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ORIGINAL ARTICLE

Repeated Dose Toxicity Study of a Live Attenuated Oral Cholera Vaccine in Sprague Dawley Rats

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Background and Aims. A live attenuated vaccine candidate against human cholera has been developed from the genetically modified *Vibrio cholerae* O1, biotype El Tor, serotype Ogawa, 638 strain. Previous single dose toxicity and local tolerance studies have demonstrated that the product is innocuous in Sprague Dawley rats by oral route and single dose. The present paper describes a repeated dose toxicity study using a further dose compared to the proposed clinical schedule.

Methods. Sprague Dawley rats (140−180 g) were treated with two doses of the vaccine candidate with a dedicated placebo formulation or were not treated at all (controls). The test products were inoculated at a 21-day interval. Animals were observed daily, body weight was determined weekly and food and water intakes were measured every other day. Three and 14 days after the last inoculation, groups of rats were humanely sacrificed, bled and macroscopically examined. Blood samples were taken for hematology, serum biochemistry and to determine the vibriocide antibody titers. A comprehensive list of tissue and organ samples was taken for microscopic studies.

Results. There was no mortality and no animal showed any clinical symptoms. Food and water intake, body weight, and hematological and biochemical variables did not show differences of toxicological and/or statistical relevance among the experimental groups. Macroscopic examination did not demonstrate any alterations and there were no histological findings of toxicological significance.

Conclusions. The vaccine was considered potentially safe for human use as indicated by the results in Sprague Dawley rats. © 2009 IMSS. Published by Elsevier Inc.

Key Words: Rat, *Vibrio cholerae*, Vaccine, Live attenuated, Oral, Repeated dose toxicity.

Introduction

Over 100,000−300,000 cholera cases are reported to the World Health Organization (WHO) yearly. However, WHO estimates that only 5−10% of cases are actually reported by health authorities, likely because governments are afraid of trading or tourism restrictions and, undoubtedly, due to a limited epidemiological surveillance (1). Human cholera primarily affects poor countries with deficient health systems, scarce means for treatment of fresh water sources and insufficient sewage.

Cholera control has been based on two main strategies: improvement of hygiene and development of effective and safe preventive vaccines. Hygienic disposal of human feces, ensuring adequate sources of fresh drinking water and good food hygiene are the essential measures in order

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to control the transmission of the disease. Nevertheless, vaccination constitutes the best cost-effective choice during outbreaks.

Three major types of vaccines have been developed by the worldwide research community: inactivated whole cell vaccines (either for oral or parenteral inoculation, the latter with aluminum hydroxide as adjuvant), the live genetically modified ones and the subunit vaccines. A Cuban cholera research group has produced two candidate vaccines: one based on inactivated whole cells presented as tablets (2), and a live attenuated vaccine. The latter was based on the strain 638 of *Vibrio cholerae* O1 El Tor, Ogawa. This is a genetically modified strain that lacks the CTXΦ phage, has an inactivated *hapA* gene and carries the marker gene *celA* that encodes for the endoglucanase A from *Clostridium thermocellum* (3).

There is a body of evidence that supports the virulence attenuation and the potential of the 638 strain as a candidate vaccine strain, explicitly: 1) deletion of CTXΦ phage impairs the expression of cholera toxin and a group of virulence factors (3), as well as avoids the horizontal transmission of virulence genes to avirulent environmental strains; 2) inactivation of the *hapA* gene impedes the synthesis of hemagglutinin/protease, which is important for the bacterial penetration of the intestinal mucus, the further detachment of bacteria and the spread of infection along the intestinal tract (4); 3) insertion of the gene that encodes for the *Clostridium thermocellum* endoglucanase A allows to readily differentiate the vaccine strain from environmental bacteria (3), fulfilling a biosafety requisite for genetically modified organisms (5); 4) likelihood of reversion to the virulent phenotype by reacquisition of the CTXΦ phage is minimal as the strain expresses very low levels of toxin-coregulated pilus (TCP), which is essential for the insertion of the phage (3); 5) significant virulence attenuation has been observed in infant mice, showing a 10,000 times increase of the median lethal dose compared to the parental strain (3,6); 6) the ileal loop model in rabbits evidenced a significant reduction of fluid accumulation after the inoculation of the 638 strain compared to a number of virulent strains; and 7) oral inoculation of hundreds of healthy human volunteers with bacterial suspensions prepared from fresh cultures has produced mild reactogenicity (3,6).

