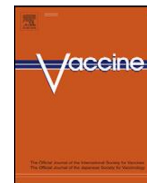
Vaccine 29 (2011) 5758–5764

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



A new oral vaccine candidate based on the microencapsulation by spray-drying of inactivated *Vibrio cholerae*

Gemma Año[a], Amaia Esquisabel[b,c], Marta Pastor[b,c], Arturo Talavera[a], Bárbara Cedré[a], Sonsire Fernández[a], Sergio Sifontes[a], Yisabel Aranguren[a], Gustavo Falero[a], Luis García[a], Rosa Lydia Solís[a], José Luis Pedraz[b,c,∗]

[a] *Finlay Institute, 27 Ave. #19805, La Lisa, Ciudad de la Habana, A.P. 16017, Cod.11600, Cuba*
[b] *NanoBioCel Group, Laboratory of Pharmaceutics, University of the Basque Country, School of Pharmacy, Paseo de la Universidad 7, 01006 Vitoria, Spain*
[c] *Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria, Spain*

ARTICLE INFO

Article history:
Received 7 January 2011
Received in revised form 25 May 2011
Accepted 27 May 2011
Available online 15 June 2011

Keywords:
Cholera vaccine
Enteric microparticles
Spray drying
Eudragit®

ABSTRACT

The aim of this work was to evaluate the microencapsulation by spray-drying of inactivated *Vibrio cholerae*, using methacrylic copolymers Eudragit® L30D-55 and FS30D. The microparticles obtained presented a particle size around 3.0 μm. The preparation temperature affected the morphology and the antigenicity of microparticles, but it did not affect the *V. cholerae* content. *In vitro* release studies showed that in acid medium less than 5% of bacteria was released, and in neutral medium, Eudragit® L30D-55 microparticles released 86% after 24 h, whereas FS30D released less than 30%. Rats inoculated with microparticles exhibited vibriocidal antibody titres. Microencapsulation by spray-drying of inactivated *V. cholerae* could be proposed as a method to obtain an oral vaccine which provides controlled release of the bacteria.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Cholera is a diarrhoeal disease still endemic in many parts of Africa and Asia, and becoming endemic in South and Central America. Moreover, disease incidence is increasing in recent years, presenting epidemics outbreaks that may occur in underdeveloped areas related to poor sanitation and hygiene and affecting the poorest and most vulnerable populations [1–3]. This bacterial disease is unlikely to ever be eradicated, and although re-hydration and antibiotic therapy could control the symptoms, these measures are quite difficult to apply in endemic areas of cholera. Therefore, control measures should be implemented, together with adequate methods of prevention, including the availability of vaccination and effective immunization programs.

To date, two cholera vaccines have been licensed, one of them is based on and attenuated live and genetically modified *V. cholerae* O1 strain (CVD 103-HgR), which although effective is not commercially available [1]. The second vaccine consists of killed whole cells oral vaccine supplemented with recombinant cholera toxin B-subunit (B-WC) and has been extensively tested in field trials [4,5]. This vaccine is administered in two doses and presents some limitations as the need of a large volume of liquid for administration which precludes its use in small children, the difficulties for storage due to the maintenance of cold chain, and the cost which remains to be high [1,6]. Due to these limitations several efforts have been carried out in order to development of an oral vaccine formulation, immunogenic and easy to administrate [7] and preferably in a single dose [8]. Another inactivated whole cell vaccine but without CTB has been locally produced and evaluated in Viet Nam [9,10]. This vaccine has provoked some controversies due to the limitations of the trial as there was not cholera cases confirmed during the first two years of the study and the amount of cells administered in could be exactly confirmed [11].

Microparticles have been widely used during the last decade for the controlled release of drug or bioactive agents, including antigens for vaccine formulation, providing potent and long-lasting immune response thanks to their inherent stimulatory properties, their ability to target gut-associated lymphoid tissue (GALT) and their capacity to protect antigens from degradation in gastric conditions [12]. However, low efficiency of microparticle uptake across the gut still remains a limiting factor and most vaccines are unable to induce immune responses when mucosally administered, requiring the use of a strong adjuvant for an effective delivery system. Traditionally, these formulations intended for oral vaccination have been based on poly-lactide-co-glycolide (PLGA) microparticles prepared by emulsion/solvent evaporation. Yeh *et al.*, have

∗ Corresponding author at: NanoBioCel Group, Laboratory of Pharmaceutics, University of the Basque Country, School of Pharmacy, Paseo de la Universidad 7, 01006 Vitoria, Spain. Tel.: +34 945013091; fax: +34 945013040.
E-mail address: joseluis.pedraz@ehu.es (J.L. Pedraz).

0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.05.098

reported the microencapsulation of *Vibrio cholerae* with PLGA polymer by this technique [13]. These polymers, although highly biocompatible and biodegradable, have some limitations, their cost is quite high and they require the use of organic solvents for their solubilization, and thus may denaturise antigens. Therefore, it is essential to develop a new, simple and effective process for oral vaccination by microparticles [14]. To avoid the use of organic solvents, alternative techniques have been reported for the encapsulation of drugs, such as ionic gelation, coacervation precipitation, and spray drying.

The spray drying technique has been widely used in the pharmaceutical, food and cosmetic industries for different purposes [15]. It is a one-step process, and offers the advantages of good production yield, reliability, reproducibility and ease to scale-up and it is only dependent on the solubility of drug and polymer [16,17]. Particularly, it can be applicable either to any type of drug, including water soluble, water insoluble and heat sensitive drugs, and recently it has gained interest due to its potential for the microencapsulation without the use or organic solvents [18]. Different polymers can be used to prepared microparticles by spray-drying. Among them, methacrylic acid copolymers (Eudragit®) appear to be interesting candidates since they are inert polymers, and are available in different presentations, including aqueous dispersions for enteric coatings [19]. Besides, they are of relatively low cost compared with other polymers used for the preparation of microparticles such as PLGA, making them attractive candidates for alternative encapsulation processes.

