Available online at www.sciencedirect.com
SCIENCE @ DIRECT®
Vaccine 24 (2006) 3381–3387
Vaccine
www.elsevier.com/locate/vaccine

Formulation in tablets of a cholera whole cells inactivated vaccine candidate

A. Talavera [a,*], G. Año [a], Y. Pino [a], J. Castaño [b], E. Uribarri [b], L. Riverón [a], S. Gil [a], S. Fernández [a], B. Cedré [a], T. Valmaseda [a], J.L. Pérez [a], J.F. Infante [a], L. García [a], G. Sierra [a]

[a] *Finlay Institut, 27 Ave. # 19805, La Lisa, Ciudad de la Habana, A.P. 16017, Cod. 11600, Cuba*
[b] *Laboratories MEDSOL, Havana City, Cuba*

Received 28 July 2005; received in revised form 8 December 2005; accepted 21 December 2005
Available online 19 January 2006

Abstract

Licensed as well as candidate cholera vaccines available at the present requires the dose preparation (included buffer) at the moment of application. The aim of this work was to evaluate the presentation in oral tablets of an inactivated cholera vaccine to avoid that inconveniences during application. We have therefore compared inactivated cultures of *Vibrio cholerae* with tablets formulation vaccine. We obtained that antigenic activity (ELISA) and immunogenicity in animal model (ELISA and vibriocidal tests) of *V. cholerae* inactivated cell remained unaltered in the final tablet formulation. The results suggest that the oral tablet formulation could be a useful pharmaceutical form in order to produce a new and affordable cholera vaccine.
© 2006 Published by Elsevier Ltd.

Keywords: Cholera; Vaccine; Oral tablets

1. Introduction

Cholera is an infectious disease characterized by severe, profuse and rice-water stools. The diarrhoea can be quite severe and can even cause death within a period of 5 h following the onset of symptoms if the patient does not receive adequate medical treatment.

Cholera infection is most common among populations inhabiting underdeveloped areas or regions affected by natural disasters, wars or other destructive events, which bring about poor sanitary conditions [1].

Antibiotic treatments and re-hydrating therapy are generally enough to reduce cholera mortality to less than 1%. On the other hand, cholera convalescence leads to strong and long-lasting protective antibacterial immunity. This specific immune protection is associated to the presence of serum vibriocidal antibodies as indicator of human protection [2,3]. The antibacterial response is mainly directed against the lipopolysaccharide (LPS, O antigen), and it was demonstrated by experimental passive protection of serum from volunteers inoculated with attenuated strain 638 of *Vibrio cholerae* O1 in animal models [4].

In the last 15 years different types of oral cholera vaccines have been developed, derived from either whole inactivated cells or from live attenuated ones that are referred subsequently:

An inactivated whole cells oral vaccine supplemented with recombinant cholera toxin B-subunit (B-WC) has been extensively tested in field trials [5–8]. Another inactivated whole cell vaccine but without CTB has been locally produced and evaluated in Viet Nam [9]. In general these vaccines are safe and well tolerated but one of them is expensive and both require more than one dose beside to provide protection.

Oral “single dose” attenuated *V. cholerae* strains constitutes another approach in the field of cholera vaccine. Several

* Corresponding author. Tel.: +53 7 2716911; fax: +53 7 2086075.
E-mail address: atalavera@finlay.edu.cu (A. Talavera).

0264-410X/$ – see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2005.12.064

live attenuated vaccine candidates have been developed and classical Inaba CVD 103 HgR constituted the most widely evaluated [10,11].

Directed to El Tor biotype, two attenuated strains have been reported as immunogenic and nonreactogenic in volunteers: 638 ($\Delta CTX\Phi$, hap::celA) [12] and Peru 15 ($\Delta CTX\Phi$,CTB^{+}, motility defective) [13].

Although above mentioned vaccines and vaccine candidates have shown usefulness, their pharmaceutical presentations present difficulties during preparation for application, because they require the dose preparation in the moment of application and co-administration of appropriated buffer solution. We present in this work the development of a oral tablet formulation of a cholera inactivated vaccine, and the evaluation of their antigenicity and immunogenicity in animal model.

2. Materials and methods

The active pharmaceutical compound was developed and obtained at Finlay Institute and the tablets were prepared at MEDSOL Laboratories. In general we described in this paper the tablet formulation with the evaluation of the possible interferences of the excipients with analytical test, interferences between the excipients and the vaccine's active compound. Finally we studied the antigenicity of the vaccine and its immunogenicity in adult rabbit model.

