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RESEARCH

Virulence in mice of a cryopreserved bank of the toxigenic strain *Vibrio cholerae* 3008 to be used as challenge in efficacy studies of vaccine candidates in humans

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ABSTRACT

Experimental models of *Vibrio cholerae* infection in humans (challenge studies) allow evaluating the efficacy of cholera vaccine candidates before carrying out phase III trials. To improve the consistency between the challenge studies, models that use an inoculum prepared directly from a cryopreserved standardized bank have been validated. To evaluate the protection conferred by vaccine strains derived from the strains of the Latin American epidemic of 1991, a challenge test was developed with the toxigenic strain *V. cholerae* 3008, prepared from a fresh culture. The main objective of the present study was to evaluate the virulence of a cryopreserved bank of the *V. cholerae* 3008 strain, to validate its use in human challenge studies. The viability of the cryopreserved strain was 1.35 ± 0.15 × 10^9 c.f.u./mL. The virulence of strain 3008 and the toxigenic controls N16961 and C7258 was evaluated in the cholera suckling mouse model of infection, confirming the death of 100 % of the inoculated animals. The administration of the bank together with two different antacid solutions it also caused a lethal effect in all the inoculated mice. These results indicate that the freezing of *V. cholerae* does not affect its virulence and that the cryopreserved bank possesses adequate power in the model used. The results achieved, together with previous experiences, support the use of lots of *V. cholerae* 3008 cryopreserved for challenge studies in humans, as has been described for other El Tor and O139 strains.

Keywords: Vibrio cholerae, challenge studies, cryoconserved bank, 3008 strain

RESUMEN

Virulencia en ratones de un banco crioconservado de la cepa toxigénica *Vibrio cholerae* 3008 para emplearse como reto en estudios de eficacia de candidatos vacunales en humanos. Los modelos de infección experimental de *Vibrio cholerae* en humanos (estudios de reto) permiten evaluar la eficacia de candidatos vacunales de cólera antes de realizar ensayos fase III. Para mejorar la consistencia entre los estudios de reto se han validado modelos que emplean un inóculo preparado directamente de un banco estandarizado crioconservado. La protección conferida por cepas vacunales derivadas de las cepas de la epidemia latinoamericana de 1991, ha sido evaluada en un ensayo de reto con la cepa toxigénica *V. cholerae* 3008, preparada de un cultivo fresco. El presente estudio tuvo como objetivos preparar un banco de trabajo crioconservado de la cepa *V. cholerae* 3008 y evaluar su virulencia en ratones, para validar su empleo en estudios de reto en humanos. La viabilidad del banco crioconservado fue 1.35 ± 0.15 × 10^9 u.f.c./mL. La virulencia del banco crioconservado y las cepas toxigénicas controles N16961 y C7258 se evaluó en el biomodelo de cólera del ratón lactante, confirmándose la muerte del 100 % de los animales inoculados. La administración del banco junto a dos soluciones diferentes de antiácido también provocó efecto letal en la totalidad de los ratones inoculados. Estos resultados indican que la congelación de *V. cholerae* no afecta su virulencia y que el banco crioconservado posee una adecuada potencia en el modelo utilizado. Los resultados alcanzados, conjuntamente con experiencias previas, avalan el empleo de lotes de *V. cholerae* 3008 crioconservados para estudios de reto en humanos, como se ha descrito para otras cepas El Tor y O139.

Palabras clave: Vibrio cholerae, estudios de reto, banco crioconservado, cepa 3008

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*I*ntroduction

Cholera, the acute diarrheic disease caused by the enterotoxigenic bacteria *Vibrio cholera*, is a serious health problem of higher incidence in poor countries, war zones and disaster areas [1, 2]. The World Health Organization (WHO) recommends the application of the oral vaccines against cholera available, together with other prevention and health management measures [3]. Particularly, killed whole-cell oral cholera vaccines have been efficacious for at least five years in endemic zones [4], but failing to protect children younger than 5 years who are more affected by the disease [5]. Therefore, additional strategies have to be developed to protect this high risk population. In this setting, live-attenuated vaccines emerge as an alternative to mimic the best possible innate immune response against the infection, thereby inducing a long lasting

