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Randomized, double-blind, placebo-controlled trial to evaluate the safety and immunogenicity of live oral cholera vaccine 638 in Cuban adults

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ABSTRACT

A randomized, double-blind, placebo-controlled clinical trial was conducted to evaluate the safety, reactogenicity and the immunogenicity of a 2×10^9 CFU dose of the 638 lyophilized live attenuated cholera vaccine for oral administration, formulated and produced at Finlay Institute, City of Havana, Cuba. Thirty-six healthy female and male adult volunteers from 18 to 40 years old were involved, clinically examined and laboratory tested after the informed consent signature. Adverse events were monitored and seroconversion rates and geometrical mean titer (GMT) of vibriocidal antibodies were tested in volunteer's sera samples. Neither serious adverse events nor other damages to the volunteers due to vaccine or placebo feeding were reported during the clinical follow-up period of this study; none of the adverse events registered within the first 72 h after inoculation were life-threatening for volunteers. Neither severe nor moderate adverse events were reported. Sixty-one percent of subjects showed mild expected adverse events in an interval lower than 24 h up to the first 72 h, 75% of these in the vaccinated group and 18% in the placebo group. Fourteen days after inoculation the GMT of vibriocidal antibodies in the vaccine group significantly increased in comparison to the placebo group. All subjects in the vaccine group (24) seroconverted (100%). Results show that this vaccine is safe, well tolerated and immunogenic in healthy female and male volunteers.

1. Introduction

Vaccination against cholera continues to be a feasible strategy against this infection. It has been demonstrated that oral immunization with genetically attenuated cholera strains stimulates an efficient mucosal response [1]. Previous studies carried out with CVD 103HgR (classic biotype) and Peru-15 (El Tor biotype) vaccines, indicate that both vaccines are well tolerated, immunogenic and protective in challenge clinical trials in humans [2–4], nevertheless their effectiveness in endemic areas still remains uncertain [5].

A group of genetically attenuated *Vibrio cholerae* strains O1 and O139 serogroups have been obtained in Cuba [6–8]. Among them, the attenuated 638 strain *V. cholerae* O1 El Tor Ogawa proved to be well tolerated and immunogenic in a single dose ranging from 10^7 to 10^9 CFU [9]. With 10^9 CFU dose, this strain is able to provide protection against the diarrhea caused by a pathogenic strain *V. cholerae* O1 El Tor Ogawa [10]. In a previous challenge clinical trial, under quarantine hospitality facilities, a group of healthy volunteers were immunized with a single dose of 10^9 CFU of the 638 strain and others received placebo as a control group. The protection conferred by previous immunization with the 638 strain against colonization and diarrhea caused by the infection with the virulent 3008 *V. cholerae* strain was also demonstrated in that study [10].

Thus, these results encouraged the pharmaceutical development of a lyophilized live attenuated vaccine. This vaccine was

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formulated under good manufacturing practice procedures and contains the 638 strain as its active pharmaceutical ingredient [11].

A phase I–II clinical trial was carried out in healthy human volunteers to evaluate safety, reactogenicity and immunogenicity of 2×10^9 CFU single dose of the live oral cholera vaccine 638.

2. Materials and methods

2.1. Study design

Thirty-six healthy female and male volunteers from 18 to 40 years working at Scientific Institutions of the City of Havana, Cuba, were enrolled in a randomized, double-blind, placebo-controlled inpatient clinical trial, conducted at the Unit for Isolation of Biological Risks at Tropical Medicine Institute "Pedro Kourí" (IPK), in the City of Havana, Cuba, after they gave an informed consent according to the guidelines of the State Regulatory Authority for Medicine Control from the Ministry of Health of Cuba. Twenty-four of them were assigned to the vaccine group, and 12 were assigned to the placebo group. This clinical trial protocol was approved by the Ethic Committee of IPK. The study was authorized and audited by the State Regulatory Authority for Medicines Control of Cuban Ministry of Health and conducted under a license from the National Center for Biological Safety which revised the protocol and inspected the facilities. Before recruitment, each volunteer received wide information about the study and subsequently they signed an informed consent as witness of his own mind to participate in the clinical trial. Each participant was to be passed a psychometric examination and a written test, to ensure a basic comprehension of the purpose of the study. After a clinical examination previous to hospital admission, a total of 36 healthy female and male adult volunteers from 18 to 40 years old were with no previous history of clinically significant diarrhea or cholera vaccination or were receiving no medication at the moment of their recruitment.

