild-type *V. cholerae* strains. Since the observed PD within 60 min was similar to the initial value, the significant increase in I_{sc} in wild-type-treated tissues can only be explained by an increase of G_t that probably reflects the effect of ZOT (zonula occludens toxin) on the tissue permeability. No variation on either J_w or electrical parameters was observed when culture supernatants from most of attenuated *V. cholerae* strains were used. The ability of supernatants from wild-type but not attenuated *V. cholerae* strain culture to increase the conductance of rabbit intestinal tissue mounted in an Ussing chamber has been previously reported [26].

In the case of CVD 109 a significant inhibition of J_w without a concomitant variation of either I_{sc} or G_t was found. These results are consistent with those reported using the parental CVD110 strain showing no change in I_{sc} in Ussing chamber assays with the rabbit ileum tissue [19] while inducing a mild diarrhea in volunteer studies [24]. Taket et al. [24] discussed the hypothesis that, as the known virulence cassette was mutated in CVD110, there is yet another toxin(s) inducing diarrhea in humans. According to these authors, the failure to detect I_{sc} variation in the Ussing chamber could be due to: (i) the use of rabbit and not human intestinal tissue; (ii) the expression of toxin(s) in vivo but not in vitro; (iii) the association of toxin(s) to the cells; (iv) the effect of toxin(s) on transport systems electrogenically silent in the enterocyte. Our experiments discard the first three possibilities and suggest that an additional toxin(s) cause inhibition of the absorptive J_w in human intestine ex vivo by a modification of the transport system electrically silent such as the Shiga toxin produced by *Shigella dysenteriae* [28]. In contrast, CVD 103-HgR is unable to modify either water or ion movements across the human intestine consistent with its non-reactogenic activity in human volunteers [22, 23]. These results show the ability of our functional test to evaluate the reactogenicity of live recombinant *V. cholerae* strains. Then, we have demonstrated that attenuated strains, 638, 413 and 251a derived from C7258, C6706 and SG 251, respectively, have lost the capacity to modify the ions and water movements across human intestine mounted in Ussing chamber.

In summary, we have standardized a functional test to evaluate diarrheagenicity of *V. cholerae* strains by assessing water and ion movements across the human intestine. Attenuated 638, 413 and 251a strains are unable to modify water transport across the human intestine indicating the low reactogenicity of these strains. Work in progress is oriented to the determination of the effects of these attenuated strains in volunteers.

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