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immune response [5]. In Cuba, a joint Project coordinated by the Center for National Scientific Research (CNIC), the Finlay Institute of Vaccines and the Institute of Tropical Medicine Pedro Kourí developed the oral live-attenuated cholera vaccine candidate CV638. It was generated from the strain *V. cholerae* 638 attenuated by genetic modification from the strain C7258 (Serogroup O1, Biotipe El Tor, Serotype Ogawa), this last a clinical isolate from the Peruvian outbreak in 1991 [6, 7]. The attenuated strain *V. cholerae* 638 and the CV638 candidate have been thoroughly evaluated in pilot, Phase I and II clinical trials (http://registroclinico.sld.cu/trials/RPCEC00000172-En, http://registroclinico.sld.cu/trials/RPCEC00000188-En), in Cuba and abroad, with around 650 subjects ages ranging 5-65 years old vaccinated. Satisfactory results have been achieved attending to safety, reactogenicity, immunogenicity and protection against cholera [3, 7-11], but the efficacy of the CV638 candidate remains to be evaluated in order to obtain the sanitary registration in Cuba and for its use in vaccination campaigns to contain future cholera outbreaks.

In this setting, the models of experimental infection with *V. cholerae* in humans, specifically in challenge studies, allow the evaluation of candidate vaccine efficacy against cholera before conducting phase III field trials in endemic areas [12]. Notably, those models have been accepted as evidence of protection for the registration of cholera vaccines by the Food and Drug Administration in US [13]. Challenge models have been developed, using toxigenic *V. cholera* serogroup O1 of biotype El Tor, as N16961 (Serotype Inaba, Bangladesh, 1975) and 3008 (Serotipe Ogawa, Argentina, 1992) [15], as virulent inoculum to evaluate efficacy against the infection by that biotype which is the prevalent in current outbreaks. In fact, strain 3008 was validated to estimate the protection conferred by biotype El Tor and Ogawa serotype vaccine strains derived from the Latin American epidemics in 1991 [16].

In these models, the inoculum of the virulent strain is prepared directly from a fresh culture [12]. In fact, the use of a fresh inoculum implies a complex logistic task in the 12 hours prior to its administration, to guarantee the adequate dose able to cause the disease in the expected number of volunteers without risking their lives (i.e., the potency of the inoculum). Besides, the use of a fresh inoculum could be a significant source of variability if the trial is done at different moments or places simultaneously. In order to improve the consistency of challenge studies run at different moments, institutions or both, a strategy has been followed in which the challenge inoculum is prepared directly from a cryopreserved standardized bank of *V. cholerae* O1 and O139 strains [16-18]. Moreover, its use minimizes the differences in attack or severity rates of the disease. However, no challenge model has been developed with *V. cholerae* 3008 using the inoculum directly prepared from a cryopreserved bank.

The estimation of the efficacy of the CV638 vaccine candidate through a challenge trial would require it run at different times and institutions, administering it to different groups of 20 volunteers each, therefore, guaranteeing its safety. To ensure the reproducibility and consistency of results, it would be necessary to have a standardized cryopreserved bank of strain *V. cholerae* 3008 from which the challenge inoculum would be prepared, as has been done for other strains [16-18]. The possible detrimental effect of freezing on bacterial virulence should be also determined. Considering all these elements, this work was aimed to prepare a cryopreserved bank of the *V. cholerae* 3008 and evaluate its virulence in mice, prior to its future use in challenge studies in humans.

Materials and methods

Strains and culture media

Bacterial strains used and their phenotypic and genotypic properties are summarized in the table.

Microorganisms were grown in the following culture media: Luria-Bertani broth (LB; 10 g/L tryptone, 5 g/L Yeast extract; 10 g/L Sodium chloride; pH 7.0), supplemented with 1.5 g/L agar; and Tryptic Soy Broth (TSB; CASO Broth; Merck, Germany). LB reagents were purchased from Oxoid (United Kingdom).