2.1. Active compound

One tablet of vaccine contain as active pharmaceutical compound heat-killed whole cells of *V. cholerae* strain C7258 (7 mg per tablet) belonging to serum group O1, serum-type Ogawa and biotype El Tor. The culture was conducted as described by Año et al. [14], but the bacterial biomass was obtained in fermentor (Chemap, Switzerland) with work volume of 20 L. Briefly, the culture media was Triptone Soy Broth (BIOCEN, batch 3500003), the cultures were done during 3–4 h at 37 °C, 400 rpm and pH controlled at 7.3. The cultures were inactivated by heat treatment for 20 min at 60 °C. After this, the bacterial cells were washing by tangential filtration against PBS.

2.2. Preformulation assays

2.2.1. Interference determination of excipients–ELISA and between excipients and the vaccine's active compound

To evaluate the compatibility between excipients and active pharmaceutical ingredient is necessary to known the excipients–ELISA interferences, if any. The evaluated excipients were; sodium carboxymethyl starch, croscaramellose, povidone, talc, magnesium stearate, titanium dioxide, silicon dioxide, corn starch and lactose.

Inactivated cells and its mixtures, including each of the different excipients, as well as separate solutions of excipients, were studied. The inactivated cells were employed as a positive control group and five replicas of each variant were carried out. In all cases the concentrations of inactivated cells were adjusted to 7 mg/mL (dry weight).

ELISA plates (Maxisorp, Nunc) were coated overnight at 4 °C with 100 μL/well of *V. cholerae* inactivated cells, excipients or an inactivated cells–excipients mixture, dissolved in phosphate buffer saline (PBS) (pH 7.4). Plates were washed with distilled water containing 0.05% (v/v) Tween 20 (Merck) and blocked with skim milk 2% in PBS. After washing an anti-LPS (4D2G5) monoclonal antibody diluted 1:8000 was dispensed in the plates and incubated 2 h at room temperature. Following another wash step, 100 μL/well of an anti-mouse IgG antibody conjugated with type VI horseradish peroxidase (Sigma Chemical Co., St. Louis, MO), diluted 1/2000 in PBS with 0.05% Tween 20 and 1% skim milk were added to plates which were incubated at room temperature for 2 h. The reaction was revealed with *ortho*-phenylene diamine (Sigma) 0.4 mg/mL, 0.04% H_2O_2 in citrate phosphate buffer (pH 5). At 15 min the reaction was stopped by addition of 50 μL/well of H_2SO_4 2.5N. The absorbance values at 492 nm were measured in a Titertek Multiskan ELISA reader. The monoclonal antibody used 4D2G5 anti-LPS Ogawa, was supplied by the Monoclonal Antibodies Department, Finlay Institute.

The antigenicity of the mixtures containing a suspension of inactivated *V. cholerae* cells and the different excipients used was determined by ELISA described before. The study was carried out at time of mixing and after 30 days of incubation at 4–6 °C. The excipients used were: lactose, starch, sodium carboxymethyl starch, croscaramellose, povidone, talc, magnesium stearate and titanium dioxide. Twelve replicas of each excipient were done.

2.3. Tablets formulation

Tablets were obtained by the humid traditional way. The mix, humectation and lubrication were performed in a Rotor Junior Zanchetta mixer. The granulated was dried in a Fluid Bed System (Viani). Physical–chemical and technological properties were determined following Standard Operation Procedures of MEDSOL.

Granulated were compressed in a Rotary Tablet Press RONCHI with a 12.7 mm (1/2 inch) normal concave to obtain nominal weight tablets of 700 mg, which were analyzed physically–mechanically and technologically following Standard Operation Procedures of MEDSOL.

2.4. Antigenicity of tablets

The antigenicity of the tablets was determined through an ELISA inhibition test aimed at detecting the lipopolysaccharide (LPS) of *V. cholerae* in the tablets.

Samples preparation: LPS from *V. cholerae* O1; Ogawa and from *V. cholerae* O139 was purified by hot phenol method [15].

Suspension cells adjusted to 7 mg/mL (dry weight) of live or inactivated cultures of *V. cholerae* C7258 strain were obtained as described before [14] and dispersed tablets, were used as samples for ELISA.

The purified LPS were diluted 1:2 in PBS from 120 to 0.12 μg/mL. Bacterial suspensions were diluted 1:5 in the same buffer. The tablets, containing 7 mg of inactivated *V. cholerae*, were dispersed by gentile agitation in 4 mL of PBS, the supernatant was centrifuged (10 000 rpm × 15 min) and finally the pellet was suspended in 400 μL of PBS and used as described for bacterial suspension.