In the present study, we aimed to investigate the feasibility of the spray drying technique for the preparation of enteric microparticles with *V. cholerae* inactivated strain prepared with Eudragit® L30D-55 and FS30D as polymers. *V. cholerae* microencapsulation might allow the development of novel vaccines, which can be administered by mucosal routes but at the same time preserving antigenicity of the bacteria. In addition, it would significantly improve patient compliance by allowing immunization to be achieved without needles. This aspect would contribute to a lower cost of the vaccination and to an easy administration thus requiring fewer doses, which is suitable for developing countries to control cholera, [20]. Finally, it would also ease the administration in paediatric populations, the most susceptible to cholera disease in endemic areas.

2. Materials and methods

2.1. Bacterial strain

The bacterial strain of microparticles was *V. cholerae* C7258 inactivated strain, El Tor Biotype, Ogawa Serotype, developed and obtained by fermentation at Finlay Institute, Havana, Cuba, as previously described [21]. A *V. cholerae* (VC) cellular suspension with known concentrations, was prepared in Phosphate Buffer Saline (PBS: ClNa, ClK, PO_4HNa_2, PO_4HK_2 pH 7.4) to be used as standard in the BCA assay (bichinconinic acid assay), and ELISA Inhibition.

2.2. Materials

Two enteric polymers available as aqueous dispersions were employed to obtain the microparticles: Eudragit® L 30 D-55 which dissolves above pH 5.5 and Eudragit® FS 30D dissolving at a higher pH (>6.5). These polymers were a kind gift of Evonik Industries (Essen, Germany). Protein assay kit (MicroBCA) was purchased from Pierce, Teknovas (Bilbao, Spain). Anti-mouse IgG antibody conjugated with type VI horseradish peroxidase, ortho-phenylene diamine was supplied by Sigma Chemical Co. (St. Louis, MO, USA). The monoclonal antibody used 4D2G5 anti-LPS Ogawa, was supplied by the Monoclonal Antibodies Department, Finlay Institute. All other chemicals were analytical grade.

2.3. Microparticle preparation

Microparticles were prepared by a spray drying technique in a Büchi 191 Mini Spray Dryer (Büchi, Labortechnik AG, Flawil, Germany) with a standard 0.7 mm nozzle. The *V. cholerae* (VC) and polymers (Eudragit® L 30 D-55 or FS 30D) were mixed at 1:10 ratio by stirring the mixture for 20 min. Feed solution was pumped continuously at a rate of 5 L/min and 600 L/h of compressed air for atomization. Three inlet air temperature values (60, 80 and 100 °C) were used in order to evaluate their influence on the microparticles prepared.

2.4. Characterization of microparticles

2.4.1. Microparticle morphology and particle size

Microparticle size, morphology and surface appearance was examined by scanning electron microscopy (JEOL. JSM 6400, Japan). Microparticles were fixed previously on a brass stub using double-sided adhesive tape and they were made electrically conductive by coating, under vacuum, with a thin layer of gold-platinum (approximately 15–20 nm). The samples were observed to a voltage acceleration of 20 kV. Particle size was analysed by laser diffractometry using a particle size analyser Sympatec HELOS/KF-VARIO equipped with Vibri Rodos T 4.1 dispersion system (Sympatec GmbH, Clausthal-Zellerfeld, Germany). Each sample was analysed in triplicate.

2.4.2. Determination of total VC loading of microparticles (encapsulation efficiency)

Total *V. cholerae* loading in microparticles was estimated using colorimetric micro bicinchoninic acid technique (Micro BCA™). This method is specific for protein quantification, so firstly, protein proportion in *V. cholerae* cell suspension should be assessed. Bovine sera albumin (BSA) was used as standard and different *V. cholerae* cell suspension dilutions were assessed. Thus, linear relationship between *V. cholerae* concentration, expressed as UFC/mL, and protein concentration was established. Once the percentage of protein respect to active ingredient was determined, encapsulation efficiency of microparticles was conducted. Ten milligrams of each variant of microparticles were weighted and resuspended with 3.0 mL of PBS pH 7.4 by shaking overnight on a rotating shaker at 37 °C. After incubation, the suspension was analysed by the microBCA protein assay. Each sample was assayed in triplicate.

2.4.3. Antigenicity evaluation

The antigenicity of *V. cholerae* microencapsulated was determined through an ELISA inhibition test aimed to detect the lipopolysaccharide (LPS) in *V. cholerae*, which is the only immunogen proven to induce a protective immune response against cholera in humans [22]. Polystyrene plates (Maxisorp, Nunc, Denmark) were coated with 100 μL/well of LPS Ogawa of *V. cholerae* at a concentration of 25 μg/mL and incubated overnight at 4 °C.

Ten milligrams of each variant of microparticles were accurately weighted and resuspended with 3.0 mL of PBS pH 7.4 by shaking overnight on a rotating shaker at 37 °C. After incubation, they were diluted in a 1:10 ratio. Serial dilutions of samples were mixed with an anti-LPS Ogawa monoclonal antibody (4D2G5: 0.625 μg/mL) at room temperature for 1 h. The monoclonal antibody used 4D2G5 anti-LPS Ogawa, was supplied by the Monoclonal Antibodies Department, Finlay Institute. The mixtures were added to plate and incubated for 1 h. Following the wash step, 100 μL/well of an anti-mouse IgG antibody conjugated with type VI horseradish peroxidase, diluted 1/2000 were added to plates. The reaction was

Table 1
Groups of rats inoculated with Eudragit® L30 D55 and Eudragit® FS30D microparticles.

