

Correlation between antibody titers in human serum and plasma prepared with EDTA for the assessment of cholera vaccine immune responses

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RESEARCH

ABSTRACT

The possibility of quantifying vibriocidal antibody activity and cholera anti-toxin IgG antibody levels using both serum and plasma samples in quantification assays is essential during the clinical evaluation of cholera vaccines. However, the anticoagulant used to obtain plasma could not interfere with the analytical results. In this work, the activity of bacterial growth inhibitory antibodies (vibriocidal antibody activity), as well as anti-toxin IgG levels, was determined in the blood of nine patients diagnosed with cholera. A strong correlation was demonstrated between antibody titers against immunologically relevant antigens during the analysis of serum and plasma samples, which were obtained concomitantly and prepared in the presence of EDTA. This result supports the feasibility of the indiscriminate use of serum or plasma in such immunological assays. Likewise, we report the preparation and characterization of reference samples that can be used as positive and negative controls in such tests.

Keywords: vibriocidal assay, cholera, *Vibrio cholerae*, vaccine, blood tests, reference sera

Biotecnología Aplicada 2017;34:2221-2225

RESUMEN

Correlación entre los títulos de anticuerpos en suero y plasma humano preparados con EDTA para la evaluación de la respuesta inmune inducida por vacunas contra el cólera. La posibilidad de utilizar tanto muestras de suero como de plasma en los ensayos de cuantificación de la actividad de anticuerpos vibriocidas y los niveles de anticuerpos IgG anti-toxina del cólera resulta útil durante la evaluación clínica de una vacuna contra el cólera. Sin embargo, el anticoagulante empleado en la obtención del plasma puede afectar los resultados analíticos. En este trabajo se determinó la actividad de anticuerpos inhibidores del crecimiento bacteriano (vibriocidas), así como los niveles de IgG anti-toxina en muestras de sangre de pacientes diagnosticados con cólera. En el análisis de muestras de suero y plasma preparado en presencia de EDTA, obtenidas concomitantemente, se demostró una fuerte correlación entre los títulos de anticuerpos contra antígenos inmunológicamente relevantes. Este resultado presupone la factibilidad del uso indistinto de suero o plasma en dichos ensayos inmunológicos. Asimismo, se informa de la preparación y caracterización de muestras de referencia que pueden ser empleadas como controles en este tipo de ensayos.

Palabras clave: ensayo vibriocida, cólera, *Vibrio cholerae*, vacuna, ensayos sanguíneos, suero de referencia

Introduction

Cholera is a diarrheal disease feared particularly by its propensity to appear as explosive outbreaks that can turn into pandemics very rapidly [1]. The disease is caused by infection with toxigenic variants of *Vibrio cholera* that colonizes the intestine and produces the cholera toxin (CT) responsible for the severe diarrhea. Infection with *Vibrio cholerae* confers long-lasting protective immunity against a second exposure [2]. It is known that cholera vaccination, together with conventional measures of drinking water supply and sanitation, can positively impact on reducing the spreads and size of epidemics, especially under conditions of deficient access to drinking water and poor sanitation during the outbreaks.

Given the non-invasive nature of this microorganism, protective immunity against cholera depends only on prevention measures against colonization, mainly those against lipopolysaccharide (LPS) [3],

while cholera toxin (CT) has no protective effect [4]. The stimulus at mucosal level produces a detectable humoral response in serum, characterized by the induction of B lymphocytes which secrete antibacterial IgA, IgM and IgG antibodies indicating a highly active mucosal response [5, 6]. In fact, the presence of bactericidal antibodies in serum of infected subjects is the standard marker of protection for both disease and colonization [7, 8]. In this case, the detected vibriocidal activity is essential due to the increase of IgM and IgG response type, specific against LPS [9].

The bactericidal or vibriocidal assay against *V. cholerae*, measures the functionality of serum antibodies capable of lysing the vibrios in the presence of exogenous blood complement. This serological analysis has been very useful since it is the only currently recognized marker of protective immunity against *V. cholerae* infection. In this sense, it has been accepted

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by the US Food and Drug Administration (FDA) as a regulatory criterion for evaluating effectiveness of vaccines in clinical trials [10]. In these studies, it is sometimes necessary to further evaluate another reliable indicator of recent infection with toxigenic *V. cholerae*. That is why the immune response of each individual against the cholera toxin is determined, specifically the anti-CT antibody levels by the ELISA technique. Therefore, the combination of these two determinations of vibriocidal and anti-CT antibodies help to establish whether a person has been recently infected by *V. cholerae* or not, and provides an eligibility criterion for the enrollment of test subjects in a clinical trial of a given cholera vaccine.