Preparation of the cryopreserved bank of strain 3008 and control strains C7258, N16961 and 638

The cryopreserved bank of *V. cholerae* strain 3008 was prepared at the Finlay Institute of Vaccines through a normalized operating procedure (NOP), as previously described for the elaboration of a working seed lot for strain 638 [19]. In this case, the bank was prepared starting from a primary bank kept frozen since 1999, which was first incubated at 37 ºC static overnight. Subsequently, it was inoculated into 100 mL of TSB medium at 0.01 optical density at 600 nm ($OD_{600\ nm}$) and cultured at 37 ºC for 4 h under 200 rpm agitation. Then, 100 mL of culture and 100 mL of 30 % Glycerol solution in TSB were mixed, manually homogenized and fractionated in 1-mL cryopreservation vials (Eppendorf, Germany). The quality control of the bank included the evaluation of purity by Gram staining, motility and viability following NOP 048, NOP 12-176 and NOP 12-160, respectively, of the Finlay Institute of Vaccines. Viability of the cryopreserved *V. cholerae* 3008 bank was determined in ten independent vials by serial dilutions in saline and plating in triplicates of 10-µL aliquots of each dilution in LB plates, which were incubated at 37 ºC overnight. The working cell banks (WCBs) of the other three control strains C7258, N16961 and 638, were prepared by culturing

Table. *Vibrio cholerae* strains used for establishing a cryopreserved bank of the toxigenic strain *V. cholerae* 3008

Strains	Characteristics	Origin [ref.]
3008	O1, El Tor, Ogawa, toxigenic reference strain	Argentina, 1992
C7258	O1, El Tor, Ogawa	Perú, 1991
N16961	O1, El Tor, Inaba	Bangladesh, 1975
VC638	C7258Δ *ctxAB*, *mshA*$^{-}$, *hap*::*celA*$^{+}$	Robert *et al*. [6]

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them similarly but on LB medium and, once reaching the 1.0 $OD_{600\ nm}$, cells were collected by centrifugation at 3000 × *g* in an Eppendorf centrifuge (Eppendorf, Germany) for 8 min. Then, the cell pellet was resuspended in LB supplemented with 15 % glycerol broth and cell concentration was adjusted to 1.0 $OD_{600\ nm}$, and fragmented into 1-mL cryopreservation vials (Eppendorf, Germany). Then, viable cells were counted in seven ten-fold serial dilutions down to 10^{-7} and 10 µL of each dilution plated in triplicates on LB solid medium, and incubated at 37 ºC overnight.

Housing and handling of laboratory animals

Virulence and colonization tests were run in male and female suckling Balb/c mice, 2 to 5 days old with weighing 1.5-2.0 g, purchased from the National Center for Laboratory Animal Production, Cuba. They were acquired with both the genetic and sanitary quality certifications. Ten suckling and two mother mice were distributed per box (Tecniplast, Italy). Animals were housed and handled following the institutional protocols issued by the Institutional Committee for the Care and Use of Laboratory Animals at CNIC, Record number 003/2016. Animals were handled according to the Ethical Guidelines for the Handling of Laboratory Animals (Habana, Cuba, 1992), the Sanitary and Environmental Safety Principles for Non-Clinical Good Laboratory Practices (Regulation No.39/04 of the Center for the State Control of Medicines, Equipment and Medical Devices, Cuba), and the related NOPs of the Center of Natural Products, CNIC, Cuba (UGC/PNT/CEI/008 and UGC/PNT/CEI/022).

Evaluation of virulence in the suckling mice model

Virulence was assessed in the cholera infection model in suckling mice, with some modifications [20]. Inoculums were prepared from working cell banks staring with a 1:100 dilution in phosphate buffered saline solution (PBS; 8 g/L sodium chloride, 1.44 g/L sodium hydrogen phosphate; 0.2 g/L potassium chloride; 0.24 g/L potassium hydrogen phosphate, pH 7.4) supplemented with Evans Blue dye (0.001 g/L). All the components were acquired from AppliChem (Germany). The dilution applied to the WCBs guarantees ~5×10^5 c.f.u. in 50 µL, the WCB dilution was further diluted 10 and 100 times, respectively. The cryopreserved cell banks of the toxigenic strains *V. cholerae* C7258 and N16961 were used as controls, the latter as challenge strain in a challenge model in human volunteers [16]. Moreover, the attenuate strain *V. cholerae* 638 was employed at a single dose of 10^6 c.f.u. as innocuousness control for inoculation procedure.

The number of viable cells was determined by serial dilutions down to 10^{-5} followed by plating in LB medium. A polyvinyl cannula was coupled to a sterile 1-mL syringe and the inoculation was done intragastrically. Groups of ten mice each were inoculated and kept with their mothers, their survival followed every 24 h for a week. The animals were separated from their mothers 3 h before inoculation and they were returned 3 h later. Data were analyzed with the aid of the GraphPad Prism software, version 5.0.1 (2007), and the Kaplan-Meier curves were estimated, together with the median survival time for each group which were compared by the Log-rank test.