A stable, lyophilized formulation based on the 638 strain was produced under Good Manufacturing Practices leading to the phase of preclinical safety and efficacy testing. Until the beginning of the present work, an oral single dose toxicity test and a local tolerance test had been carried out in Sprague Dawley rats (unpublished results). They both demonstrated that the product was potentially safe for human use. The present paper describes a repeated dose toxicity test performed in Sprague Dawley rats inoculated two times with the same absolute dose suggested for human immunization.

Table 1. Vaccine composition

Source	Constituent	Amount
Lyophilized product	*V. cholerae*, 638 strain	$1-5 \times 10^9$ CFU
	Sodium chloride	9 mg
	Meat peptone	40 mg
	Skim milk	23 mg
	Sorbitol	40 mg
Buffer solution	Sodium bicarbonate	53 mg
	Ascorbic acid	33 mg
	Lactose	4 mg
	Fresh water	2 mL

The candidate vaccine is presented as a lyophilized formulation dispersed in a buffered solution. A human immunizing dose is planned to contain $1-5 \times 10^9$ viable bacteria.

Materials and Methods

Animals

Five- to six-week-old Sprague Dawley rats weighing 140–180 g and produced and supplied by CENPALAB (Havana, Cuba) were used after 2 weeks of quarantine. Five rats were randomly allocated in each cage (Type 4, polycarbonated, 1800 cm^2 floor area, Tecniplast, Casale Lita, Italy) and treatment groups were then assigned to cages at random. Room temperature and relative humidity were controlled at 20–25°C and 60–65%, respectively. A cycle of 12 h light–12 h darkness was maintained during the experiment. Food and drinking water were supplied *ad libitum*.

Test Substance

The test substance consisted of the lyophilized candidate vaccine developed from the genetically modified *V. cholerae* O1, biotype El Tor, serotype Ogawa, 638 strain plus the buffer solution used to dissolve the powder. The final constituents of the vaccine are listed in Table 1. The placebo formulation contained the same substances as the vaccine, except the attenuated bacteria. Lyophilized vaccine or placebo products were kept at −20°C from the manufacture date to the day of administration and were then dissolved in 2 mL of buffer solution and allowed to stand until reaching room temperature.

Experimental Design

The experiment consisted of a comprehensive clinical and pathological study of the rats treated with two oral doses of the test product, a further dose compared to the schedule proposed for the clinical study (7). The experimental groups and the number of animals used are summarized in Table 2. Two milliliters of the vaccine or placebo solution was administered to overnight-starved rats using an intragastric cannula. Rats were treated with the dose proposed for humans ($1-5 \times 10^9$ CFU), which covers a substantial

Table 2. Experimental design

Treatment	Sex	*n*	Sacrifice time (days)	
			3	14
Control	Female	20	10	10
	Male	20	10	10
Placebo	Female	20	10	10
	Male	20	10	10
Vaccine	Female	20	10	10
	Male	20	10	10

Twenty male and female rats were inoculated with two doses of either vaccine or placebo formulations. Likewise, control rats did not receive any treatment. Clinical observation was conducted during the experiment and groups of 10 rats were sacrificed on days 3 and 14 after administration of the second dose in order to perform hematological, biochemical and histopathological studies.

margin considering the body weight and the allometric relationships between both species (8).

Despite the fact that the immunization schedule with this candidate vaccine is for only one dose, a repeated dose toxicity study is still appropriate. The safety profile can be further explored by maximizing exposure of the animal model to the test product and the potential toxicity due to the accidental revaccination under field conditions could also be studied in the biomodel at the preclinical stage (9).