Polystyrene plates (Maxisorp, Nunc, Denmark) were coated with 100 μL/well of LPS Ogawa of *V. cholerae* at a concentration of 25 μg/mL, dissolved in a phosphate buffer solution (PBS), and incubated overnight at 4 °C, all incubation were done in a wet chamber. The plates were washed and as described in Section 2.2.1. Following the wash, plates were blocked with 150 μL/well of skim milk at 1% in a PBS, and incubated for 1 h at 37 °C.

The samples were incubated for 1 h at room temperature in solutions with a dilution ratio of 1:2 in anti-LPS Ogawa monoclonal antibodies (4D2G5, 25 μg/mL) dissolved in a PBS plus 0.05% Tween 20. One hundred microlitres of mixtures were added per well, incubate for 2 h at room temperature. After wash, the reaction was revealed, stopped and measured as described in Section 2.2.1.

2.5. Immunogenicity

Vaccine preparation immunogenicity was evaluated using adult rabbit intraduodenal inoculation model [16]. Locally supplied, 2–2.5 kg New Zealand White rabbit were used. The experiments and procedures were approved by Finlay Institute's Committee for the Care and Use of Laboratory Animals. After a simple laparotomy, the inoculum was administered in two doses, at cero and 14 days, in the duodenum luminal space. Five animals received vaccine inoculum from each one of five tablets batches, five rabbits received inactivated cultures of C7258 strain (active compound, adjusted to 7 mg/mL dry weight) as positive control and three were used as negative control and received phosphate-buffered saline, PBS, instead dissolve tablet. Suspensions containing the tablet formulation, and inactivated cells of *V. cholerae* El Tor Ogawa C7258 strain, were prepared as we described before and used as samples.

Blood was collected on days 0, 7, 14, 21, 28, 35 and 42 and antibacterial serum antibodies were measured by ELISA [17] with LPS of *V. cholerae* Ogawa and vibriocidal titter, as it is described elsewhere [18,19]. For both methods we used 1:2 as dilution factor. The first method used purified LPS as solid-phase antigen and horseradish peroxidase anti-rabbit IgG conjugate (1:2000) (Sigma Chemical Co., St. Louis, MO). The antibody titter was considered to be the interpolated dilution of the sample giving an absorbance value of 0.4 above background.

Serum vibriocidal antibody titters were measured in a microassay with *V. cholerae* Ogawa serotype VC12 strains as reference and human complement diluted 1:5. Vibriocidal titter was calculated as the highest dilution of serum causing complete inhibition of bacterial growth.

2.6. Statistical analysis

In the study of interferences of excipients with ELISA test was done by Box and Whisker Plot. To determine the interference between excipients with the vaccine's active compound was carried out the variance analysis followed by a test of comparison of multiple range and calculated the percentages of the average ELISA test obtained at 30 days respect to 0 day. The acceptance criterion was ±10% or less. In the immunogenicity assay, logarithmic transformation was used in base 10 of the inverse of the titter, it was verified the normal distribution and variance equality. For the comparison among groups were applied ANOVA and the test of multiple ranges (LSD) as well as Kruskall–Wallis.

The tests were carried out using the statistical package Statgraphics Plus for Windows 2.1, with a level of significance of $p < 0.05$.

3. Results and discussion

The prevention of cholera by vaccination is recommended in endemic areas (developing countries) or in disasters, in both scenarios, the hygienic-sanitary conditions, health infrastructure, communications and cold chain for products are very poor. For that reasons, a vaccine against this illness, besides being oral, sure and effective, it should be of easy dosage and application, as well as to tolerate high environmental temperatures. In the developed pharmaceutical forms, capsules and tablets are those that better possibilities to fulfill the requirements before mentioned and among them, the tablets, in general, are of smaller costs. For it, we evaluate the possibility to achieve a vaccine against the cholera in tablets, maintaining their fundamental characteristic of antigenicity and immunogenicity.

3.1. Preformulation assays

3.1.1. Determination of excipients–ELISA interferences

There are not many examples of vaccine formulation in tablets. For this reason is not possible to determine the compatibility between active compound and excipients by the same techniques used in pharmaceuticals products. In order to complete this objective was proposed to carry out ELISA technique.