Virulence and colonization assays of the cryopreserved bank of strain 3008 in the presence of antacid

For this, inoculums of ~ 5×10^3 c.f.u. were incubated in the presence of an antacid solution (22.2 g/L sodium bicarbonate, 6.6 g/L citric acid, 0.75 g/L tartaric acid, 0.3 g/L sodium stearyl fumarate) containing either 0.75 g/L polyvinylpyrrolidone (variant A) or 0.75 g/L kollidon VA-64 (variant B) as agglutinating agent, as previously described. All the components of both antacid variants were purchased from Merck (Germany). The inoculum for the attenuated strain *V. cholerae* 638 consisted on 10^6 c.f.u., and it was used as innocuousness control.

After freezing, strain colonization was evaluated by inoculating five mice with *V. cholerae* 3008 and 638 suspensions prepared on antacid variants A and B. Twenty-four hours later, mice were sacrificed and the intestines were extracted and homogenized [20]. The number of vibrio per intestine (total recovered c.f.u.) were quantified by viable cell counting in serial dilutions with PBS and extension on LB medium.

Results and discussion

Viability and quality control of the cryopreserved bank of strain 3008

Once evaluated bacterial concentration of the WCB, it was found of $1.35 \pm 0.15 \times 10^9$ c.f.u./mL as expected (approximately $1.0 \pm 0.15 \times 10^9$ c.f.u./mL), consistent with subsequent determinations. This guarantees that a single vial could suffice for the virulence of the challenge in more than 100 human volunteers, considering that the challenge dose could be ~10^6 c.f.u./mL during the trial [3].

Moreover, the colonies isolated from counting plates were homogeneous, round, smooth and translucid, consistent with the expected appearance of *V. cholerae* colonies. Microscopic analysis of Gram stainingdone to the bank as quality control revealed the presence of pure, comma-shaped Gram-negative bacilli cultures. Similarly, motility analysis evidenced the presence of constantly moving short colorless bacilli, consistent with *V. cholerae* performance.

Virulence of the cryopreserved bank of strain 3008

The number of viable cells determined in every dose administered to mice for virulence evaluation in the experimental suckling mice model was within the expected range for *V. cholerae* strains 3008 (~6.3×10^3; ~7.6×10^4 and ~6.5×10^5 c.f.u.) and N16961 (~6.1×10^3; ~8.4×10^4 and ~5.8×10^5 c.f.u.). That was not the case for *V. cholerae* strain C7258, with values an order higher than expected (~2.3×10^4; ~3×10^5 and ~3.8×10^6 c.f.u.). Then, the experiment continued since viable counts for each dose were determined a day after, hence, results were recorded and processed as valid.

Survival analysis of inoculated mice indicated that there were no deaths in the control group receiving the control attenuated strain *V. cholerae* 638 (Figure 1). This corresponded to the attenuation of the strain

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and the adequate inoculation procedure. Toxigenic *V. cholerae* strains 3008, C7258 and N16961 caused the death of all the animals inoculated with all the three evaluated doses (Figure 1A, 1B and 1C, respectively). Further comparison of the median survival time of the groups of mice inoculated with the three doses of strains *V. cholerae* 3008, C7258 and N16961 did not show any statistical differences (Log-rank; $p = 0.3974$; $p = 0.6629$ and $p = 0.1553$, respectively). Overall, these results indicated that the lowest dose tested, prepared directly from the cryopreserved bacteria, was enough as to cause a lethal effect in all the inoculated animals, consistent with previous reports on using fresh culture inoculums [9].

Afterwards, the virulence of the different *V. cholerae* strains was assessed by comparing the survival time median values of mice receiving equal doses. Only *V. cholerae* strains 3008 and N16961 doses ~ 5×10^3 c.f.u. were compared since strain C7258 was inoculated at higher doses. Median survival values showed statistically significant differences (Log-rank, $p = 0.0070$) for mice receiving *V. cholerae* strain 3008 (2 d median value) as compared to those inoculated with strain N16961 (3 d median value), since these last lived longer (up to five days, Figure 1), suggesting that N16961 displayed a lower virulence at the dose tested.