Group	Inoculum
I	Phosphate buffer saline
II	*Vibrio cholerae* (VC)
III	Eudragit® L30 D55 empty microparticles
IV	Eudragit® FS30 D empty microparticles
V	Eudragit® L30 D55:FS30D (50:50) (60 °C) empty microparticles
VI	Eudragit® L30 D55 (60 °C) – VC microparticles
VII	Eudragit® FS30 D (60 °C) – VC microparticles
VIII	Eudragit® L30 D55:FS30 D (50:50) (60 °C) – VC microparticles
IX	Eudragit® L30 D55 (60 °C) – VC microparticles (half dose)

revealed with *ortho*-phenylene diamine 0.4 mg/mL, 0.04% H_2O_2 in citrate phosphate buffer (pH 5). At 15 min the reaction was stopped by the addition of 50 μL/well of H_2SO_4 2.5 N and measured at 492 nm with an automatic microplate reader (Multiscan EX, Labsystems, Helsinki, Finland). A *V. cholerae* cellular standard was employed in order to obtain a calibration curve to calculate the concentration of the sample by interpolations in the calibration curve. The sample results were expressed as the per cent of the cellular standard antigenicity. Each sample was assayed in triplicate.

2.4.4. In-vitro release of V. cholerae from microparticles

The gastrointestinal tract conditions were simulated following USP XXV. A series of tubes, each containing 10 mg of microparticles dispersed in 1.0 mL of 0.1 N HCl, were incubated at 37 °C and continuously shaken on a rotational incubator. At 0, 1 and 2 h, sample tubes were centrifuged (2000 g, 5 min), the supernatant was completely removed to analyse the protein content by microBCA and fresh medium was added to the microparticles. After 2 h, the HCl was replaced by 1.0 mL of 0.05 M phosphate buffer ($Na_3PO_4.H_2O$, HCl: pH 6.8). Samples were centrifuged (2000 g, 5 min) at 2.5, 3, 4, 5, 7, and 24 h, the supernatant was removed and evaluated by microBCA. At each time point, fresh PBS was added to the microparticles. Results were expressed as % of *V. cholerae* released from microparticles compared to total *V. cholerae* encapsulated. The amounts of *V. cholerae* were corrected with the percentage of protein in *V. cholerae* cellular suspension. Release profiles were calculated in terms of cumulative release percentage versus incubation time. Each sample was assayed in triplicate.

2.4.5. Immunogenicity

The immunogenicity of microparticles was evaluated using an oral inoculation adult rat model (0.15–0.2 kg, female Sprague Dawley). The experiments and procedures were approved by Finlay Institute's Committee for the Care and Use of Laboratory Animals. The inoculum was orally administered in two doses, at days 0 and 14.

Animals were randomly distributed into nine groups, 10 rats in each one. Immediately before administration, the required dose of microparticles was weighted and resuspended in 1 mL of PBS. The characteristics of microparticles used for immunization are shown in Table 1. Eight groups of rats were orally given 1 ml PBS solution, 10^{10} CFU/rats of inactivated VC in PBS solution (equivalent to 7 mg of dry weight), 60 mg Eudragit® L30D55 – VC microparticles, 60 mg Eudragit® FS30D – VC microparticles, 60 mg Eudragit® L30D55 – Eudragit® FS30D (50:50) – VC microparticles and empty microparticles as control, respectively. One additional group of 30 mg Eudragit® L30D55 – VC microparticles (corresponding to 3.5 mg of VC) was included in order to assay the immunogenic effect of reduced dose of the microencapsulated *V. cholerae*. Taking account results of microparticles morphology and antigenic analysis, rats were inoculated with microparticles obtained at 60 °C. Blood samples were collected at the day of immunization (just before dosing) and at 7, 14, 21, 28, 35 and 42 days by retro-orbital sinus puncture. Serum was separated by centrifugation at 1300 g for 15 min and stored at −20 °C until assay. Serum vibriocidal antibodies were determined as described previously (23). Briefly, 50 μL of two-fold dilutions of decomplemented sera in PBS were mixed with 50 μL of a *V. cholerae* VC12 (serotype Ogawa) suspension containing 10^8 CFU/mL in PBS supplemented with human complement (w/v) and placed in 96-well microtitre plate. The mixture was incubated for 1 h at 37 °C and subsequently supplemented with 100 μL of Brain Heart Infusion Broth containing 2% (w/v) dextrose and 0.02% (w/v) bromocresol purple. The plates were incubated at 37 °C for 3 h. Bacterial growth was determined by color change of medium, which indicated bacterial dextrose consumption. Vibriocidal titre was calculated as the inverse of the highest dilution of serum causing complete inhibition of bacterial growth (no change of color in medium). The results were expressed as log 10 titre [23].

2.4.6. Dose–response assay

Microparticles dose–response was evaluated using a rat model. Rats were randomly divided into eight groups, 10 rats in each group. The inoculum was administered in two doses, at cero and 14 days, by oral route. Before inoculation, 60, 30, 15 and 7.5 mg of microparticles-VC were weighted per rat and resuspended in 1.0 mL of PBS immediately before administration. The other four groups were inoculated with 7, 3.5, 1.75 *y* 0.875 mg of VC, respectively. Blood samples were collected at day 21 of inoculation by retro-orbital sinus puncture. Serum was separated by centrifugation at 1300 g for 15 min and stored at −20 °C until assay. Levels of vibriocidal antibodies were analysed by the vibriocidal technique as described previously.

2.5. Statistical analysis

The yield of the microencapsulation process, particles size, total *V. cholerae* loading of microparticles results, antigenicity and immunogenicity were assayed by ANOVA and significant differences were tested at $p < 0.05$ with the statistical package Statgraphics Plus 5.0.

