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Process development for a Cuban cholera vaccine based on the attenuated strain *Vibrio cholerae* 638

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Abstract

Genetically modified *Vibrio cholerae* strain 638 (biotype El Tor, serotype Ogawa) has previously been shown to be immunogenic in animal models and in human trials. Our objective in the work reported herein was to describe the process development methods for the production of the 638 attenuated cholera vaccine. Cell seed bank, culture of biomass, lyophilization and final formulation were processes were developed. The results show kinetics of culture that fulfils a logistical model. The microbiological properties, colonizing capability, immunogenicity and non-toxigenicity of the final product were indistinguishable from the properties of the working seed lot. We conclude that the non-reactogenic, immunogenic and protective strain 638 is robust and can withstand the fermentation processes required for large-scale production of a vaccine.

Keywords: Vaccine; Cholera; Process

1. Introduction

Vibrio cholerae is the infectious agent responsible for cholera. Only *Vibrio cholerae* O1 and O139 serogroups are known to cause epidemics of cholera. Isolates of *V. cholerae* serogroup O1 are classified into two biotypes, El Tor and classical, on the basis of several phenotypic characteristics. Currently, the El Tor biotype is responsible for virtually all of the cholera cases throughout the world and classical biotype isolates have virtually disappeared worldwide. In addition, *V. cholerae* O1 is classified into two main serotypes, Inaba and Ogawa, based on agglutination test using polyvalent antiserum against lipopolysaccharide (LPS) antigens. A third serotype, Hikojima, has been described, but it is very rare. The immunity due to previous infection of *V. cholerae* is serogroup specific. There are other serogroups of *V. cholerae* (for example, serogroups O5 and O37) that can cause isolated cases of watery diarrhea but they do not cause epidemics. The current seventh pandemic of cholera due to *V. cholerae* O1 biotype El Tor has been reported from all regions of the world [1].

Some strains of *V. cholerae* O1 show resistance to multiple antibiotics including to ampicillin, furazolidone, tetracycline and trimethoprim/sulphamethoxazole, drugs that are recommended by the WHO for treatment of clinical cholera. Massive prophylaxis has been shown to be unsuccessful and is discouraged. Although the organism remains susceptible to nalidixic acid (not used for the treatment of cholera but used as a proxy for sensitivity to all fluoroquinolones), minimum inhibitory concentrations are increasing [2].

Two oral cholera vaccines that are already licensed have recently been evaluated for their effectiveness and practicality as public health tools in controlling cholera outbreaks in developing country populations at high risk of cholera [1,3,4]. These experiences suggest that mass vaccination with oral cholera vaccines can be a useful adjunct tool for controlling outbreaks [3,4], particularly if they are implemented early, in association with other standard control measures.

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Genetically modified *V. cholerae* strain 638 (biotype El Tor, serotype Ogawa) has previously been shown to be a good colonizer of the intestine and is strongly immunogenic and protective, as well as being non-virulent and non-toxigenic "in vitro" and in animal models and small scale human clinical trials [5]. Recently, it was shown to confer a high protection in an experimental challenge study involving human volunteers [6]. In a definitive vaccine formulation strain 638 also showed good viability "in vitro" and retained the capacity to colonize neonatal mice [6].

Herein we describe the process development methods for the consistent, potentially large-scale production of attenuated cholera vaccine strain 638. Methods for the working seed lot, culture of biomass, lyophilization and preparation of a final formulation were developed. The yield and process consistency, identity, potency, safety and stability of the product were documented.

2. Materials and methods

2.1. Working seed lot

A lyophilized 638 strain from the reference seed lot was cultivated in Tryptone Peptone Broth at 37 °C, with shaking at 200 rpm for 4 h. Each 0.3 mL of this culture was mixed (v/v) with skim milk (10%) and glycerol (20%) and distributed in cryopreservation tubes (1.5 mL) and finally frozen at −70 °C, until use.

Viability was determined in three tubes every 6 months by enumeration of colony forming units (CFU). Morphology studies were done by optic microscopy with Gram stain and by electron microscopy using negative staining [8].

Biochemical and serological tests for identity were done according to OPS/OMS/CDC/NCID [9]. These included the string test, kligler iron agar, lysine iron agar, voges proskauer, oxidase, catalase, arginine dehydrolase and ornithine and lysine decarboxylase tests.

2.2. Colonization test

The colonization studies were done in neonatal mice (Balb/c, 2–4 days) [10]. Each animal was orally inoculated with 50 μL of bacterial suspension (10^5 CFU/mL) containing Evan's blue dye (0.01%). After 24 h, the animals were sacrificed and the CFU/g of intestine was determined. Adherence of vaccine strain to intestinal mucosa of mice was observed by direct immunoperoxidase method [11]; mice were inoculated as previously described and the intestinal samples were taken at 24 h post inoculation.