The results showed that sodium carboxymethyl starch presented a strong interference with ELISA. The cornstarch and PVP were on the acceptation limit. When the other excipi-

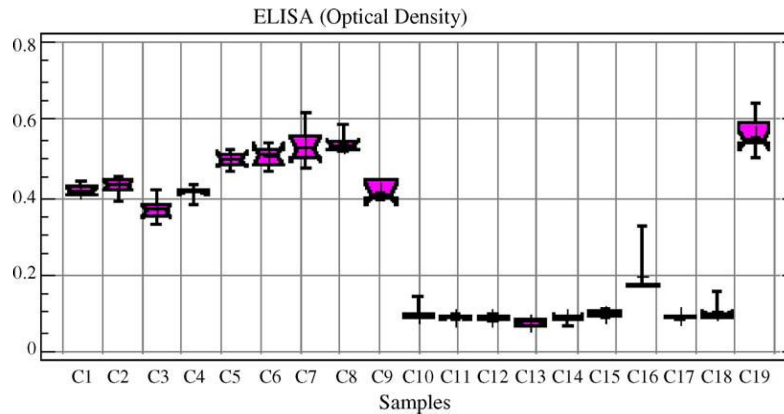

Fig. 1. Study of possible interferences of tablet excipients using the ELISA technique to evaluate the LPS of *Vibrio cholerae*. From C01 to C09: mixture of excipients with inactivated cells; C10–C18: excipients alone; C19: inactivated cells alone. Excipients used in each of the samples—C01 and C10: lactose; C02 and C11: corn starch; C03 and C12: sodium carboxymethyl starch; C04 and C13: talc; C05 and C14: povidone (PVP); C06 and C15: sodium croscaramellose; C07 and C16: magnesium stearate; C08 and C17: titanium dioxide; C09 and C18: siliceous dioxide 1500; C19: inactivated cells.

ents evaluated were mixed with the vaccine active compound showing similar results to the single inactivated cells used as positive control, while the unmixed excipients offered significantly lower values as shown in Fig. 1. Most excipients did not showed interference with the ELISA. These results permit us the utilization of the referred ELISA in order to evaluate the compatibility of the active compound with different excipients.

3.1.2. Interference between excipients and the vaccine's active compound

The lipopolysaccharide antigenicity of the inactivated cells was no statistically affected when were mixed with the excipients. The ELISA values of different evaluated mixtures were maintained below 10% of difference throughout 30 days of incubation at 4–6 °C (Fig. 2). These results permitted the selection of excipients needed to elaborate the final formulation with technological criteria.

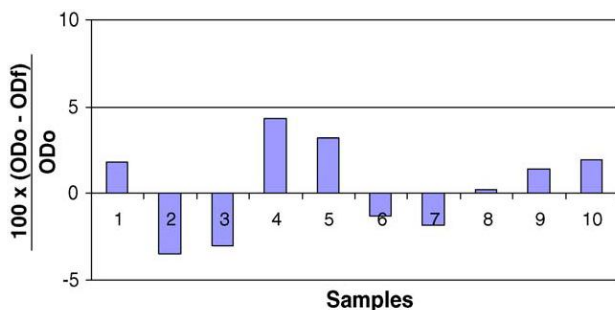

Fig. 2. Determining possible effects of the excipients on the antigenicity of the vaccine's active complex. The graph represent the percentages of the average ELISA result values for samples incubated at 4–6 °C for 30 days, with respect to the ELISA result values for the same samples evaluated at time 0. The evaluated excipients were: (1) lactose; (2) corn starch; (3) sodium carboxymethyl starch; (4) sodium croscaramellose; (5) povidone (PVP); (6) talc; (7) magnesium stearate; (8) titanium dioxide; (9) inactivated cells. The criteria of acceptance was ±10% or below. OD_0: initial optical density of ELISA lectures; OD_f: final optical density of ELISA.

Similar results were described before using different antigens as purified O antigen [20] or inactivated whole cell of Gram positive bacteria [21].

3.2. Tablets formulation

Granulated and tablets fulfill all traditional control parameters of wide use in the tablets production technology such as: flow speed, repose angle, distribution of particle size, medium diameter, porosity and others one.

The obtained granulated (Table 1) showed good fluid characteristics by Carr index values classified as excellent for values between 5 and 15%. A homogeneous distribution was achieved of particle size and that influences in the solubility and therefore in the product absorption and its therapeutical effect.

Densities show values inside established limit, that permit an efficient full of dies during the compression process. In general, all parameters show acceptable values for granulates.

In the other hand, the compression process was carried out adequately and tablets with good mechanical properties (weight, thickness, hardness, friability and disintegration) were obtained (Table 2).

In the evaluated properties, for this formulation, low variability in the measured parameters was appreciated and there was reproducibility in the results. These allow a dependability index, due to in all cases values in the specification ranges were observed.