3. Results and discussion

Cholera is one of the oldest and best known diseases in the world, yet it continues to cause considerable suffering and needless deaths. Outbreaks are linked to crowd living conditions, inadequate or unprotected water supply and poor sanitation. These conditions are out of control in many of the developing countries, making almost all of them vulnerable to cholera outbreaks. An effective oral vaccine would mitigate the disease spread and to stabilise endemic areas [24]. It should be economic, of easy dosage and administration, as well as withstand high environmental temperatures. For this reason, the development of a satisfactory oral formulation remains a challenge. Among the possible strategies, microencapsulation represents an exciting approach to enhance the uptake and transport of orally administered vaccines.

3.1. Preparation and characterization of microparticles

We evaluated the feasibility of using a microencapsulation process by spray drying to achieve a vaccine against cholera, maintaining their antigenicity and immunogenicity properties. Table 2 summarises the average value of microparticles recovery (percentage of weight of the obtained microparticles taking as reference the total amount of product used for the preparation), as well as the outlet temperatures reached in each inlet temperature studied, for the microparticles prepared with Eudragit® L30D55 and FS30D polymers.

Table 2
Microparticle recovery, outlet temperatures reached and particle size obtained in the microencapsulation process of *V. cholerae* by spray drying.

Inlet T (°C)	Outlet T (°C)		Yield ± S.E. (%)		Particle size ± S.E. (μm)	
	L30 D55	FS30D	L30 D55	FS30D	L30 D55	FS30D
60	40	40	78.4 ± 4.2	72.6 ± 5.3	2.67 ± 0.05	2.91 ± 0.11
80	50	50	86.8 ± 6.5	73.9 ± 5.8	2.88 ± 0.08	3.16 ± 0.03
100	64	64	87.2 ± 6.3	74.2 ± 5.4	3.02 ± 0.05	3.41 ± 0.04

As reported by Esposito and co-workers [15], temperature of air-drying plays a major role in the dimensions of the microparticles obtained and in the production yield of the spray-drying process. However, to the best of our knowledge, there are few studies reporting the influence of air-drying temperature on the recovery rate of microparticles. Unlike the results shown by Esposito, we obtained the higher yields at high temperatures for the microparticles prepared with both types of Eudragits® (87.2 ± 6.3% for Eudragit® L30 D55 microparticles and 74.2 ± 5.4% for Eudragit® FS30D microparticles, both prepared at 100 °C), finding statistically significant differences at 60 °C with respect to 80 °C and 100 °C. Kusonwiriyawong et al. [25] also obtained higher yields when increasing inlet drying air temperature, reporting that in their study, this was the only parameter that affected the yield percentage. Besides that, similar studies of microparticles prepared by a spray-dried process have shown lower product yields than those we obtained [26–28]. A temperature increase in the nozzle head produces a higher heating at the desiccating chamber, thus facilitating a better evaporation of the aqueous polymers and decreasing microparticle humidity, reducing the loss by particle adhesion to chamber walls, and increasing the final product yield. Obviously, the temperature employed in the spray-drying process has to be compatible with the material to be dried and with the solvent used. Process developing at high temperatures and/or the use of organic solvents would guarantee a bigger recovery of microparticles, but could affect the active compound by altering the antigenic properties of *V. cholerae* cells.

Microparticle morphology was influenced by the temperature variation and polymer used. Only in the case of microparticles prepared with Eudragit® L30D-55 polymer at the lowest temperature (60 °C), particles appeared more spherical and had a smooth surface, as shown in Fig. 1(A). The rest of batches prepared at different temperatures (80 °C and 100 °C) or with Eudragit® FS30D (Fig. 1(B–F)) gave rise to crumbled, collapsed and irregular shaped microparticles.

The microparticle size is a key factor that controls the absorption of particulates across the oral mucosal tissue [29]. As reported by De Magistris [30], microparticles of a diameter <10 μm can be taken up by antigen presenting cells such as dendritic cells *in vitro* and *in vivo* and might have more opportunity for endocytosis in gut associated lymphoid tissue (GALT) to thus elicit an immune response for *V. cholerae* [31,32].

Air-drying temperature is also an important parameter that affects particle size, showing a significant effect ($p < 0.05$) for the microparticles prepared with the two polymers studied Eudragit® L30D-55 and FS30D. As displayed in Table 2, *V. cholerae* microparticle size was found to be between 2.67 and 3.41 μm. These microparticles would have a suitable size to allow uptake by M cells and thus might favour the antigen targeting to Peyer's patches in the intestine following oral administration [28].

With regards to the *V. cholerae* loading of the microparticles, a similar content was obtained in the Eudragit® L30D55-VC and FS30D-VC microparticles (Fig. 2). In all cases, 50–55 μg of VC was entrapped per each mg of polymer, showing encapsulation efficiencies of around 98.5%. The group of Yeh [13,33] reported similar values in PLG or PLA/PEG-blended microparticles prepared using the w/o/w solvent evaporation method, presenting encapsulation efficiencies between 82.6 and 97.8% depending on the type of polymer used.

3.1.1. Antigenicity studies

Several studies have shown that the protective mechanism against cholera is directed against both bacteria and cholera toxin. The antibacterial response is mainly against the lipopolysaccharide (LPS). It has been reported that most of vibriocidal antibodies can be absorbed by *V. cholerae* LPS, indicating its importance in the development of a protective immune response [22,34]. For this reason, the preservation of *V. cholerae* antigenicity in the microspheres, mainly the LPS, is an important factor to take into account when designing an oral vaccine against cholera.