The experimental design was in concordance with current perspectives on the preclinical testing of vaccines that recommend episodic instead of daily administration and the inoculation of the number of clinical doses plus one, in the present case, two doses (10,11). Dosing interval (21 days) was based on the decline of serum vibriocide antibodies as observed in previous experiments (see supplemental figure) following a single oral dose of the same magnitude as the one used in the present study. The observations carried out 72 h after the last administration were planned to reveal early side effects. On the contrary, those performed 2 weeks later intended to show either long-term alterations or the reversibility of early changes.

Variables

Clinical symptoms (daily examination), body weight (measured weekly), food and water intake (every other day), hematology, blood chemistry, serology and post-mortem examination (necropsy and histopathology) were the groups of variables under study. Animals were observed carefully in order to record clinical alterations. Evidence of symptoms indicative of general toxicity such as lethargy, laborous breathing, lack of motility, salivation, hair bristling and signs of local gastrointestinal alterations such as diarrhea were given special attention. Blood and tissue samples were taken on days 3 and 14 after the last inoculation. Animals were anesthetized with intraperitoneal sodium pentobarbital (35 mg/kg for female and 40 mg/kg, male rats) and then bled to death. EDTA was used as anticoagulant for the hematological studies. Blood chemistry and the vibriocide assays were performed on serum samples obtained by centrifuging (2100 $\times$ g, 10 min) blood collected without anticoagulant. Serum vibriocide titers were determined as described by Benítez et al. (3).

Hematology included hemoglobin, hematocrit, total leukocytes and differential white blood cell counts. Blood chemistry variables were comprised of glucose, urea nitrogen, uric acid, creatinine, alkaline phosphatase (ALP), protein, triglycerides, cholesterol, direct bilirubin, creatine phosphokinase (CPK), alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT). All biochemical parameters were analyzed using diagnostic kits (CENTIS Diagnostics, Havana, Cuba). One normal and one pathological control serum sample (CENTIS Diagnostics) were analyzed for every group of 10 test samples in order to control the performance of the techniques.

Pathological examination included necropsy of all animals as well as the sampling and histological study of the following organs and tissues: tongue, parotids, esophagus, stomach, pancreas, liver, duodenum, jejunum, ileum, cecum, colon, rectum, submaxillary, parotid and mesenteric lymph nodes, heart, aortic artery, thymus, larynges, trachea, lungs, sternum, spleen, kidneys, urinary bladder, ovaries, oviduct, uterus, vagina, testicle, epididymus, seminal vesicles, prostate, thyroid, pituitary, Harderian, mammary and adrenal glands, eyes, cerebrum, cerebellum, spinal cord, skin and bone marrow. The liver, spleen, lungs, heart, thymus and kidneys were weighed to calculate their relative weight (%) in relation to the body weight.

Tissue samples were fixed in 10% neutral buffered formalin except reproductive organs, eyes, pituitary and Harderian glands that were fixated in Bouin's solution. The standard techniques of wax inclusion and hematoxylin-eosin stain were performed. A double-blind assessment of tissues and a detailed description of histological changes were carried out.

Statistical Analysis

A three-way (treatment/sex/time) analysis of covariance was used to compare body weights, as it is difficult to start the experiment with age-mated rats of different sex and similar body weight. The weight at the beginning of the assay was utilized as covariate. Observations of food and water intakes were grouped in weeks and compared by means of factorial (treatment-sex-time) analysis of variance (ANOVA). Serum vibriocide titers were transformed into decimal logarithms and compared by ANOVA. Hemoglobin, hematocrit and total leukocytes were also analyzed by factorial ANOVA. The differential white blood cell counts were compared by using the Kruskal-Wallis test. Blood chemistry variables were analyzed by means of a factorial ANOVA, and in the case of cholesterol, *post-hoc* comparisons were carried out by using Dunnett's and Least Significant Difference tests. The