Another essential aspect while testing vaccine candidates in clinical trials comprises the availability of positive and negative serum reference materials as controls, to be used for the selection of volunteers and to further assess the immunological potential of the vaccine. In fact, it is recommended to include the same control sera in both analytical procedures.

Traditionally, human serum has been employed in cholera serological tests in the clinical and epidemiological settings. Serum differs from plasma in that fibrinogen and coagulation factors have been removed, and, unfortunately, plasma samples obtained for clinical trials or those for routine research are often discarded. It would be beneficial if serological studies of cholera could be performed indistinctly with serum and plasma samples. This is advantageous in infants and young children settings, where small volumes of blood have to be extracted, these age groups considered relevant for its inclusion at different stages of the clinical development of vaccine products against cholera. This appraisal is based on the fact that obtaining plasma offers a higher availability of supernatant (15-20 % more than serum). Aside of the practical recovery of plasma, a technical difficulty arises from the use of EDTA as anticoagulant for plasma obtainment. This compound prevents the formation of thrombin by Ca^{2+} chelation, which is necessary for the blood clotting cascade, while complement activation by blocking the formation of the membrane attack complex [11]. Moreover, the chelating capacity of EDTA-bound divalent cations such as Ca^{2+} and Mg^{2+} is associated with the interruption of the outer membrane of Gram-negative bacteria [12].

Therefore, in this work, it was assessed the usefulness of plasma obtained with EDTA as an anticoagulant for performing vibriocidal antibody assays and to quantify anti-CT antibody levels, as compared to the respective serum samples. Likewise, positive and negative reference human serum samples were obtained and characterized in both types of assays during the clinical assessment of a Cuban vaccine candidate against cholera.

Materials and methods

Reagents and bacterial strain

The commercially available young rabbit serum supplied by Invitrogen (USA) was used as an exogenous complement source in the bactericidal assay.

The reference strain *Vibrio cholerae* VC12 (*Vibrio cholerae* O1, Ogawa Serotype, Classic Biotype) was

used as a target in the bactericidal assay. It was stored frozen at $-80\text{ }^{\circ}\text{C}$ in Luria-Bertani (LB) broth supplemented with glycerol at 20 % [13].

Serum samples

Experiments were run with human biological samples provided by human subjects who provided their approved informed consent, in agreement with Helsinki guidelines [14]. Blood samples were extracted by medical professionals, with disposable sterile material and following all measures of asepsis. Serum was obtained from nine individuals recovering from cholera, who were residents in Havana city in 2013. Additionally, blood was collected by venipuncture from six out of these nine human subjects for plasma extraction, in venipuncture-specific test tubes (Vacutainer, Becton Dickinson, USA) containing EDTA 5 mM as an anticoagulant agent. An aliquot of each sample was taken and stored at $4\text{ }^{\circ}\text{C}$ for immediate use and the rest was stored at $-20\text{ }^{\circ}\text{C}$. In all cases, blood was centrifuged for 10 min at 5000 rpm to separate the cells from the serum or plasma.

Serum obtained from a patient diagnosed with cholera during the 2012 outbreak in Manzanillo, Granma province, was used as a positive control. Similarly, serum with no detectable bactericidal or anti-CT antibodies and obtained from a patient diagnosed with Acute Diarrheal Disease (ADD) during the same outbreak and time, was used as negative control. Negative control plasma was extracted from samples of O+ blood provided by the Havana Province Blood Bank.

Vibriocidal assay

The vibriocidal antibody assay was performed as previously described [15]. Briefly, serum or human plasma samples were subjected to serial paired dilutions with saline solution (0.85 % NaCl) in sterile microtitration plates (Greiner, Germany). Starting from a 1:20 to 1:40 960 dilutions. Each sample was evaluated in duplicates. *V. cholerae* VC12 was cultured in Brain Heart Infusion medium (BHI) at $37\text{ }^{\circ}\text{C}$ for 4 h. Then, biomass was collected in physiological saline solution, adjusted to a concentration of approximately 10^7 c.f.u./mL and mixed with the same volume of young rabbit complement diluted 1:5 in saline solution. Sera were mixed with equivalent volumes of the bacterium-complement suspension and the plates were further incubated at $37\text{ }^{\circ}\text{C}$ for 1 h. The positive and negative control serum samples and a suspension control were included on each plate, mixed with serum-free complement. Right after the complement-dependent cell lysis reaction, 150 μL of indicator medium (LB supplemented with glucose 1 % and purple bromocresol 0.003 %) were added. Then plates were incubated for 3 h and the reaction read by visual inspection. The titer of vibriocidal antibodies was expressed as the reciprocal of the highest dilution at which complete inhibition of bacterial growth was achieved, detected by the absence of color change in the culture medium.