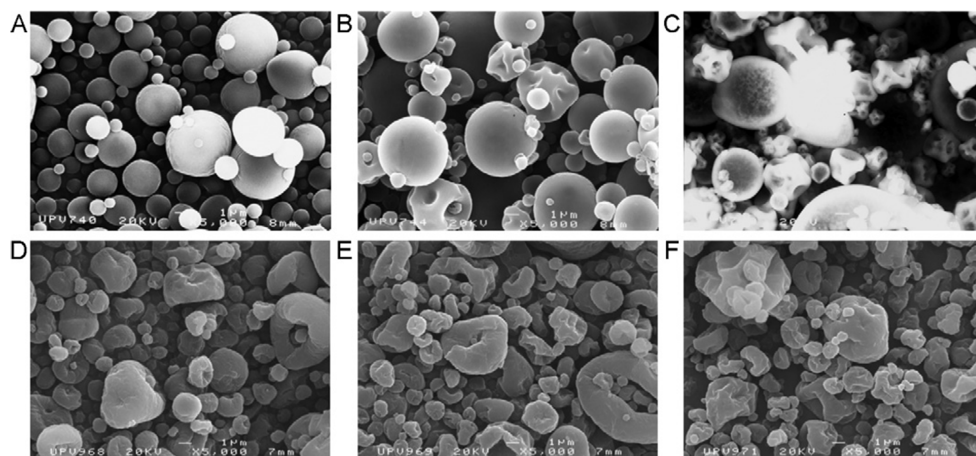


Fig. 1. Scanning electronic microscopy photographs of microspheres obtained by spray drying with Eudragit® L30D55 (A: 60 °C, B: 80 °C, C: 100 °C) and Eudragit® FS30D (D: 60 °C, E: 80 °C, F: 100 °C).

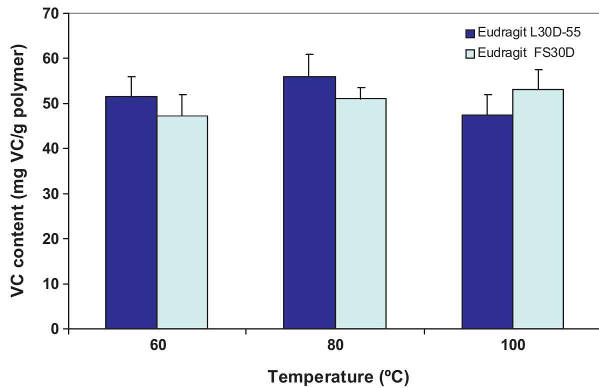


Fig. 2. *Vibrio cholerae* content in Eudragit® L30D-55 and FS30D microparticles, obtained at 60 °C, 80 °C and 100 °C.

The influence of microencapsulation process on the *Vibrio* antigenicity was determined by comparing the VC in the microspheres with a cellular standard. The LPS antigenicity determined in Eudragit® L30D55 – VC microspheres, at 60 °C and 80 °C represented approximately a 98% of the LPS antigenicity of cellular standard, whereas at 100 °C only represent an 88% (Fig. 3), results that were statistically significant ($p < 0.05$). On the other hand, antigenicity of *V. cholerae* encapsulated in Eudragit® FS30D was not maintained to the same extent (54–68%), although we did not observed statistically significant differences as a function of the temperature used in the preparation process (60 °C, 80 °C or 100 °C).

3.1.2. In vitro release study

The cumulative release profiles of VC-loaded microparticles are shown in Fig. 4. In acid medium (0.1 N HCl), less than 3% of VC was released from L30D55 and FS30D microparticles after 2 h of incubation. However, in intestinal medium (PBS pH 6.8), *V. cholerae* from Eudragit® L30D-55 microparticles prepared at 60 or 80 °C was slowly released reaching a 70% at 7 h and 86% at 24 h. Alternatively, less than 30% of *V. cholerae* was released from Eudragit® FS30D microparticles, at 24 h, regardless of the temperature used in the preparation procedure. Moreover, for both types of microparticles, a much lower release was observed when the air drying temperature of the spray-drying process was 100 °C.

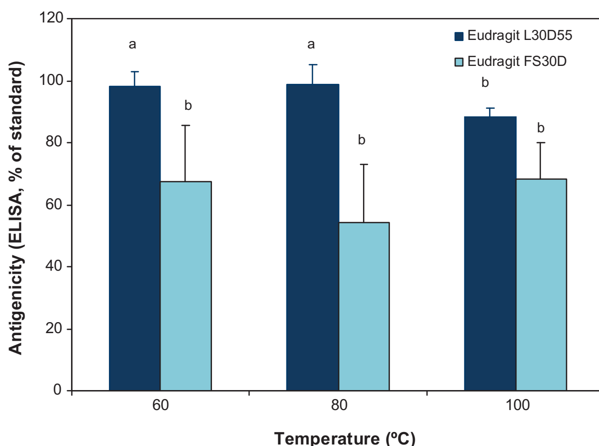


Fig. 3. *V. cholerae* LPS concentration in Eudragit® L30D-55 and FS30D microparticles obtained at 60 °C, 80 °C and 100 °C, respect to cellular standard, measured by ELISA Inhibition with MAb anti LPS 4D2G5. Different letters (a or b) indicate significant differences ($p < 0.05$).

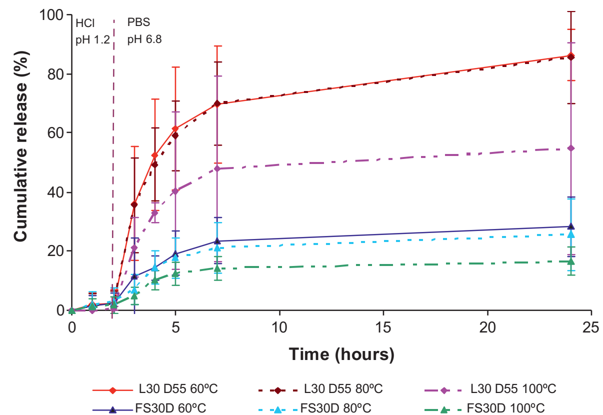


Fig. 4. *Vibrio cholerae* cumulative release profiles from Eudragit® L30D-55 – VC and Eudragit® FS30D – VC microparticles ($n = 3$).