The presence of abnormal results in clinical laboratory tests, positive stool cultures for an enteric pathogen, recent antibiotic use, positive serological test results for human immunodeficiency virus antibodies, hepatitis B antigen, hepatitis C antibody or a pregnancy positive test result, failure to pass the written examination, or psychological incompatibility with accepting quarantine conditions, impaired admission of any volunteer in this study.

2.2. Vaccine and placebo formulations

Vaccine and placebo were manufactured at Finlay Institute, from Havana, Cuba. The vaccine vial contains a lyophilized product, in which the pharmaceutical active ingredient is the 638 live attenuated cholera strain with a single dose of 2×10^9 CFU mixed with several lyoprotectors: skimmed milk (0.06 g), peptone (0.02 g) and sorbitol (0.02 g) [11]. This strain has been modified by engineering techniques to be non-toxinogenic (Δ CTX-hapA::celA), it was obtained at the National Center for Scientific Research, Cuba [7]. The placebo vial contained only the lyoprotectors. Each vial was reconstituted with 2 ml of natural water processed by "El Glacial" factory at the City of Havana, Cuba. The buffer sachet, manufactured and packaged by the pharmaceutical enterprise "Reynaldo Gutierrez" at the City of Havana, Cuba, and containing sodium bicarbonate (2.65 g), ascorbic acid (1.65 g), manitol (0.28 g), anhydrous lactose (0.20 g), sodium saccharine (0.015 g) and polyvinylpyrrolidone (0.20 g) was added to 98 ml of natural water and gently mixed with vaccine or placebo; previously reconstituted with 2 ml of natural water. The resulting suspension was considered an oral dose.

2.3. Randomization

Vaccine and placebo vials were packaged and coded at random with identical appearance. The code remained unbroken until the end of the study.

2.4. Vaccination

Subjects were randomized in a double-blind way to receive vaccine or placebo. When the vaccine or placebo was about to be fed to volunteers, it was first reconstituted with 2 ml of natural water, after that the antiacid was unpacked and added to 98 ml of natural water until it was dissolved, finally both dissolutions were gently mixed. Volunteers were fasted for 90 min before and after vaccine or placebo feeding.

2.5. Antibiotic treatment

Day 5 after vaccination, 300 mg of doxycycline was given to each volunteers being released from quarantine only after the third negative *V. cholerae* coproculture.

2.6. Specimens

All stools were collected in disposable plastic bedpans and weighted, stools consistency was graded on a 5-point scale (grade 1, formed; grade 2, soft but formed; grade 3, thick liquid; grade 4, opaque watery; and grade 5, rice water). The total volume of diarrhea was determined including loose stools after doxycycline administration. Venous blood collections were performed the day of inoculation and 7 days later in order to evaluate any biochemical changes in volunteers after vaccine administration. Sera were also collected on days 14, 28 and 42, at sixth month and one year after treatment to determine the titers of vibriocidal antibodies directed against Ogawa serotype.