2.3. Detection of choleric toxin

In order to demonstrate the absence of choleric toxin genes (*ctx*), we applied a polymerase chain reaction (PCR) method [12]. For amplification we used two primers synthesized in the Centre of Genetic Engineering and Biotechnology. These primers defined a sequence of 564 pair bases (pb) complementary to gene *ctxA*. The sequences used as primers were: initiator ctx 2686: 5′ CGG GCA GAT TCT AGA CCT CCT G 3′, of the position 73 at 94 initiator ctx 2687: 5′ GCA TGA TCT TGG AGC ATT CCC AC 3′, of the position 611 at 636.

As positive control we used the amplification product from enterotoxigenic *V. cholerae* O1 strain 569B. A functional test for toxin activity was performed by inoculating rabbit ileal loops (New Zealand, 2–2.5 Kg of body weight) [13].

2.4. Immunogenicity

Immunogenicity was determined by intraduodenal inoculation of control or bacterial cell suspension (10^9 CFU) in rabbits [14]. Serum samples obtained 14 days after inoculation were assayed for titers of vibriocidal antibody activity by colorimetric method [15] and for antibodies (IgG) against LPS by ELISA [16].

2.5. Production flow

The production process had five steps (Fig. 1): (1) preparation of work seed lot; (2) fermentation; (3) harvest; (4) formulation and filling; (5) lyophilization. Each step was followed by quality control tests. Briefly, purity was determined by Gram stain and growth on tryptone soy agar plates (TSA). Growth rate was calculated by optical density determination each half hour. Yield (CFU/mL of culture) was estimated by dilution method and colony counting on TSA plates. Identity was done by agglutination test using polyvalent O1 and monovalent (Ogawa and Inaba) diagnostic sera. The genetic marker (Endogluconase A) was identified as described Robert, 1996 [17]. The absence of *ctxA*$^-$ was determined by PCR as described above.

3. Results and discussion

3.1. Working seed lot

The 638 strain deep frozen (−70 °C) for more than for 4 years maintained the same order of viability (10^8 CFU/mL) as the initial value ($p = 0.07$). Morphology studies showed gram-negative, highly motile and curved rods with a single polar flagellum. The biochemical and serological results were typical for *V. cholerae* O1, El Tor Ogawa [9]. The PCR amplified a band of 564 bp from the hypertoxigenic 569 B strain that was used as a positive control; this corresponds to *ctxA*, which encodes the subunit A of cholera toxin. This band was not observed in the chromosomal DNA of the 638 strain.

The ileal loop technique in rabbits showed 0.25 ± 0.15 mL/cm of accumulated fluid, i.e., lack of enterotoxic activity. The assessment of colonization capability in neonatal mice demonstrated $2.4 \times 10^7 \pm 1.73 \times 10^7$ CFU/g of intestine. Moreover, by immunoperoxidase method it

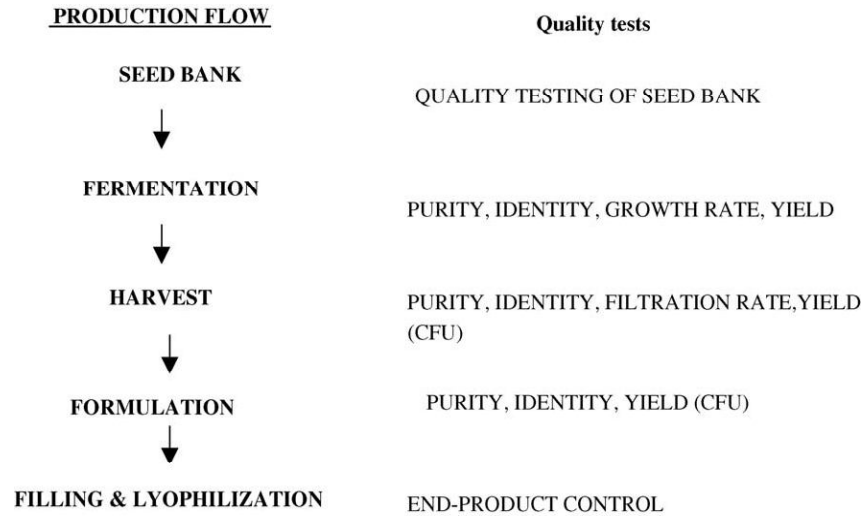

Fig. 1. Flow-chart of the attenuated vaccine of *V. cholerae* based on 638 strain.

was shown that the vaccine bacteria adhered to the mucosal layer of the mouse intestine. The serum antibodies showed a vibriocidal titer of 2.9 ± 0.4 ($\log T^{-1}$) and an ELISA titer of IgG against LPS of 3.2 ± 0.8 ($\log T^{-1}$). All these results are very similar to the results obtained with the original 638 strain, as previously described; accumulated fluid 0.28 mL/cm [14], intestinal colonization in neonatal mice 1×10^6 UFC/g [7], vibriocidal titer 2.75 ($\log T^{-1}$) [14] and ELISA titer (IgG against LPS) 2.64 ($\log T^{-1}$) [14].