For the ~ 5×10^4 c.f.u., the mean survival time values for mice receiving *V. cholerae* strains 3008 (2.5 d median value), N16961 (2 d median value) and C7258 (1.5 d median value) did not show statistically significant differences (Log-rank, $p = 0.0788$). The same result was obtained for the ~ 5×10^5 dose (strains 3008 and N16961, 2 d median value each; strain C7258, 1 d median value; Log-rank, $p = 0.1279$).

Consequently, these results indicated that the virulence of the three *V. cholerae* strains were equivalent. Moreover, the values obtained for the strain C7258, specifically for c.f.u. inoculum doses in the orders of 10^4 and 10^5, coincide with those previously reported during virulence assessment in the experimental suckling mice model with direct fresh culture inoculums [9]. Advantageously, this makes comparable the applications of fresh inoculums to those using preparations cryopreserved at –70 ºC as to assess the virulence of *V. cholerae* strains in this mice biomodel. Overall, our results indicate that freezing does not affect the virulence capacity of the *V. cholerae* strains tested and that they display an adequate potency. Therefore, the *V. cholerae* strain 3008 inoculum directly prepared from a cryopreserved suspension could display a virulence similar to the inoculum prepared from a fresh culture [8, 21]. This is also consistent with the performance of other *V. cholerae* strains obtained from cryopreserved inoculums and tested in experimental infection models in humans [16-18].

Virulence and colonization of the cryopreserved bank of strain 3008 in the presence of antacids

Validated cholera challenge models comprise administering the virulent *V. cholerae* strain in a sodium bicarbonate buffered solution to neutralize the stomach acidity. This last is the main barrier interfering with the adequate colonization and infection of the gastrointestinal tract by *V. cholerae* due to its

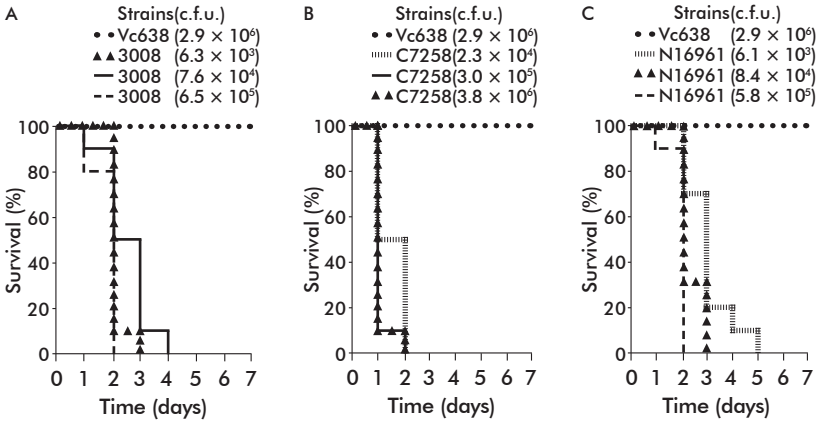

Figure 1. Kapplan Meier curves of survival over time of Balb/C suckling mice (10 mice per group) inoculated with different doses (c.f.u.) of *Vibrio cholera* strains. A) Control strain 638 and challenge strain 3008. B) Control strains 638 and C7258. C) Control strains 638 and N16961. Inoculums were prepared from cryopreserved bacterial suspensions. Data were processed with the aid of GraphPad Prism version 5.0.1 (2007).

sensitivity to acidic conditions [22]. Hence, the virulence of the *V. cholerae* 3008 cryopreserved cell bank was evaluated in the presence of two antacid solutions, providing a final 1.33 % sodium bicarbonate concentration according to previous challenge studies [8]. A dose of ~ 5×10^3 c.f.u. was assayed, since it was the lower dose causing the death of all the inoculated animals. Viable cell counts were within the expected range, showing ~4.5×10^3 c.f.u. for *V. cholerae* strain 3008 with antacid solution variant A (containing 0.75 g/L polyvinylpyrrolidone) and ~3.2×10^3 c.f.u. with variant B (0.75 g/L kollidon VA-64). Values for *V. cholerae* 638 were ~3.8×10^6 c.f.u. with antacid variant A and ~2.5×10^6 c.f.u. with variant B.