2.7. Clinical surveillance

The incidence of adverse events was followed up during 30 days after treatment feeding and recorded by a team of clinical researchers. All volunteers were monitored during the first hour and daily during the next 5 days after treatment feeding, under an active surveillance to detect the occurrence of solicited adverse event, defined as those symptoms and signs as malaise, headache, fever, borborygmus, abdominal cramps, nausea, vomiting, and diarrhea, which could occur after treatment feeding and were solicited to report. Diarrhea was defined as the passage of two or more loose stools of at least 200 g (grades 3–5) within 48 h or a single loose stool of 300 g or greater [9,10], vomiting was defined as one or more episodes of emesis, and fever as an axillary temperature ≥37.5 °C. Intensity of the most of adverse events was classified as mild if it did not interfere with daily activities, moderate if it interfered with but did not impair daily activities, or severe if it impaired daily activities. The intensity of diarrhea, vomiting and fever were classified as follows: diarrhea was mild if in 24 h, stools weighed less than 3 kg, moderate if stools weighed between 3 and 5 kg, or severe if stools weighed more than 5 kg. Vomiting was classified as mild if the frequency of emesis in 24 h was less than 2 without dehydration, moderate if it was more than 2 with a mild dehydration, or severe if it was more than 2 and lead patient to a dehydration. Fever was classified as mild if temperature in 24 h was between 37.5 and 38.0 °C, moderate if it was between 38.0 and 38.5 °C or severe if it was over 38.5 °C.

2.8. Serology

Vibriocidal antibodies titers in sera were determined by means of a colorimetric microassay method using a commercial freeze-dried complement (*Pel Freez/31038.100*) [12,13]. The titer was defined as the highest serum dilution that inhibited bacterial growth, as determined by visual color examination. Seroconversion rate was defined as the fourfold increase in vibriocidal antibodies titers after the vaccine or placebo administration with respect to the initial titer.

2.9. Bacteriology

After vaccination, all stools were plated directly onto thiosulfate–citrate–bile-salt–sucrose (TCBS) vibrio selective agar (Merck) and also inoculated into alkaline peptone water (Merck) during an overnight incubation before plating onto TCBS agar.

The determination of CFU of *V. cholerae* strains per gram of stool, was made by dispersion of 1 g of each fecal specimen in 1 ml of 0.9% NaCl, serial dilution, and plating onto TCBS agar. Suspicious colonies from TCBS agar were confirmed as 638 strain by means of the agglutination with specific antisera, by the resistance to polymyxin B, and by the expression of *cel*A marker gene [7].

2.10. Statistical analysis

Calculations were performed to determine the sample size by means of the "bpower" and "bpower.sim" functions from the "hmisc" library, R implementation in the S system [14,15]. Seroconversion percentages and the geometrical mean estimation of vibriocidal antibodies titers between vaccines and placebos were compared using the Fisher Exact Test and the Wilcoxon Rank Sum Test respectively [16].

3. Results

3.1. Clinical response to 638 vaccine

No significant changes were seen on volunteer's Hematological and Biochemical Laboratory parameters tested after vaccine or placebo feeding. No clinical manifestations were observed or reported during the first hour after vaccine administration. Nobody had serious adverse event during the clinical follow-up time of this study. None of general adverse events registered during the entire follow-up period of 30-day treatment feeding were life-threatening for volunteers involved in this study.

Tables 1–3 show reactogenicity results. In Table 1, adverse events were reported in 22 out of 36 subjects, (61%), being notably higher in the vaccinated group; in which 18 subjects reported adverse events (75%) in contrast to the placebo group with only 4 (33%). None of these adverse events were classified as severe or moderate and those from the vaccinated group maintained a correspondence with solicited adverse events. The solicited adverse events registered in the vaccinated group were headache (17%), general discomfort (8%), nausea (33%), abdominal pain (38%), borborygmus (38%) and diarrhea (25%), while in the placebo group were only headache (8%), nausea (8%) and borborygmus (33%) (Table 2).

Table 1
Incidence of general adverse events after vaccination, with a single dose of the attenuated oral cholera 638 live vaccine or placebo.

No. (%) of subjects with adverse events		
Total, $N = 36$	Vaccine, $n^a = 24$	Placebo, $n^b = 12$
22 (61)	18 (75)	4 (33)

N: Subjects.
[a] Subjects in the vaccine group.
[b] Subjects in the placebo group.