Cholera anti-toxin antibody detection by ELISA

Alternate wells of Maxisorp 96-well microtitration plates (Nunc, Denmark) were coated with 100 μL of 1 $\mu\text{g}/\text{mL}$ CT (Sigma) in carbonate buffer (0.05 M, pH 9.6). Uncoated wells were filled with 100 μL

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of 0.01 M phosphate-buffered saline, pH 7.2, plus 0.05 % Tween (PBS-T). Plates were incubated overnight at 4 °C, subsequently washed three times with PBS-T and blocked with PBS supplemented with skimmed milk at 5 %. Then, the 1:200 diluted sera were added in PBS-T and paired serial dilutions were performed. Plates were incubated at 37 °C for 1 h. After washing with PBS-T, an anti-human IgG-peroxidase conjugate preparation (CIGB-Sacti Spiritus, Cuba) was added. Reactions were developed by adding 50 µL of 1 mg/mL 3,3',5,5'-Tetramethylbenzidine substrate (Sigma Chemical Co., Germany) and H₂O₂ 0.0064 % in sodium acetate buffer 0.11 M, pH 5.5. Reactions were stopped by adding 50 µL of 2.5 N H₂SO₄ and the plates were read on a DIALAB microplate reader (Austria) at a wavelength of 450 nm. The cutoff value was established as the mean reading of the negative control serum plus 3 times the standard deviation. The anti-CT antibody titer was determined as the reciprocal of the highest dilution at which the optical density (OD) was greater than or equal to the cutoff value.

Statistical analysis

The statistical analysis was performed using the statistical package GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). A statistical significance level of 0.05 % was used for all comparisons. Results of the vibriocidal antibody titers and the level of anti-CT-specific antibodies in the relevant serum and plasma samples were plotted, and the correlation between values of both assays was examined using the Pearson correlation coefficient (r).

Results and discussion

Correlation of vibriocidal response and anti-CT antibodies in human serum and plasma samples

The alternative use of either serum or plasma samples in antibody detection assays has been reported, but few studies have correlated antibody titers against microbial antigens between both types of samples obtained concomitantly [16, 17]. Hence, we examined the activity of bacterial growth inhibitory antibodies (vibriocides) as well as anti-CT IgG antibody levels in the blood of patients diagnosed with cholera, and its correlation. Samples were taken generally from 18 to 28 days after patient discharge. Considering that the in-hospital period ranged from 3 to 10 days after the onset of disease symptoms, most samples were older than 28 days from the start of infection. Only in one case the sample was obtained earlier, at 14 days. Blood samples were collected from nine patients, from which serum was prepared and only six samples were also processed for plasma obtainment. All serum and plasma samples were positive for vibriocidal and cholera anti-CT antibodies (Table).

The high antibody titers detected in serum indicate the effective stimulation of the immune system against both the somatic antigen and CT in all the patients. The relative increase of anti-CT titers regarding unstimulated individuals (below 200) was far higher than those observed for bactericidal antibodies, probably due to the slower decline kinetics. Anti-CT IgG antibodies reach their maximum value from 21 to 28

Table. Response of vibriocidal and anti-cholera toxin (anti-CT) IgG antibodies in serum and plasma samples of nine Cuban cholera patients*

Sample	Bactericidal titer**		Anti-CT IgG titer**	
	Serum	Plasma	Serum	Plasma
1	1280	NA	12 800	NA
2	320	NA	6400	NA
3	3620	5120	409 600	409 600
4	320	320	6400	6400
5	320	320	6400	6400
6	640	640	6400	6400
7	640	NA	12 800	NA
8	1280	1280	2263	1600
9	640	640	4525	6400

* Serum was obtained from nine individuals recovering from cholera, who resident in Havana city, Cuba, in 2013. Plasma was obtained by venipuncture from six out of the nine patients. **Titer values were expressed as the geometric mean of the titer of two independent determinations. NA: Plasma not available.

days after the onset of the disease and can remain at detectable levels for up to two years after infection. Nevertheless, maximum values of bactericidal antibodies (mainly IgM) are reached from 7 to 10 days after and decrease significantly thereafter [17-19].