3.3. Antigenicity of tablets

The antigenicity studies of tablets (Fig. 3) showed a high level of inhibition when the tablet suspension was utilized in the competence ELISA against monoclonal antibody (anti-LPS), with similar values to those obtained when the live cells, the inactivated cells or the LPS Ogawa were used. No inhibition was observed in the case involving the LPS O139.

Table 1
Technological characteristics of granulates used in the compression process for vaccine tablets

Parameter	Lot 1	Lot 2	Lot 3	Medium	Limit
Apparent density (g/cm^3)	0.57	0.57	0.54	0.56	0.4–0.7
Tapped density (g/cm^3)	0.64	0.61	0.62	0.62	0.4–0.7
Flow speed (g/segcm^2)	24.12	20.28	27.1	23.83	>7
Repose angle (°)	25	31.9	31.8	29.57	<30
Powder medium diameter (μm)	336.44	392.7	283.2	337.45	–
Powder. fine dust (%)	7.60	16.00	14.10	12.57	–
Residual humidity (%)	1.42	0.94	0.88	1.08	0.8–1.4
Real density (g/cm^3)	1.4	1.5	1.5	1.47	>1
Index Carr (%)	10.90	6.55	12.90	10.12	5–15
Index Hausner (%)	1.12	1.07	1.09	1.09	<1.25
Porosity	59.20	62.00	62.00	61.07	<60

This demonstrates that the LPS antigenic activity of the inactivated cells remained unaltered in the final tablet formulation (up to dilution 1:3125) after the technological process.

3.4. Immunogenicity of tablets

Protective immunity against cholera is generally considered as being strictly antibody mediated. Antibodies against cholera toxin (which causes the characteristic diarrhoea) are believed to have minimal contribution to protection. The best assessment of protection against cholera is the presence of antibacterial serum vibriocidal antibodies, after mucosal vaccine administration, the latter are believed to correlate with intestinal s-IgA associated with protection by preventing the adherence [22].

No single animal model can readily reproduce all the aspects of human disease [23]. However, several of them have been used to evaluate genetically attenuated strains virulence and colonizing capability [24]. One of them is the intraduodenal inoculation model to evaluate the immunogenicity. This model is easy to use and these inoculations are capable of stimulating an immune response without to need the neutralization of gastric acid. Despite the fact that this method does not reproduce natural human mechanisms, it is of election in order to know if the products or samples themselves are capable to induce an immune response without gastric interference [16].

The results of immunogenicity evaluation of tablets using adult rabbit intraduodenal inoculation model are presented in

Table 2
Mechanical properties of obtaining tablets

Parameters	Lot 1 (S.D.)	Lot 2 (S.D.)	Lot 3 (S.D.)
Weight (mg)	708.72 (13.97)	704.03 (9.46)	710.07 (11.45)
Thickness (mm)	5.63 (0.24)	5.61 (0.28)	5.39 (0.14)
Hardness (kg FM)	5.68 (0.73)	5.74 (0.73)	7.16 (2.18)
Friability (%)	0.14	0.15	0.15
Desintegration time (min)	10	10	13

S.D.: standard deviation for 20 tablets tested for each batch.

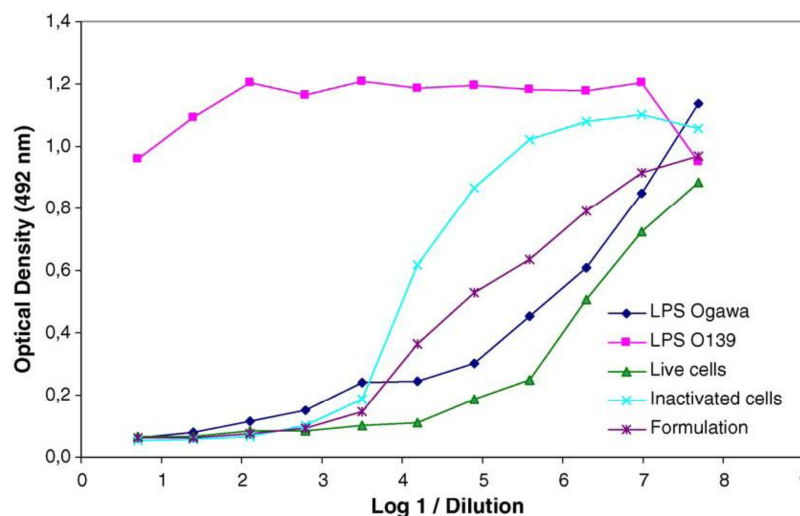

Fig. 3. Determining the antigenicity of the lipopolysaccharide (LPS) of *Vibrio cholerae* in the final tablet formula by ELISA monoclonal antibody anti-LPS inhibition test. Purified LPS were diluted 1:2 from 120 to 0.12 μg/mL. Bacterial suspensions were serial diluted 1:5 from 7 mg/mL dry weight. Tablets were suspended by gently agitation in PBS, the supernatants were centrifuged and the final pellets were dissolved in 400 μL of PBS and serial diluted 1:5. All samples were incubated with anti-LPS Ogawa monoclonal antibodies.