Most of the registered solicited adverse events began in the first 72 h, headache, abdominal pain, borborygmus and diarrheas had a duration range from 24 to 48 h, and only two recorded between 48 and 72 h (one nausea and one diarrhea episodes) (Table 3).

3.2. Immune response to 638 vaccine

The peak of the immune response occurred at day 14 after vaccination in the vaccine group, in which the 100% of subjects raised the vibriocidal antibodies titers (152 times average), with a reciprocal geometric mean vibriocidal antibody titer of 3320. The 88% of subjects in this group reached vibriocidal antibodies titers equal to or greater than 1280 (Table 4). The remaining volunteers of this group showed titers between 160 and 320.

On day 14 after vaccination no subject of the placebo group had seroconverted and the GMT of vibriocidal antibody kept the similar low baseline levels observed as before vaccination (Table 4) with 80 being the maximal vibriocidal antibody titer reached in this group.

3.3. Kinetics of immune response

Seroconversion percentage was kept up to 90% until day 42, falling down almost a 60% at sixth month, and proximate to 30% at one year after vaccination. Baseline of seroconversion in placebo group always kept under 17%.

The GMT of vibriocidal antibodies in the placebo group was kept at low level before and until one year after vaccination. In the vaccine group, the GMT of vibriocidal antibodies decreased rapidly on day 28, even more on day 42 and they were very low at 6 months, though they were significantly higher than in the placebo group. One year after vaccination the GMT of vibriocidal antibodies has fallen near to the baseline (see Fig. 1).

3.4. Bacteriological isolation of 638 strain

Vaccine strain was detected in the feces of 20 of the 24 subjects (83%) who ingested the vaccine. The mean of incubation period of excretion was 3 days (range, 1–5). The peak of the geometrical mean of the number of organisms excreted was 1.84×10^4 CFU/g of stool (range, 1×10^2–1.60×10^6). Vaccine strain was never detected in the stools of volunteers receiving placebo. Vibrios isolated from stools of volunteers produced endogluconase A, indicating that *cel*A reporter gene was stably maintained during growth in the human intestine.

Table 2
Solicited adverse events after vaccination with a single dose of the attenuated oral cholera 638 live vaccine or placebo.

Group	N^a	Number and percentage of subjects with solicited adverse events					
		Headache, n^b (%)	General discomfort, n^b (%)	Nauseas, n^b (%)	Abdominal pain, n^b (%)	Borborygmus, n^b (%)	Diarrheas, n^b (%)
Vaccine	24	4 (17)	2 (8)	8 (33)	9 (38)	9 (38)	6 (25)
Placebo	12	1 (8)	0	1 (8)	0	4 (33)	0

[a] Subjects.
[b] Subjects with at least one solicited adverse events reported.

Table 3
Beginning and duration of mild solicited adverse events in the vaccine group during the hospital surveillance period after vaccination, with a single dose of the attenuated oral cholera 638 live vaccine or placebo.

Solicited adverse events (grade[a] 1)	Number and percentage of vaccinated subjects (N^b = 24)					
	Total, n^c (%)	Duration (h), n^d (%)			Beginning, n^e (%)	
		≤24	24–48	48–72	First 30 min	First 72 h
Headache	4 (17)	3 (12)	1 (4)	0	0	4 (17)
General discomfort	2 (8)	2 (8)	0	0	0	2 (8)
Nausea	8 (33)	7 (29)	0	1 (4)	0	3 (13)
Abdominal pain	9 (38)	7 (29)	2 (8)	0	0	6 (25)
Borborygmus	9 (38)	7 (29)	2 (8)	0	0	6 (25)
Diarrheas	6 (25)	3 (13)	2 (8)	1 (4)	0	6 (25)

[a] Intensity of the adverse events: 1: mild, 2: moderate and 3: severe.
[b] Subjects.
[c] Subjects with at least one solicited adverse events reported.
[d] Subjects with at least one solicited adverse events during the first 72 h.
[e] Subjects with solicited adverse events which began within the first 30 min or within the first 72 h.