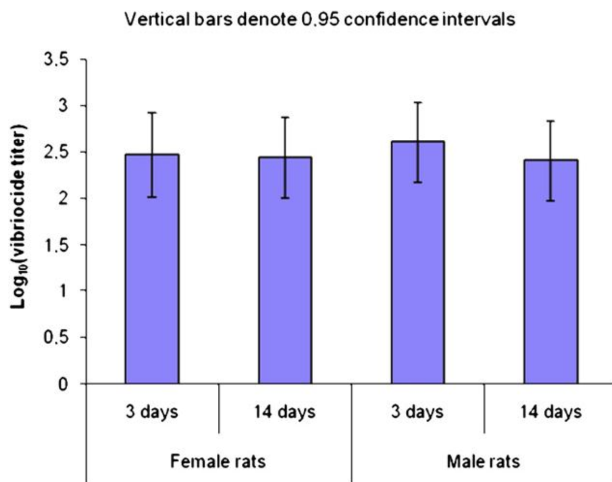


Figure 1. Serum vibriocide titers after the second dose. Serum samples were obtained by centrifuging blood collected by femoral vein incision on days 3 and 14 after the second dose of either vaccine or placebo. Vibriocide antibody titers were determined as previously described (3) and the $\log_{10}$ (titers) compared by analysis of variance. Similar results were shown by rats of either sex at any of the sample times, indicating that a fast secondary immune response was induced. Color version of this figure available online at www.arcmedres.com.

frequency of histological changes was compared by log-lineal analysis and Fisher's exact test. Statistical analysis was performed with the STATISTICA 6.1 software [StatSoft, Inc. (2003). STATISTICA (data analysis software system), version 6. www.statsoft.com]. Statistical significance was assumed at p values <0.05.

Results

Although rats have not extensively been used for experimental human cholera research, they are capable of mounting a proper serum vibriocide immune response against *V. cholerae* after oral inoculation (12). In previous studies, we also found that Sprague Dawley rats notably responded to the oral inoculation of the present vaccine candidate (unpublished results). In this study, vibriocide antibodies were measured 3 and 14 days after a second dose (Figure 1). Control and placebo-treated rats had no detectable vibriocide serum antibodies. On the contrary, all vaccinated rats showed a high vibriocide response. The antibody titers were comparable between sexes and sampling times. The similarity of titers 3 and 14 days after that second dose suggests either that a fast, secondary immune response was induced by the vaccine or that the primary, local, intestinal immunity blocked the attachment of bacteria and impeded the development of a secondary immune response. Although the latter has been reported in clinical studies of the combined *Vibrio cholerae* CVD103-HgR and *Salmonella typhi* ty21a live oral vaccines (13), we did demonstrate a secondary response in rats with the candidate

Table 3. Average body weight (mean and 95% confidence intervals)

Sex	Treatment	*n*	0	4 days	7 days	14 days	21 days	24 days	35 days
Female	Vaccine	17	147 [142–151]	171 [165–177]	184 [177–191]	202 [194–211]	220 [210–230]	221 [204–239]	327 [310–343]
	Control	19	150 [145–154]	176 [170–182]	189 [182–195]	208 [200–216]	225 [215–234]	223 [206–239]	349 [334–365]
	Placebo	19	147 [142–151]	176 [171–182]	186 [179–192]	207 [199–215]	226 [217–235]	228 [211–244]	327 [311–342]
Male	Vaccine	19	179 [175–184]	222 [216–227]	247 [241–254]	290 [282–298]	332 [323–342]	250 [234–267]	410 [394–425]
	Control	20	178 [173–182]	223 [217–228]	249 [242–255]	291 [284–299]	335 [326–344]	258 [242–273]	397 [382–413]
	Placebo	20	179 [175–184]	224 [219–230]	251 [245–257]	295 [288–303]	338 [328–347]	261 [246–277]	414 [398–429]

Body weight was measured individually on the inoculation day and 4, 7, 14, 21, 24 and 35 days afterwards. Measurements performed on days 24 and 35 were carried out after overnight starvation prior to sacrifice. As a result, a false body weight decrease may be noticed in some experimental groups. There were no statistical differences among any of the experimental groups ($p > 0.05$).