Identical values of vibriocidal antibody titers and anti-CT IgG were detected in paired analysis of serum and plasma samples, in 5/6 and 4/6 samples, respectively. Variations were within the range of error allowed for each technique (± 1 dilution). Noteworthy, a statistically significant correlation was found between the titers of vibriocidal antibodies of the respective serum and plasma samples ($r = 0.9974$, $p < 0.0001$, Figure 1), as well as in the level of specific antibodies against CT ($r = 0.9934$, $p < 0.0001$, Figure 2). This indicates that neither the anticoagulant nor the remaining components present in plasma cause significant variations in vibriocidal and anti-CT titers, as compared to the titer of the respective serum samples.

Simultaneously, during the preparation of this report, another research group published results somewhat differing from ours [20]. They found that heparin

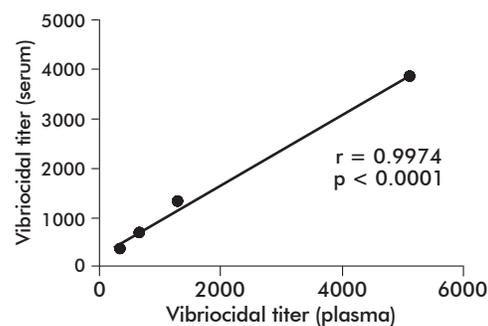


Figure 1. Correlation between vibriocidal antibody titers from serum and plasma samples. Plasma and serum samples from six cholera patients were evaluated for bactericidal activity. Serum vibriocidal antibody titers were compared with those obtained by using EDTA as anticoagulant. The diagonal line indicates the correlation line between values of both types of samples. The Pearson correlation coefficient (r) between the titers detected in the respective plasma and serum samples was obtained from two independent assays. Values were considered as statistically different for $p < 0.05$.

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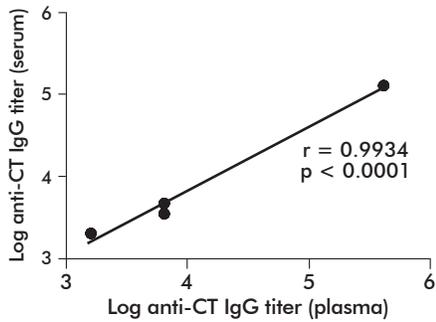


Figure 2. Correlation between anti-cholera toxin (CT) IgG antibody titers detected in serum and plasma samples. The respective plasma and serum samples from six cholera patients were analyzed to determine anti-CT IgG antibody titers by using EDTA as anticoagulant. The diagonal line indicates the correlation between values of both types of samples. The Pearson correlation coefficient (r) between the titers detected in the respective plasma and serum samples was obtained from two independent assays. Values were considered as statistically different for $p < 0.05$.

is an anticoagulant adequate for blood collection and to measure vibriocidal activity and to quantify antibody levels against different *V. cholerae* antigens in plasma samples. Nonetheless, they report that the use of EDTA in plasma production does not affect the quantification of specific antibodies but interferes in the determination of bactericidal activity.

In this regard, in the history of bactericidal assays for cholera, different methodologies have been developed, with different endpoints and methods for the determination of vibriocidal titers. Analysis based on microtitration plates has been widely used to assess the effectiveness of vaccines against *V. cholerae* [15, 21, 22]. The two variants most commonly used for determining the bactericidal titer are: spectrophotometric determination and visual observation using a chromogenic substrate as an indicator of pH changes [23] or redox changes [24].

Cedr e *et al.* developed a colorimetric method by adding glucose and a pH indicator to the culture medium for growing the surviving cells, which has been used in the clinical assessment of vaccine candidates against cholera developed in Cuba [15]. This method is based on the fact that vibrios surviving the bactericidal action grow at the expense of nutrients, leading to culture medium acidification that causes the color change of the indicator from purple to yellow. In this method the titer corresponds to 98 % or more bacterial lysis detected for the serum-free control [15, 25]. In the abovementioned study [20], a bactericidal procedure was used in microtitration plates, the end point established by spectrophotometric determination after 4 h of growth, and the highest dilution that completely inhibited bacterial growth was set as the vibriocidal titer [20]. The method of choice to establish the cutoff criterion determines the sensitivity of each technique and the ability to detect the effect that a given factor may have on the kinetics of bactericidal activity. The observed differences as for the influence of the anticoagulant, in this case EDTA, on the final result of the assay could be attributed to methodological differences

between both experimental settings, mainly due to differences for determining the endpoint. In this case, the advantage of having a simple and fast method, through visual inspection of the pH indicator color change, is associated to a lower sensitivity. Altogether, our results support the use of plasma samples obtained in the presence of EDTA for the assessment of the vibriocidal response with the colorimetric method developed in Cuba. The influence of other anticoagulants on the results shown here should be further evaluated. It was also evidenced that the variety of techniques implemented by different laboratories worldwide to measure the vibriocidal response complicates the extrapolation and comparison of results.