Table 4
Geometrical mean vibriocidal antibody titer and numbers of volunteers whom seroconverted 14 days after vaccination with a dose of 2×10^9 CFU of Ogawa *Vibrio cholerae* O1 638 vaccine strain or placebo.

Group	Subjects	Geometrical mean titer of vibriocidal antibodies (CI to 95%)		Subject (%) with ≥4-fold rise (CI to 95%)	Subject (%) with ≥1:1280 titer (CI to 95%)
		Before vaccination	Day 14 after vaccination		
Vaccine	24	29 (22–38)	3320 (2807–6996)	24 (100) (86–100)	21 (88) (69–96)
Placebo	12	28 (21–39)	32 (21–47)	0	0

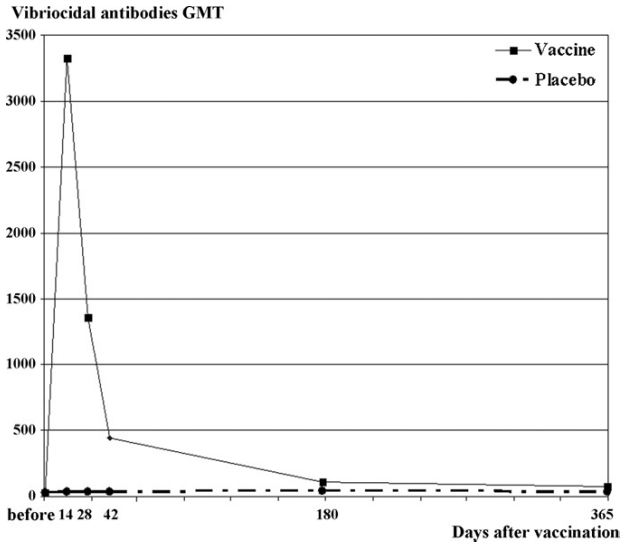

Fig. 1. Kinetics of the vibriocidal antibodies response to live *V. cholerae* 638 vaccine strain, during one year after vaccination. Subjects received either vaccine (2×10^9 CFU) or placebo on day 0.

4. Discussion

In this study, the absence of serious or severe adverse events or significant changes in volunteer's Hematological and Biochemical Laboratory parameters shown that the 638 vaccine is safe and confirms data from previous studies [8–10]; where a fresh culture of the 638 strain was used as oral the inoculum. Considering all subjects were clinically examined and exhaustively selected to participate in this study, we expected a higher incidence and an earlier beginning of adverse event in the vaccine group than in the placebo group; directly related to vaccine feeding and due to the effect of the lyophilized vaccine strain colonization in the small intestine. As in previous clinical studies with the fresh culture of the 638 strain, the registered adverse events in the vaccine group were mild and transient without any treatment needed. All these adverse events were coincident with those solicited adverse events. This is consistent with what others authors have reported. On the other hand, this behavior has been also found in previous studies conducted with one oral fresh cultured 2×10^9 CFU dose of the 638 strain [8–10].

Borborygmus, abdominal pain, nausea and diarrhea were typical most frequent adverse events and they were more clinically related to the bacteriological response of the 638 strain feeding than the antiacid used as part of the vaccine formulation [8,9].

In the placebo group the borborygmus incidence was as elevated as in the vaccine group. This event has been present in 638 fresh cultures studies [8–10] and has been also reported in studies with the CVD 103HgR strain. In all cases the cause of this adverse event could be the action of sodium bicarbonate used, directly or contained into the buffer to neutralize gastric acidity. Other authors have reported a close relation between sodium bicarbonate used in oral vaccine formulations and borborygmus incidences [17]. Accordingly to this the antiacid used as part of the vaccine formulation which also contains sodium bicarbonate, might also contribute to the manifestation of this adverse event. Taken together, all these evidences allow us to conclude that the 638 vaccine is safe and non-reactogenic after a single oral dose.