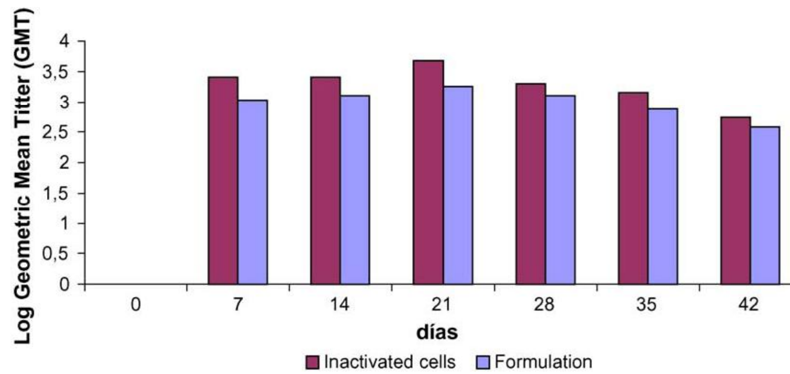

Fig. 4. Titters anti-lipopolysaccharide *V. cholerae* O1, Ogawa, detected by ELISA in serum of rabbits intraduodenal inoculated with inactivated cells or with the tablet vaccine. Five animals received vaccine inoculum from each one of five tablets batches, five rabbits received inactivated cultures of C7258 strain and three were used as negative control and received phosphate-buffered saline, PBS, instead dispersed tablet. Suspensions containing the tablet formulation, and inactivated cells of *V. cholerae* El Tor Ogawa C7258 strain, were prepared as we described in the text before and used as inoculums. The serums of each rabbit were processed by ELISA IgG against purified LPS-Ogawa. The antibody titter was considered to be the interpolated dilution of the sample giving an absorbance value of 0.4 above background. It is showed as geometric mean of Log 1/titter.

Figs. 4 and 5. Two doses were applied by the same surgical procedure and seven serum samples were taken from inoculation day up to 42 days later. Serum vibriocidal titter and ELISA IgG anti-Ogawa LPS antibodies were measured. For both methods there was a rapid titter increase in the first 7 days and the values remained high during the rest of the study. No significant differences between active compound and tablets immunogenicity were found. There was not an increase of immune response after second dose (14 days after the first one) by any of two methods. In challenge studies in volunteers inoculated orally with attenuated strain of *V. cholerae*, a booster response in vaccines after the virulent strain inoculation, have not been clearly identified, may be due to immune response evoked by vaccine inoculation [22,24]. On the other hand, for orally administered inactivated cholera vaccines, at least two doses are recommended [6,9,28]. We have not previous report of the application of this animal model for more than one dose.

Previously we have published the use of intraduodenal inoculation model to evaluate the immunogenicity of attenuated *V. cholerae* as compare with its parental ones [16]. Specifically the vaccine candidate strain 638 (*V. cholerae* O1 El Tor Ogawa ΔCTXϕ, hap::celA) attenuated strain showed a very similar antibodies titters and response kinetic than results presented in this paper. In 14 days serum sample vibriocidal titter and ELISA (IgG) geometric mean titter were 3.2 and 3, respectively, meanwhile this same figures in the firs report were 3.0 and 2.17. The immunogenicity evaluation of 638 strains was the last preclinical study prior conduction healthy volunteers reactogenicity, immunogenicity and protective capacity trials. After that, in several controlled clinical trials, this strain demonstrates that in one single dose, it is well tolerated, immunogenic and able to afford 1 month protection (period of time evaluated in challenge trials) [12,25].