In the control group receiving the attenuated strain 638, all the animals survived for the seven days of the experiment (Figure 2). On the contrary, the strain 3008 in the presence of either antacid killed all the animals 48 h upon administration (Figure 2). A median survival time of 2 d was found for both antacid variants, with no statistically significant differences

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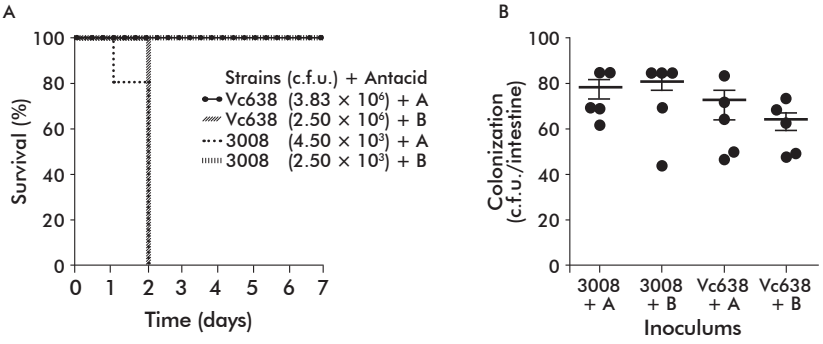

Figure 2. Virulence and colonization of *V. cholerae* strains 638 and 3008 in the suckling mice model. Strains inoculums were prepared from the cryopreserved bank and coadministered with antacid solutions containing either 0.75 g/L polyvinylpyrrolidone (variant A) or 0.75 g/L kollidon VA-64 (variant B). Data were processed with the aid of GraphPad Prism version 5.0.1 (2007). A) Kapplan-Meier curves of survival over time of Balb/C suckling mice (10 mice per group) inoculated with different doses (c.f.u.) of Vibrio cholera strains 638 or 3008. B) Intestinal colonization in Balb/C suckling mice (N = 5), inoculated either with ~103 c.f.u. of the enterotoxigenic strain 3008 or ~10^6 c.f.u. de 638. Error bars stand for the standard deviation.

found neither between them (Log-rank, p = 0.3404) nor when compared to the group of animals receiving the same dose but in PBS (Log-rank; p = 0.8544). Therefore, the virulence of the cryopreserved bank of the *V. cholerae* strain 3008 remained unaffected in the presence of both antacids. This indicates that these could be applied in future challenge studies in humans.

The biomodel used has also been well established to evaluate the colonization of *V. cholerae*, and it was found very useful to identify genes facilitating or conditioning the intestinal colonization by *V. cholerae* of the human gastrointestinal tract [23-25]. In this study, strains 3008 and 638 completely colonized the intestinal tract of the inoculated mice (Figure 2), reaching up to 10^6 c.f.u. This was in agreement with previous reports on the increase in the number of recovered c.f.u. in more than one order as compared to the initial inoculum for *V. cholerae* enterotoxigenic strains, 24 h post-inoculation [20]. Similar results were attained for the *V. cholerae* strain C7258 inoculated at a similar dose (~1×10^3 c.f.u.) from a fresh culture [9].

Moreover, no statistically significant different were found in total c.f.u. counts for mice inoculated with strain 3008 neither in antacid solution A nor B, with no statistically significant differences attending to colonization (Kruskal-Wallis; p = 0.3916). Therefore, it can be interpreted that the colonization by *V. cholerae* strain 3008 remained unaffected in the presence of antacid solutions A or B, and it was similar to that of the strain C7258 inoculated from fresh cultures. Therefore, it can be concluded that the virulence of the cryopreserved bank of the toxigenic *V. cholerae* strain 3008 was comparable to that of other reference strains (C7258 and N16961) and it was protected by the antacid solutions tested.

Conclusions

In summary, the results presented herein evidenced, together with previous experiences with fresh culture of this biomodel, that cryopreservation did not affect the virulence of *V. cholerae* 3008. They support the testing of the cryopreserved bank of this strain in future challenge trials in humans, as described for other *V. cholerae* strains such as El Tor and O139. There will be necessary to study the stability of viability and virulence of the cryopreserved bank over time, by using as specification of virulence its capacity to cause death in all the suckling mice inoculated with 10^3 c.f.u. of *V. cholerae* by the intra-gastric route in the subsequent five days. Overall, this is the first report on the virulence of a cryopreserved bank of the *V. cholerae* 3008 strain, for which an experimental challenge model in humans using it has not been established to date. The cryopreserved bank obtained could also be used in efficacy trials of the CV638 vaccine candidate, to improve the consistency and reproducibility of challenge trials conducted in different institutions and at different times.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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