According to these results, pH release dependence was evidenced for microparticles prepared with both type of polymers, indicating that *V. cholerae* could remain unaltered in gastric conditions, being released after the disintegration of the microparticle once in contact with intestinal fluids. Several authors have used Eudragits® for preparing microparticles presenting gastrorresistant properties [14,18,35]. Lin et al. [14] obtained similar results in a release study of *Micoplasma hyponeumoniae* from Eudragit® L30D-55 microparticles, showing that the microorganism was released after 2 h of incubation at pH 7.4. On the other hand, Eudragit® FS30D is a polymer soluble above pH 7.0, which explains the lower release of *V. cholerae* from microparticles at pH 6.8.

3.2. Immune response

Serum vibriocidal antibodies are not considered the immune response real mediators [23,36], as confirmed by the World Health Organisation in its “Guidelines for the production and control of inactivated oral cholera vaccines”. Moreover, vibriocidal antibody levels have been proven to be the best correlation with the protection against the colonization and disease [37], and elevated titres of serum antibodies have proven to confer protection for oral immunization of cholera [38]. As reported by Lavelle and O’Hagan [12], orally administered microparticles containing vaccines can induce an enhanced serum and mucosal antibody response, with the production of secretory IgA antibodies at mucosal sites.

For the *in vivo* study, several groups of rats were orally administered with Eudragit® L30D55 – VC microparticles, Eudragit® FS30D – VC microparticles, or Eudragit® L30D55: FS30D (50:50) – VC microparticles. Animals inoculated with *V. cholerae* or Eudragit® L30D55 – VC microparticles did not show statistically significant differences in sera vibriocidal activity (up to 28 days) whereas animals inoculated with Eudragit® FS30D – VC and Eudragit® L30D55:FS30D – VC microparticles showed a lower immune response ($p < 0.05$) compared with non-encapsulated VC and Eudragit L30D55 at the same times of the study (Fig. 5). At day 21, a sharp increase was observed in all groups inoculated with VC microencapsulated or VC alone, which correspond with the antibody response to the second dose administered. Vibriocidal antibodies were not detected in control animals sera inoculated with empty microparticles or PBS.

Although *V. cholerae* antigenic capacity is, in general, well preserved after the microencapsulation by spray drying, a different immune response was observed between Eudragit® L30D-55 and FS30D microparticles. The lower immunogenicity of FS30D microparticles could be explained by the slower release rate and

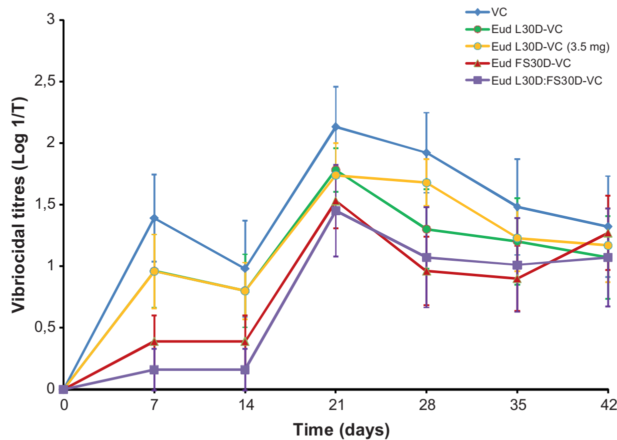


Fig. 5. Serum vibriocidal antibodies in groups of Sprague Dawley rats following oral immunization (7 mg at 0 and 14 days). Vibriocidal antibodies were not detected in control animals (PBS or empty microparticles). Data are presented as mean ± S.E.

by the lower antigenicity maintained after the microencapsulation process. Moreover, as this polymer dissolves at higher pH values (pH: 7), microparticles containing *V. cholerae* would remain intact when passing through the duodenum and could be uptaken by the Peyer's patches. This would explain the higher increase observed in the FS30D microparticles after the boosting dose administered at day 14, compared with the other groups. Besides that, a slight increase in the vibriocidal titres was observed in this group between day 35 and 42, which could be due to a controlled release of the cholera from these microparticle. However, Eudragit® L30D55 is dissolved in the upper region of the small intestine, precisely the target site where the immune response against cholera is produced [39].

Regarding the dose–response study, vibriocidal antibodies at day 21 after the administration of VC in different doses showed proportional declines with decreasing doses (Fig. 6). However, vibriocidal titres in rats inoculated with Eudragit® L30D-55-VC microparticles were similar even when dose was reduced from 7 to 3.5 mg and, except for the 7 mg dose, were higher than the titres obtained when administering the same doses of VC alone without microparticles, especially for the 3.5 mg dose. These levels also correlate with the vibriocidal titres observed in Fig. 5, in which rats inoculated with the 3.5 mg dose showed a similar profile to rats administered the 7 mg dose either alone or microencapsulated. It seems that with the 7 mg dose the amount of *V. cholerae* that remain unaltered after the passage through the stomach is high enough to induce a suitable immune response. At lower doses, VC alone is not protected enough and, therefore, it is not able to induce an immune response. By the administration of 3.5 mg of VC microencapsulated, an appropriate protection of the bacteria is achieved which leads to the production of high levels of vibriocidal titres, which could suggest the possibility of using lower doses of *V. cholerae* microencapsulated in order to achieve a suitable immune response.