Some of cholera vaccines are applied with protection to gastric barrier, mainly the attenuated vaccine [26] and one of

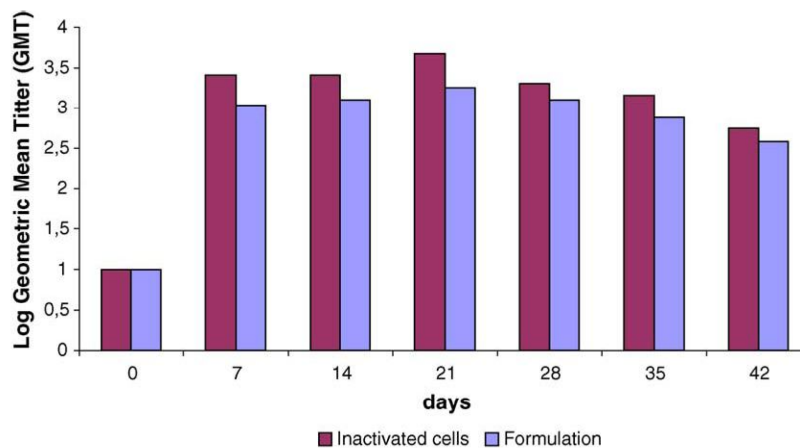

Fig. 5. Serum vibriocidal titters of rabbits intraduodenal inoculated with inactivated cells or with the tablet. The samples and animals inoculated are the same as described in Fig. 4. The vibriocidal tests were done in a microassay with *V. cholerae* Ogawa serotype VC12 strains as reference and human complement. Vibriocidal titter was calculated as the highest dilution of serum causing complete inhibition of VC12 strain growth and it is showed as geometric mean Log 1/titter.

whole cell inactivated vaccine with B-subunit cholera toxin (BS-WC) [27]. In the first ones type, it is necessary to achieve bacterial viability in the intestine and in the second one, to maintain the immunological activity of recombinant cholera toxin. However, particularly, the whole cell inactivated vaccine (WC) developed in Viet Nam was safe and immunogenic and showed similar results when it was administrated with or without buffer for protection against gastric acid [28] and in general the inactivated whole cell vaccine may not require a buffer for delivery [29]. In this way, our vaccine formulated in tablet is a WC without BS, in this sense; it would be possible that it would not need gastric protection for application, nevertheless, it will be necessary evaluate in clinical trail.

In general, as our results shown, the lipopolysaccharide antigenicity of the inactivated cells was not affected by the presence of different excipients, the capability to obtain tablets by standard technology using inactivated *V. cholerae* suspension as active compound, the antigenicity and the immunogenicity (in terms of ELISA IgG and vibriocidal test) in animal model of LPS of the inactivated cells are present in the final tablet formulation, we concluded that the tablet formulation could be a useful pharmaceutical form in order to produce whole cell inactivated vaccine against cholera, and recommend preclinical and stability studies and clinical trail including enteric coating necessity studies.

References

[1] Word Health Organization. Health Topics. Cholera, 2005. http://www.who.int/topics/cholera/.

[2] Glass RI, Svennerholm AM, Stoll BJ, Khan MR, Hossain KM, Huq MI, et al. Protection against cholera in breast-fed children by antibodies in breast milk. N Engl J Med 1983;308:1389–92.

[3] Jertborn M, Svennerholm AM, Holmgren J. Evaluation of different immunization schedules for oral B subunit vaccine in Swedish volunteers. Vaccine 1993;10:130–2.

[4] Pérez JL, García LG, Talavera A, Oliva R, Balmaceda T, Año G, et al. Passive protection of serum from volunteers inoculated with attenuated strain 638 of *Vibrio cholerae* O1 in animal models. Vaccine 2000;19:376–84.

[5] Svennerholm AM, Holmgren J. Oral combined B subunit-whole cell cholera vaccine 1986. In: Holmgren J, Lindberg A, Mollby R, editors. Development of vaccines and drugs against diarrhea, proceedings of the 11th novel conference. 1985. p. 33–43.

[6] Holmgren J, Svennerholm AM, Lonroth I, Fall-Persson M, Markham B, Lundback H. Development of improved cholera vaccine based on subunit toxoid. Nature 1977;269:602–4.

[7] Sánchez JL, Holmgren J. Recombinant system for overexpression of cholera toxin B Subunit in *Vibrio cholerae* as basis for vaccine development. Proc Natl Acad Sci USA 1989;86:481–5.

[8] Lucas ME, Deen JL, von Seidlein L, Wang XY, Ampuero J, Puri M, et al. Effectiveness of weight oral cholera vaccination in Beira, Mozambique. N Engl J Med 2005;24(8):757–67.

[9] Trach DD, Clemens JD, Ke NT, Thuy HT, Son ND, Canh DG, et al. Field trial of a locally produced, killed, oral cholera vaccine in Vietnam. Lancet 1997;349:231–5.