Table 4. Water and food intake (mean and 95% confidence intervals)

Variable	Female rats			Male rats		
	Vaccine	Control	Placebo	Vaccine	Control	Placebo
Water intake (mL)	29.9 [29−31]	33.3 [32−34]	31.3 [30−32]	45.3 [44−46]	42.9 [42−44]	44.6 [44−46]
Food intake (g)	18.3 [18−19]	19.6 [19−20]	19.7 [19−20]	27.2 [27−28]	26.9 [26−27]	27.3 [27−28]

Water and food intakes were measured every second day and grouped by weeks to ease statistical analysis. A slightly lower food intake was found in vaccinated female rats ($p = 0.031$) compared to placebo and control ones, but differences were not considered relevant from the biological point of view.

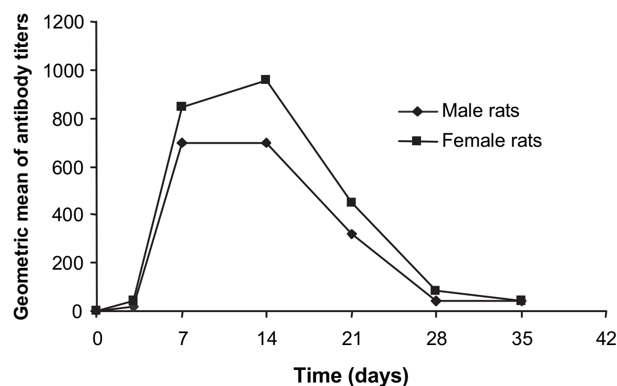
vaccine under study by administering two doses 14 days apart (unpublished results).

The induction of specific vibriocide antibodies by the candidate vaccine after oral inoculation in Sprague Dawley rats validates them as a relevant animal model for the toxicological testing of this vaccine because they may be sensitive not only to its intrinsic toxicity, but also to the potential toxicity linked to the immune response elicited by the vaccine. Given that toxicokinetics is not usually performed during the course of vaccine toxicity tests, immunological studies are also a way to confirm the vaccine administration and serves as an additional proof of concept of the vaccine in the animal model (14).

No animal died or showed clinical symptoms after the administration of the vaccine. Body weights of the three treatment groups were similar ($p > 0.5$) and a progressive increase was noticed after the inoculation of the first and second doses (Table 3). As expected, male rats had a higher growth rate compared to female rats ($p < 0.001$). There was no significant interaction among the factors under study (treatment, sex and time) regarding body weight. According to data presented, a decrease of body weight seemed to occur in certain groups (mainly male rats) during the first 3 days after the second dose, but figures corresponded to measures performed following overnight starvation as opposed to the ones carried out previously.

The average water intake (Table 4) did not show statistically significant differences ($p > 0.5$) among the experimental groups. However, there was a significant interaction ($p < 0.001$) between sex and treatment because vaccinated and placebo female rats consumed more water than control ones, but the opposite happened with male rats. Nevertheless, differences were not biologically relevant as they were in the range of 2−3 mL per animal/day. Likewise, food intake was similar among male rats. Vaccinated female rats ate less food ($p = 0.031$) than control and placebo ones. Again, differences were not significant from the biological point of view (1.1−1.3 g/animal).

Hematological studies demonstrated similar ($p > 0.5$) hemoglobin concentrations (Table 5) among treatment groups as well as between genders. Average values corresponded to those reported for rats (15−18). Hematocrit values were correlated with hemoglobin, as expected when there were no alterations of mean corpuscular hemoglobin concentration. From the toxicological viewpoint, hemoglobin and hematocrit can reveal systemic adverse effects, damage to target organs such as bone marrow and liver and direct toxic effects on erythrocytes leading to hemolysis.