Obtainment of control serum as positive and negative reference material for the evaluation of the immune response against *V. cholerae*

Considering the presence of antibodies against *V. cholerae* in the serum samples available, corresponding to nine cholera-convalescent subjects, they were pooled in a single sample set as positive control for the evaluation of the immune response against this microorganism. The presence of bactericidal and anti-CT antibodies was evaluated in this sample, with titers of 1280 and 25 600, respectively, what make it available as positive control serum for both analytical techniques.

Similarly, a negative control reference sample was generated by evaluating plasma obtained from healthy blood donors, the mixture was found to have undetectable levels of both vibriocidal and anti-CT antibodies. Hence, it was established as negative control in the clinical trial.

To generate reference samples of greater stability, batches were prepared of each control (positive and negative), by fractionating the reference samples in aliquots that were lyophilized in the presence of 6.6 % bovine serum albumin. After lyophilization, 3 vials were processed from each batch per day (vials from the same batch processed in the same plate and in duplicate), which were reconstituted and the bactericidal activity and anti-CT titers reassessed (Figure 3).

Following lyophilization, a statistically significant reduction was observed in vibriocidal titers (median: 640, Mann Whitney U-test, $p = 0.0025$) and anti-CT

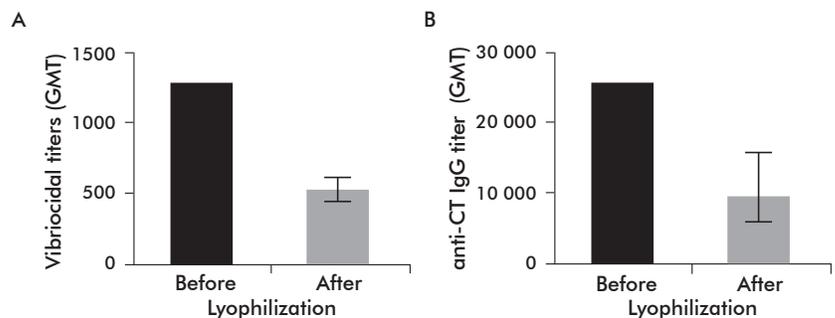


Figure 3. Effect of lyophilization on the bacterial activity and cholera anti-toxin antibody titers in pooled sera of cholera patients. A) Geometric mean titer (GMT) of antibodies with bactericidal activity in a sample of pooled sera from nine convalescent cholera patients, evaluated before and after lyophilization. B) Geometric mean titer (GMT) of cholera (CT) anti-toxin IgG antibody titers of the same pooled sera, before and after lyophilization. Error bars indicate the 95 % confidence interval of the mean.

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IgG titers (median: 12 800, Mann Whitney U-Test, $p = 0.0179$). In the case of the determination assay for anti-CT antibody test in human serum, a titer of 400 is considered positive, while seroconversion criterion for bactericidal antibodies is established based on a four-fold increase in titer values in respect to that of pre-immune serum, which is usually in a range between 20 and 80 in individuals without prior exposure to *V. cholerae* [9]. These elements determined that both the anti-CT and vibriocidal antibody titers in the analyzed reference sample are considered unequivocally positive and it is ready to be used as control material in both assays. On the other hand, the negative control reference sample maintained the antibody levels detected before lyophilization, with assigned titers of 10 (vibriocidal titer) and 100 (anti-CT antibody titer), respectively. These results allow us to conclude that both reference samples can be used as positive and negative controls, respectively, in assays to determine vibriocidal and anti-CT antibodies present in human serum samples. Moreover, these provide us an advantage for the research, since the

homogeneity and stability of samples is guaranteed during the clinical evaluation of vaccine candidates. They are very valuable reference quality control tools that allow detecting any deviation of the results during the execution of each technique, as well as to harmonize the results among different tests.

Conclusions

In summary, our results indicate that serum and plasma samples obtained in the presence of EDTA at the concentration tested can be used interchangeably to perform the quantification assay of anti-CT and vibriocidal antibody levels. Notably, the correlation between the vibriocidal titers corresponding to each type of sample will depend on the characteristics of the test used. It is also reported the collection and evaluation of human sera and plasma reference samples positive and negative against anti-cholera antibodies. These can be applied as controls in assays aimed to determine cholera anti-toxin antibodies and vibriocidal activity during the clinical assessment of vaccines against cholera.

Received in October, 2016.

Accepted in April, 2017.