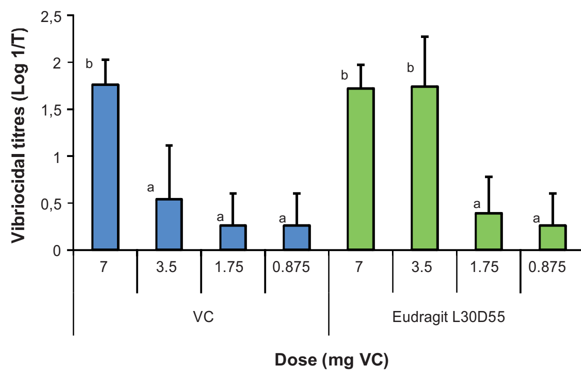


Fig. 6. Serum vibriocidal antibodies at day 21st in groups of 10 Sprague Dawley rats following oral immunization at day 0 and 14, with different doses of *Vibrio cholerae* (VC) or Eudragit® L30D-55-VC microparticles (prepared at 60 °C). Vibriocidal antibodies were not detected in control animals (PBS). Different letters (a, b) indicate significant differences ($p < 0.05$).

The group of Talavera in Cuba [3,7,40,41] has been working during the last decade in the development of a cholera vaccine suitable to avoid some of the inconveniences of current available vaccines, such as the use of a strict cold chain for storage, the lack of efficacy in young children (under 2 years of age) or the inconveniences of the administration procedure. In the previous studies, they have obtained similar immune responses to the vaccine candidates tested in this work, although the vibriocidal titres are slightly higher to the ones obtained in our study. These differences can be explained by the type of variant used, since, as it has been reported, cholera attenuated vaccines provide higher immune response than inactivated vaccines [1]. Nevertheless, in the development of novel delivery systems for oral vaccine administration, for safety reasons, it would be desirable to use non-living carrier systems, rather than live attenuated vaccines [12].

4. Conclusion

We have demonstrated the feasibility of the *V. cholerae* microencapsulation by spray drying. Characterization of microparticles revealed that an increase in the temperature produced changes the microparticle morphology and in antigenicity capability, confirming that an inlet temperature of 60 °C using Eudragit® L30D55 as polymer lead to suitable and immunogenic microparticles. This would open the possibility to use other polymers that will increase the immune response, and administrate the inactivated cholera vaccine in a single dose. However, further research is needed in order to find the right combination of microparticles which would provide high and sustained vibriocidal titres. Finally, the microencapsulation by spray-drying of inactivated *V. cholerae* could be proposed as a method to obtaining an oral vaccine, providing a low cost, easy to produce vaccine, which, besides that, would be feasible to be administered to young children.

Acknowledgements

G. Año and M. Pastor thank the Basque Government (Department of Education, Universities and Research) for the fellowship grant. Authors also acknowledge the technical support and advice provided by SGIker (UPV/EHU, MICINN, GV/EJ, ESF) on scanning electron microscopy. This project was partially supported by the Basque Government (Consolidated Groups, IT-407-07).

References

[1] WHO. Oral cholera vaccine use in complex emergencies: What next? World Health Organization, 2005. [cited 2010 December 22]. Available from http://www.who.int/cholera/publications/cholera_vaccines_emergencies_2005.pdf.
[2] Zuckerman JN, Rombo L, Fisch A. The true burden and risk of cholera: implications for prevention and control. Lancet Infect Dis 2007;7(8):521–30.
[3] Talavera A, Perez EM. Is cholera disease associated with poverty? J Infect Dev Ctries 2009;3(6):408–11.
[4] Lucas ME, Deen JL, von SL, Wang XY, Ampuero J, Puri M, et al. Effectiveness of mass oral cholera vaccination in Beira, Mozambique. N Engl J Med 2005;352(8):757–67.
[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. Stockholm, 1985: 21st Nobel Conference; 1986. p. 33–43.
[6] Kabir S. Cholera vaccines. Lancet Infect Dis 2007;7(3):176–8.