[10] Holmgren J, Svennerholm AM. Vaccines against diarrheal diseases. In: Perlmann P, Wigzell H, editors. Vaccines. Berlin: Springer-Verlag; 1999. p. 290–328.

[11] Richie E, Punjabi NH, Sidharta Y, Peetosutan K, Sukandar M, Wasserman SS, et al. Efficacy trial of single-dose live oral cholera vaccine CVD 103-HgR in North Jakarta, Indonesia, a cholera-endemic area. Vaccine 2000;18:2399–410.

[12] Benítez JA, García L, Silva A, García H, Fando R, Cedré B, et al. Preliminary assessment of the safety and immunogenicity of a new CTXϕ-negative, haemaglutinin/protease-defective El Tor strain as a cholera vaccine candidate. Infect Immun 1999;67:539–45.

[13] Kener JR, Coster TS, Taylor DN, Troffa AF, Barrera-Oro M, Hyman T, et al. Peru-15, an improved and attenuated oral vaccine candidate for *Vibrio cholerae* O1. J Infect Dis 1995;72:1126–9.

[14] Año G, Balmaceda T, Cedré B, Pino Y, Ancheta O, Pérez JL, et al. Antigenicity and immunogenicity of inactivated *Vibrio cholerae* strain. Biotecnología Aplicada 2003;20:9–13.

[15] Westphal O, Jann K. Bacterial lipopolysaccharides: extraction with phenol–water and further applications of this procedure. Methods Carbohyd Chem 1965;5:83–91.

[16] García L, Oliva R, Cedré B, Balmaceda T, García H, Talavera A, et al. Intrauodenal inoculation of adult rabbits for evaluating the immunogenicity of genetically attenuated *Vibrio cholerae* strains. Lab Anim Sci 1998;48(5):538–41.

[17] Cedré B, Año G, García H, Pérez JL, Talavera A, González I, et al. Validation of an ELISA assay to determine antibodies against LPS of *Vibrio cholerae*. Vaccimonitor 2003;12(1):11–7.

[18] Cedré B, García H, García L, Talavera A. Estandarización del ensayo Vibriocida modificado para la determinación de anticuerpos contra *Vibrio cholerae*. Revista Cubana de Medicina Tropical 1999;51(3):56–9.

[19] Cedré B, Rodríguez T, Año G, Pino Y, García H, González I, et al. Validation of colorimetric vibriocidal assay to determine serum antibodies against vaccine candidate strains of *Vibrio cholerae*. Vaccimonitor 2003;12(1):23–30.

[20] Djatilov IA, Eliseev JJ, Gromova OV, Taranenko TM, Dzhaparidza MN, Kireev MN, et al. Oral chemical vaccine for protecting against cholera. Patent RU2159128. I.A., 2000.

[21] Clancy RL. Non-adyuvanted vaccine. US Patent 4,873,090, 1989.

[22] Dietrich G, Griot-Wenk M, Metcalfe IC, Lang AB, Viret JF. Experience with registered mucosal vaccines. Vaccine 2003;21:678–83.

[23] Word Health Organization. Guidelines for the production and control of inactivated oral cholera vaccines, WHO Technical Report, Series No. 924, Annex 3, 2004: p. 129–149.

[24] García L, Díaz M, García HM, Rodríguez BL, Fernández R, Año G, et al. The vaccine candidate *Vibrio cholerae* 638 is protective against cholera in healthy volunteers. Infect Immun 2005;73(5):3018–24.

[25] Cedré B, García LG, García HM, Fariñas M, Talavera A, Infante JF. Intestinal colonization of the infant mouse model by attenuated and virulent *Vibrio cholerae* strains. Arch Med Res 1998;29(3):231–4.

[26] Sack DA, Shimko J, Sack B, Gomez JG, MacLeod K, O'Sullivan D, et al. Comparison of alternative buffer for use with a new live oral cholera vaccine, Peru-15, in outpatient volunteers. Infect Immun 1997;65(6):2107–11.

[27] Clemens JD, Jertborn M, Sack D, Stanton B, Holmgren J, Khan MR. Effect of neutralization of gastric acid on immune response to an oral B-subunit, killed whole cell cholera vaccine. J Infect Dis 1986;154:175–8.

[28] Trach DD, Cam PD, Ke NT, Rao MR, Dinh D, Hang PV, et al. Investigations into the safety and immunogenicity of killed oral cholera vaccine developed in Viet Nam. Bull World Health Org 2002;80(1):2–8.

[29] Walker RI. Considerations for development of whole cell bacterial vaccines to prevent diarrheal diseases in children in developing countries. Vaccine 2005;23:3369–85.