Supplementary Figure 1. Development of vibriocide antibody titers after single oral dosing of Sprague Dawley rats with the attenuated cholera vaccine based on the 638 strain of *Vibrio cholerae*. Overnight-starved Sprague Dawley rats were immunized with a single dose of the attenuated cholera vaccine. Blood samples were taken from the retro-orbital sinuses at several time-points and the serum obtained was used to determine vibriocide antibody titers as described elsewhere (3). Figures are the geometric mean of 10 rats per sex. The highest serum vibriocide activity was demonstrated from days 7−14 after immunization, declining thereafter to reach stable low titers by day 28.

Total leukocyte count was similar ($p > 0.05$) among experimental groups and slightly higher in male rats ($p < 0.05$). We have not found any reference of leukocytosis linked to *V. cholerae* infection in humans or rats, thus an increase of leukocytes due to the inoculation of live bacteria was unexpected. However, absence of changes in leukocyte quantities reveals the innocuousness of the final product, i.e., the active ingredient, the rest of the constituents, impurities and the contaminants acquired during the manufacturing process. Leukocytosis has been reported among the side effects of vaccines during clinical trials in some instances (19−22). On the other hand, immunotoxic effects are usually related to depletion in the number of leukocytes (23).

The differential count of neutrophils, eosinophils, basophils, lymphocytes and monocytes was comparable among the experimental groups. Lymphocytes were the dominant cells followed by neutrophils, and the rest of the cell types were rare, as described for healthy rats (14,17).

Table 5. Hematological studies

Variable	Female			Male			p
	Vaccine	Placebo	Control	Vaccine	Placebo	Control	
Hemoglobin (g/L)	155 [151–160]	159 [155–163]	155 [151–159]	159 [155–163]	159 [155–163]	157 [153–161]	0.42
Hematocrit (mL/100 mL)	52.1 [50.6–53.7]	53.3 [51.8–54.8]	51.7 [50.2–53.2]	53.3 [51.8–54.8]	53.1 [51.6–54.5]	52.5 [51.1–53.9]	0.34
Leukocytes (10^3/mm^3)	5.7 [5.2–6.1]	5.3 [4.9–5.7]	5.8 [5.4–6.2]	6.3 [5.9–6.7]	5.8 [5.4–6.1]	5.9 [5.5–6.3]	0.089
Neutrophils (%)	32 [27–37]	32 [27–35]	32 [26–38]	34 [27–40]	32 [28–37]	30 [26–34]	0.65
Lymphocytes (%)	67 [63–71]	68 [65–73]	68 [62–72]	63 [55–73]	68 [63–72]	69 [64–74]	0.63

Hematological studies were conducted on animals sacrificed 3 and 14 days after the second inoculation. Similar results ($p = 0.54$) were obtained at the two sampling times; therefore, average values are presented instead of those corresponding to each sampling time. Figures presented for hemoglobin, hematocrit and leukocytes are mean and 95% confidence intervals, whereas medians and 25–75% intervals are provided for the differential count of neutrophils and lymphocytes. Lymphocytes and neutrophils were the predominant cells, whereas basophils and eosinophils were in a very low proportion in most of the samples examined. No statistically significant differences ($p < 0.1$) were demonstrated among the experimental groups for any of the hematological variables included.

Table 6. Blood chemistry analysis (mean and 95% confidence intervals)