The results on seroconversion and GMT of vibriocidal antibodies demonstrate a very good response of the vaccinated subjects elicited by attenuated *V. cholerae* strain contained into the vaccine at the same level that it had been observed during previous studies with the 638 fresh culture oral administration [8–10]. The seroconversion results in this study are comparable to others found with CVD 103HgR (92%) and lyophilized Peru-15 (98%) vaccines in US healthy adult volunteers [3,17].

Fig. 1 shows the intensity of the vibriocidal antibodies response during one year after vaccination. In the vaccinated group the vibriocidal antibodies titers were only able to reach their maximal magnitude around day 14, falling down immediately on days 28 and 42, almost reaching at the same level of the placebo group at the sixth month and at one year after treatment feeding. Other authors report similar results in which they state that vibriocidal antibodies level in sera is short-lived after the immunization with *V. cholerae*, and it is only an indirect consequence of the colonization of *V. cholerae* in the small intestine, but the most important fact is that a long term local immunity is assured and it is independent from the presence of serum antibodies [1,2,17].

Accordingly to seroconversion criteria in cholera, the significant differences detected between vaccinated and placebo groups ($p < 0.0001$) indicate first; that the vaccine efficiently overcomes the gastric barrier and efficiently reaches the intestinal mucosa and secondly it activates the immune system to produce an immune response including an indirect vibriocidal antibodies response [18]. This finding mainly indicates that an active homotypic response has been established in a vaccinated group as a consequence of the proper colonization of the intestinal epithelia by the 638 strain [8–10].

The immune response and the excretion results observed in this study support the idea that repetitive dosing could not be necessary [18]. Like the natural infection, a single dose of 638 vaccine, as the other oral live cholera vaccines, stimulates the local immunity and could assure a lifelong protection. This is a consistent advantage of live vaccines over killed vaccines.

The significant increment of the GMT and the 100% of the seroconversion rates of vibriocidal antibodies reached after the day 14 of vaccination in the vaccine group suggest a high immunity conferred by the inoculum. Though there is no a global consensus about a protection correlate in cholera following the level of vibriocidal antibodies, in this study it was found that the 88% of the vaccinated subjects having vibriocidal antibodies titers of 1280 or higher could be potentially protected against an episode of cholera infection [19,20]. In addition, considering that in a previous challenge study, most of the volunteers with vibriocidal antibodies titers over 320 were protected against the diarrhea caused by a virulent *V. cholerae* strain [10], is possible that vaccinated subjects in this study with vibriocidal antibodies titers from 320 to 640, could also be protected; at least against the most severe manifestations of the disease. Nevertheless there is no consensus on a surrogate vibriocidal antibody level of protection against cholera [20,21].

An advantage of this lyophilized live attenuated vaccine which contains the 638 strain as its active pharmaceutical ingredient is that the pharmaceutical development did not affect its growth rate *in vitro* and *in vivo*. Our current results confirm the previous report [8–10] that indicates that the attenuated 638 strain effectively colonizes the intestinal tract of humans. However, it is considered necessary the occurrence of an active colonization of *V. cholerae* of the small intestine, in order to produce a protective local mucosal immune response [18]. *V. cholerae* is frequently isolated in feces of infected subjects and in fact it could explain the relative increment of the solicited adverse event incidence in vaccine group.

The lyoprotectors and the antiacid used into the vaccine formulation were suitable to make the 638 vaccine, safe, well tolerated and immunogenic in healthy adult volunteers in Cuba where there is no endemic exposure to *V. cholerae*. That could be useful for the control of endemic or epidemic cholera, but the 638 vaccine still should be tested in advance under phase I–II clinical trial in presumed-healthy adults and children volunteers from endemic areas with additional different environmental conditions and move forward to a phase III clinical trial to assess the potential efficacy of this vaccine.

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