[7] Talavera A, Año G, Pino Y, Castano J, Uribarri E, Riveron L, et al. Formulation in tablets of a cholera whole cells inactivated vaccine candidate. Vaccine 2006;24(16):3381–7.
[8] Hill DR, Ford L, Lalloo DG. Oral cholera vaccines: use in clinical practice. Lancet Infect Dis 2006;6(6):361–73.
[9] Thiem VD, Deen JL, von SL, Canh dG, Anh DD, Park JK, et al. Long-term effectiveness against cholera of oral killed whole-cell vaccine produced in Vietnam. Vaccine 2006;24(20):4297–303.
[10] 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(9047):231–5.
[11] Kabir S. Questionable merits of the field trial of an oral killed whole cell cholera vaccine in Vietnam during 1998–2003. Vaccine; 25(8):1353–4.
[12] Lavelle EC, O'Hagan DT. Delivery systems and adjuvants for oral vaccines. Expert Opin Drug Deliv 2006;3(6):747–62.
[13] Yeh M, Chiang C. Inactive *Vibrio cholerae* whole-cell vaccine-loaded biodegradable microparticles: in vitro release and oral vaccination. J Microencapsul 2004;21(1):91–106.
[14] Lin JH, Weng CN, Liao CW, Yeh KS, Pan MJ. Protective effects of oral microencapsulated *Mycoplasma hyopneumoniae* vaccine prepared by co-spray drying method. J Vet Med Sci 2003;65(1):69–74.
[15] Esposito E, Roncarati R, Cortesi R, Cervellati F, Nastruzzi C. Production of Eudragit microparticles by spray-drying technique: influence of experimental parameters on morphological and dimensional characteristics. Pharm Dev Technol 2000;5(2):267–78.
[16] Esposito E, Cervellati F, Menegatti E, Nastruzzi C, Cortesi R. Spray dried Eudragit microparticles as encapsulation devices for vitamin C. Int J Pharm 2002;242(1–2):329–34.
[17] Harikarnpakdee S, Lipipun V, Sutanthavibul N, Ritthidej GC. Spray-dried mucoadhesive microspheres: preparation and transport through nasal cell monolayer. AAPS Pharm Sci Technol 2006;7(1):E12.
[18] Ribeiro Rattes AL, Pereira Oliveira W. Spray drying conditions and encapsulating composition effects on formation and properties of sodium diclofenac microparticles. Powder Technol 2007;171(1):7–14.
[19] Evonik Röhm GmbH. Guidelines for Formulation Development and Process Technology for Enteric Coatings, 2009. [cited 2010 December 22]. Available from http://eudragit.evonik.com/sites/dc/Downloadcenter/Evonik/Product/EUDRAGIT/Guidelines%20for%20Formulation%20Development%20and%20Process%20Technology%20for%20Enteric%20Coatings.pdf.
[20] Flick-Smith HC, Eyles JE, Hebdon R, Waters EL, Beedham RJ, Stagg TJ, et al. Mucosal or parenteral administration of microsphere-associated *Bacillus anthracis* protective antigen protects against anthrax infection in mice. Infect Immun 2002;70(4):2022–8.
[21] Año G, García HM, Valmaseda T, Cedré B, Pino Y, Ancheta O, et al. Antigenicidad e inmunogenicidad de una cepa de *Vibrio cholerae* inactivada. Biotecnología Aplicada 2003;20:9–13.
[22] Provenzano D, Kovac P, Wade WF. The ABCs (antibody, B cells, and carbohydrate epitopes) of cholera immunity: considerations for an improved vaccine. Microbiol Immunol 2006;50(12):899–927.
[23] Cedré B, García HM, García L, Talavera A. Estandarización y evaluación del ensayo vibriocida modificado. Rev Cubana Med Trop 1999;51(3):156–9.
[24] Eko FO, Schukovskaya T, Lotzmanova EY, Firstova VV, Emalyanova NV, Klueva SN, et al. Evaluation of the protective efficacy of *Vibrio cholerae* ghost (VCG) candidate vaccines in rabbits. Vaccine 2003;21(25–26):3663–74.
[25] Kusonwiriyawong C, Pichayakorn W, Lipipun V, Ritthidej GC. Retained integrity of protein encapsulated in spray-dried chitosan microparticles. J Microencapsul 2009;26(2):111–21.
[26] Benchabane S, Subirade M, Vandenberg GW. Production of BSA-loaded alginate microcapsules: influence of spray dryer parameters on the microcapsule characteristics and BSA release. J Microencapsul 2007;24(6):565–76.
[27] Raffin RP, Jornada DS, Re MI, Pohlmann AR, Guterres SS. Sodium pantoprazole-loaded enteric microparticles prepared by spray drying: effect of the scale of production and process validation. Int J Pharm 2006;324(1):10–8.
[28] Uddin AN, Bejugam NK, Gayakwad SG, Akther P, D'Souza MJ. Oral delivery of gastro-resistant microencapsulated typhoid vaccine. J Drug Target 2009;17(7):553–60.
[29] Delie F, Blanco-Prieto MJ. Polymeric particulates to improve oral bioavailability of peptide drugs. Molecules 2005;10(1):65–80.
[30] De Magistris MT. Mucosal delivery of vaccine antigens and its advantages in pediatrics. Adv Drug Deliv Rev 2006;58(1):52–67.
[31] Hillery AM. Microparticulate delivery systems: potential drug/vaccine carriers via mucosal routes. Pharm Sci Technol Today 1998;1(2):69–75.
[32] Byrd W, de Lorimier A, Zheng ZR, Cassels FJ. Microencapsulated subunit vaccine approach to enterotoxigenic *Escherichia coli* and other mucosal pathogens. Adv Drug Deliv Rev 2005;57(9):1362–80.
[33] Yeh MK, Liu YT, Chen JL, Chiang CH. Oral immunogenicity of the inactivated *Vibrio cholerae* whole-cell vaccine encapsulated in biodegradable microparticles. J Control Release 2002;82(2–3):237–47.
[34] Perez JL, Garcia L, Talavera A, Oliva R, Valmaseda 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(2–3):376–84.
[35] Lin SY, Kao YH. Tablet formulation study of spray-dried sodium diclofenac enteric-coated microcapsules. Pharm Res 1991;8(7):919–24.
[36] Saha D, LaRocque RC, Khan AI, Harris JB, Begum YA, Akramuzzaman SM, et al. Incomplete correlation of serum vibriocidal antibody titer with protection from *Vibrio cholerae* infection in urban Bangladesh. J Infect Dis 2004;189(12):2318–22.
[37] Benitez JA, Garcia L, Silva A, Garcia H, Fando R, Cedre B, et al. Preliminary assessment of the safety and immunogenicity of a new CTXPhi-negative, hemagglutinin/protease-defective El Tor strain as a cholera vaccine candidate. Infect Immun 1999;67(2):539–45.
[38] M. Corbel, D. Garcia, E. Griffiths. Guidelines for the production and control of inactivated oral cholera vaccines. 2004. WHO Technical Series Report No. 924.
[39] Flach CF, Qadri F, Bhuiyan TR, Alam NH, Jennische E, Lonnroth I, et al. Broad up-regulation of innate defense factors during acute cholera. Infect Immun 2007;75(5):2343–50.
[40] Garcia L, Jidy MD, Garcia H, Rodriguez BL, Fernandez 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.
[41] Talavera A, Año G, Garcia H, Moreira T, Delgado H, Riveron L, et al. Process development for a Cuban cholera vaccine based on the attenuated strain *Vibrio cholerae* 638. Vaccine 2006;24(18):3746–9.