Variable	Female			Male			p
	Vaccine	Placebo	Control	Vaccine	Placebo	Control	
ALT (U/L)	51.5 [40–63]	58.9 [48–70]	67.5 [57–78]	64.4 [54–75]	65.2 [55–76]	61.9 [51–72]	0.53
AST (U/L)	132 [112–152]	140 [119–160]	130 [111–150]	160 [141–180]	168 [149–187]	154 [135–173]	0.49
ALP (U/L)	245 [204–285]	236 [196–277]	276 [236–315]	391 [351–431]	362 [323–400]	399 [360–437]	0.10
CPK (U/L)	1338 [883–1792]	1309 [854–1763]	1268 [825–1710]	2255 [1813–2697]	2143 [1712–2575]	1741 [1310–2172]	0.44
Creatinine (mmol/L)	64.6 [58–71]	70.5 [64–77]	73.7 [67–80]	64.8 [58–72]	63.2 [57–70]	65.2 [59–72]	0.31
Triglycerides (mmol/L)	1.16 [0.8–1.5]	0.92 [0.6–1.3]	0.93 [0.6–1.3]	0.65 [0.3–1.0]	1.14 [0.8–1.5]	0.61 [0.3–0.9]	0.30
Cholesterol (mmol/L)	1.17 [1.0–1.4]	1.26 [1.1–1.4]	1.49 [1.3–1.7]	1.04[a] [0.9–1.2]	1.24[a] [1.1–1.4]	1.16 [1.0–1.3]	0.042
Urates (µmol/L)	75.5 [62–89]	78.7 [65–92]	69.7 [56–83]	61.9 [49–75]	66.4 [54–79]	72.5 [60–85]	0.86
Urea (mmol/L)	9.8 [9–11]	9.3 [8–10]	9.3 [8–11]	8.5 [7–10]	8.9 [8–10]	8.7 [8–10]	0.96
Glucose (mmol/L)	9.9 [9–11]	9.0 [8–10]	9.0 [8–10]	10.0 [9–11]	10.4 [9–12]	10.0 [9–11]	0.79
Total proteins (g/dL)	6.1 [5–7]	6.0 [5–7]	5.8 [5–7]	5.6 [5–6]	5.2 [4–6]	5.5 [5–6]	0.82
Direct bilirubin (mg/dL)	0.09 [0.04–0.2]	0.07 [0.02–0.1]	0.13 [0.08–0.2]	0.07 [0.02–0.1]	0.06 [0.01–0.1]	0.11 [0.06–0.2]	0.064

Blood chemistry analyses were performed on serum samples collected 3 and 14 days after the last inoculation. No statistical difference was found for any of the variables measured except cholesterol. Male rats treated with the vaccine formulation had lower cholesterol values than placebo rats ($p < 0.05$); however, figures were statistically similar to those found in control rats.

[a]Significant statistical differences between vaccine and placebo groups at $p < 0.05$.

Table 7. Organ to body weight ratios

Variable	Female rats			Male rats			p
	Vaccine	Placebo	Control	Vaccine	Placebo	Control	
Thymus	0.21 [0.19–0.23]	0.22 [0.20–0.23]	0.21 [0.19–0.22]	0.17 [0.15–0.18]	0.16 [0.14–0.17]	0.16 [0.14–0.17]	0.97
Right lung	0.31 [0.29–0.33]	0.27 [0.26–0.29]	0.28 [0.26–0.30]	0.24 [0.23–0.26]	0.25 [0.23–0.27]	0.25 [0.23–0.27]	0.19
Left lung	0.18 [0.16–0.19]	0.18 [0.17–0.20]	0.19 [0.18–0.21]	0.14 [0.13–0.16]	0.14 [0.13–0.16]	0.14 [0.13–0.16]	0.72
Heart	0.41 [0.39–0.42]	0.38 [0.36–0.39]	0.37 [0.35–0.39]	0.39 [0.37–0.40]	0.36 [0.34–0.38]	0.37 [0.35–0.38]	0.32
Liver	3.77 [3.6–3.9]	3.53 [3.4–3.7]	3.64 [3.5–3.8]	3.57 [3.4–3.7]	3.34 [3.2–3.5]	3.34 [3.2–3.5]	0.084
Spleen	0.25 [0.24–0.26]	0.24 [0.23–0.25]	0.25 [0.24–0.26]	0.21 [0.20–0.22]	0.21 [0.20–0.22]	0.21 [0.20–0.22]	0.74
Right kidney	0.38 [0.37–0.40]	0.36 [0.35–0.38]	0.38 [0.37–0.39]	0.37 [0.36–0.38]	0.37 [0.36–0.38]	0.37 [0.35–0.38]	0.13
Left kidney	0.38 [0.36–0.39]	0.37 [0.35–0.38]	0.39 [0.38–0.40]	0.37 [0.35–0.38]	0.36 [0.35–0.38]	0.36 [0.35–0.38]	0.12

Rats were starved overnight and their body weights determined just before sacrifice. Major organs were carefully removed and their weight measured to calculate the organ to body weight ratios (%). Data presented are mean values and 95% confidence intervals. No statistically or biologically significant difference was found among the experimental groups for any of the variables under analysis.