3.2. Process

The fermentation showed a culture kinetics that fulfils the logistical model, with a growth rate of $1.6\,h^{-1}$ (Fig. 2) and a consistent yield of $2.6 \times 10^{10} \pm 6.5 \times 10^9$ CFU/mL of culture. The final product kept the same morphological, biochemical, serological and genetic markers as the cultures of the working seed lot. The final product also maintained the colonizing capability, 1.1×10^6 (2×10^5 to 1.6×10^6) CFU/g, and its immunological capacity tested in rabbits, vibriocidal titers of $\log T^{-1}$ 3.3 (2.5–3.7) and ELISA titers of $\log T^{-1}$ 3.1 (1.7–3.4). In general, after the production process the strain in the final product kept the relevant characteristics of the original strain 638 conserved in the working seed lot, without any divergence.

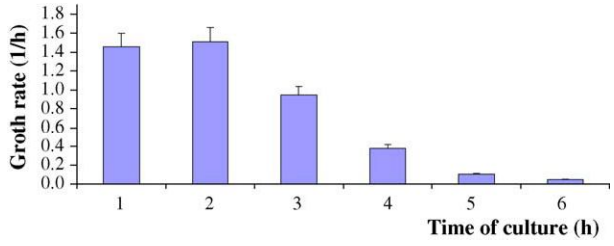

Fig. 2. Growth rate (h^{-1}) of 638 strain at different cultured times in the fermentation process.

Finally, the stability test of the final products was more than 80% of viability, for at least one year.

4. Conclusion

The non-reactogenic, immunogenic and protective live oral cholera vaccine strain 638 is robust and strain able to withstand the various production processes necessary to reliably and consistently manufacture an industrial formulation of this cholera vaccine.

References

[1] WHO. Initiative for Vaccine Research (IVR). State of the art of new vaccines: research and development. 2002.
[2] Keddy KH. Cholera update. Communicable Diseases Surveillance Bulletin, NICD, 2004. March.
[3] Calaine P, Chaine J-P, Johnson E, Hawley M-L, O'Leary M, Oshitani H, et al. Can oral cholera vaccination play a role in controlling a cholera outbreak? Vaccine 2004;22:2444–51.
[4] Lucas ME, Deen JL, von Sedlein L, Wang XY, Ampuero J, Puri M, et al. Effectiveness of mass cholera vaccination in Beira, Mozambique. N Eng J Med 2005;352:757–67.
[5] Benitez J, Garcia L, Silva A, Garcia H, Fando R, Cedré B, et al. Preliminary assessment of the safety and immunogenicity of a new CTXϕ-negative, hemagglutinin/protease-defective El Tor strain as a cholera vaccine candidate. Infect Immun 1999;67(2):539–45.
[6] García L, Fando R, Díaz M, García H, Rodríguez B, Fernández R, et al. Controlled volunteer challenge study to protective efficacy of a single oral dose of strain Vibrio cholerae, a Cuban cholera vaccine candidate. Vaccine for enteric diseases, Montego Bay, Jamaica: April 2004; 28–30.
[7] Talavera A, Moreira T, Año G, Cedré B, Delgado H, García H, et al. Viabilidad in vitro y colonización en ratones neonatos de una formulación vacunal de la cepa 638 de *Vibrio cholerae*. Revista CENIC 2000;31(2):110–2.
[8] Richardson K. Roles of motility and flagella structure in pathogenicity of *Vibrio cholerae*: analysis of motility mutants in th ere animal models. Infect Immun 1991;59:2727–36.

[9] OPS/OMS/CDC/NCID.Curso de Métodos de Laboratorio en el diagnóstico de *Vibrio cholerae*. Procedimientos de laboratorio. Caracas, Venezuela: Octubre 1994; 22–25.

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

[11] Vacca LL. Laboratory Manual of Histochemestry. New York: Raven Press Books; 1985.

[12] Morris JG, Losonsky GE, Johnson JA, Tacket CO, Nataro JP, Panigrahi P, et al. Clinical and immunological characteristics of *Vibrio cholerae* O139 Bengal infection in North American volunteers. J Infect Dis 1995;171:903–8.

[13] Ichinose Y, Yamamoto K, Nakanose N, Tanabe NJ, Takeda T, Miwanati TK, et al. Enterotoxicity of the El Tor-like hemolisin of non-O1 *Vibrio cholerae*. Infect Immun 1987;55:1090–5.

[14] García L, Oliva R, Cedré B, Valmaseda T, García H, Talavera A, et al. Intraduodenal inoculation of adult rabbit for evaluating the immunogenicity of genetically attenuated *Vibrio cholerae* strains. Lab Animal Sci 1998;48(6):538–41.

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

[16] Pino Y, Valmaseda T, Medina Y, Cedré B, Año G, García H, et al. Validation of an ELISA assay to determine antibodies against LPS of *Vibrio cholerae*. Vaccimonitor 2003;12(1):11–7.

[17] Robert A, Silva A, Benítez JA, Rodríguez BL, Fando R, Campos J, et al. Taggin a *Vibrio cholerae* El Tor candidate vaccine strain by disruption of its hemagglutinin protease gene using a novel reporter enzime. Clostridium Thermocellone Endogluconase A: Vaccine 1996;14:1517–22.