Table 8. Summary of histological findings

Histological changes	Vaccine (n = 40)	Placebo (n = 40)	Control (n = 40)
Peyer's patch lymphoid hyperplasia	8	6	3
Focal interstitial nephritis	2	0	0

Ten rats of each sex and treatment group were sacrificed on days 3rd and 14th after the second inoculation and a comprehensive list of organ and tissue samples were taken during necropsy in order to conduct histological studies. Log-lineal analysis of data demonstrated a similar ($p > 0.05$) frequency of histological changes in the three experimental groups.

Blood chemistry studies (Table 6) did not show statistically significant differences among the experimental groups for any of the variables measured, except for cholesterol. Male rats differed in terms of cholesterol levels; however, the magnitude of the differences was not biologically relevant and vaccinated rats did not differ statistically from control ones. The mean values and ranges recorded for all the variables were, in general, similar to reference figures reported in the scientific literature. They were also different from those found in animals suffering from adverse events during toxicological studies (24–37).

Organ to body weight ratios (Table 7) were statistically similar among the experimental groups ($p > 0.05$). An overall analysis of organs and body weights suggests that these variables were not affected by treatments and were in the range reported for rats of a comparable age (14,17). Body weight has been considered in several toxicological tests as a sensitive general indicator of toxicity. Similarly, the relative weight of major organs may be useful in identifying target organs. An organ weight increase may be associated with congestion, edema, inflammation, hyperplasia, hypertrophy, neoplastic changes or due to the intracellular or extracellular deposit of substances. On the contrary, the organ to body ratio decreases during atrophy, fibrosis, hypoplasia and degenerative processes (38,39).

Necropsy did not show any observable lesion. Histopathological study (Table 8) demonstrated lymphoid hyperplasia of Peyer's patches; however, the changes were mild and almost as frequent in vaccinated as in placebo and control rats. Two cases of focal interstitial nephritis were found in vaccinated rats, whereas no control or placebo rat showed such tissue change ($p = 0.1561$). This lesion is not an unusual finding in rats (40) and was considered incidental.

Because no animal died or showed clinical symptoms indicative of local or systemic toxicity, and because clinical observations, blood chemistry, hematology and anatomopathological studies did not reveal any relevant differences among the experimental groups, the candidate vaccine developed from the 638 strain was considered innocuous for Sprague Dawley rats and potentially safe for human use.

A single dose toxicity test, a local tolerance test and a repeated dose toxicity test have been conducted so far.

These three preclinical studies have demonstrated that the candidate vaccine is locally and systemically safe for Sprague Dawley rats, a biomodel traditionally used for toxicological studies and capable of mounting a vibriocide immune response against *V. cholerae* comparable to that found in humans (3,6,41,42). This oral live attenuated candidate vaccine against human cholera is proposed to initiate clinical trials, taking into consideration the vast evidence on the strain virulence attenuation and the satisfactory results of the preclinical tests conducted on the vaccine formulation.

Bearing in mind that this vaccine is conceived for people of all ages, the risk of inadvertently vaccinating pregnant women makes testing for embryo/fetal and perinatal toxicity necessary (9–11). Nevertheless, the hypothesis that this candidate vaccine does not pose a further risk for the *conceptus* and the pregnant woman is quite reasonable considering: 1) that there is no association between cholera disease and abortion; 2) the nature of the immune response to the vaccine and after the natural infection is similar; 3) all the auxiliary substances that constitute the vaccine are of common pharmaceutical use, and 4) the absence of histological changes of the reproductive system in preclinical studies.

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