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A semi-automated vibriocidal assay for improved measurement of cholera vaccine-induced immune responses

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Abstract

Vibriocidal antibody assay has been a surrogate standard assay in the evaluation of cholera vaccine efficacy because it has a good correlation with protection. Although the optical density-based vibriocidal assay in a 96-well microtiter-plate format is widely used in clinical trials, it has limitations as vibriocidal titers are altered by incubation time and samples with the same end-point titers could have potentially different vibriocidal kinetics. In the present study, we developed an improved agar-plate assay coupled with an automated colony counting system. Through testing 30 pairs of human sera from vaccinees administered with a cholera vaccine or placebo, these two assays showed good correlations for the vibriocidal titers and fold increases in titers between pre- and post-vaccinated sera as determined by the Pearson correlation coefficient and the Regression coefficient. Notably, the newly-developed semi-automated assay demonstrated that serum samples with the same end-point titers turned out to have distinct vibriocidal kinetics that were not distinguishable by the microtiter-plate assay. The semi-automated assay responded specifically to *Vibrio cholerae* but not to irrelevant bacteria such as *Salmonella typhi* and *Escherichia coli*. These results demonstrate that the semi-automated assay provides better sensitivity, accuracy, and stability of the assay results with minimized efforts than conventional microtiter-plate assay and could provide a useful tool as an *in vitro* surrogate assay for the evaluation of cholera vaccine efficacy.

Keywords: *Vibrio cholerae*; Improved vibriocidal assay; Cholera vaccine

1. Introduction

Cholera is an acute intestinal infectious disease caused by the gram-negative bacterium *Vibrio cholerae*. It produces an enterotoxin that, without proper treatment, can cause watery diarrhea in the host and lead to death. This life-threatening disease is the cause of several million global cases of infection with over 100,000 deaths annually (Vu et al., 2003). *V. cholerae* is grouped into O1 and O139 serogroups. O1 serogroup is further classified as classical or El Tor biotype by its physiological properties. Each biotype is then classified into serotypes Inaba, Ogawa, or Hikojima by serologic differences in their O antigen structures of lipopolysaccharide (Chatterjee and Chaudhuri, 2003). Although hygienic and sanitary control is the best way to prevent *V. cholerae* acquisition, vaccination is still considered to be an effective tool in protecting individuals from the disease, especially in the less developed countries where access to high-quality sanitary conditions is limited (Quick et al., 1996).

A killed injectable vaccine was developed earlier, but it was not practically applicable because it only conferred a period of protection of about 6 months. In addition, reactogenicity issues with painful inflammation, combined with a limited protective ability, did not encourage continuous use of the vaccine and did not prevent the disease from spreading (Mosley et al., 1972).

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Next, oral vaccines were introduced. These vaccines were formulated with either killed whole cells supplemented with cholera toxin B subunit (Holmgren et al., 1989) or a live-attenuated non-virulent mutant strain of *V. cholerae* (CVD103HgR) (Tacket et al., 1999). Although the former was found to be efficacious and has been used in many countries, research and development are still on-going in order to develop new or improved vaccines with better protective efficacy and lower reactogenicity.

In order to develop effective vaccines, a reliable assay that can effectively evaluate vaccine efficacy is a necessity. The vibriocidal assay which measures the ability of antibodies to kill *V. cholerae* has been widely used because it has a good correlation with the protective efficacy of cholera vaccines (Levine, 1992; Svennerholm et al., 1984). The first vibriocidal assay was an agar plate-based assay (Finkelstein, 1962), although it is no longer used due to its labor-intensive and time-consuming processes. Next, an agar-plaque method was developed but a serious problem occurred where agglutination of viable cells took place by antibodies (Holmgren et al., 1971). The third assay developed was a tube assay using bacteria culture broth. Unfortunately, this assay was capable of testing only a few samples at a time (Benenson et al., 1968; Feeley, 1976). More recently, a microtiter-plate-based assay was introduced and has been used widely to evaluate vaccine efficacy against *V. cholerae* since it is easy to perform with acceptable reproducibility and specificity (Attridge et al., 2002).

Although the microtiter-plate assay is commonly used among the aforementioned methods in the evaluation of cholera vaccine efficacies, it has limitations yet to be overcome. For instance, the microtiter-plate assay may not be appropriate in testing serum samples as having different vibriocidal kinetics but with the same end-point vibriocidal titer. Moreover, vibriocidal titer changes in proportion to incubation time. In order to overcome such limitations and to further improve the assay, we have developed a semi-automated vibriocidal assay. This assay combines the agar-based assay and the microtiter-plate assay with a semi-automated colony counting system that has been used in the opsonophagocytic-killing assay for the evaluation of pneumococcal vaccines (Burton and Nahm, 2006; Kim et al., 2003). The semi-automated colony counting system contains two key components, that are an overlay-agar containing 2,3,5-triphenyl tetrazolium chloride (TTC) dye to colorize the bacterial colonies and an automated colony counter to efficiently enumerate the colonies.

2. Materials and methods

2.1. Reagents

Brain Heart Infusion (BHI) was obtained from DIFCO (San Jose, CA, USA), and the TTC dye was purchased from Sigma-Aldrich (St. Louis, Missouri). *V. cholerae* O1 El Tor Inaba strain, T19479 was kindly provided by Prof. Jan Holmgren (Gothenbug University, Sweden). Guinea pig complements purchased from Rockland (Gilbertsville, PA, USA) were aliquoted and frozen at −80 °C until used. When an aliquot was thawed and used, any remaining parts were discarded. Fetal bovine serum (FBS) and phosphate-buffered saline (PBS) were obtained from Hyclone (Logan, UT, USA) and Mediatech Inc. (Herndon, VA, USA), respectively.

2.2. Serum samples

Blood was drawn from Vietnamese individuals who had been vaccinated with a bivalent whole-cell killed cholera vaccine produced by VABIOTECH, Vietnam, through the clinical phase II trial in SonLa, Vietnam, from May to June 2005 (Anh et al., 2007). The sera have been prepared from the blood and stored at −80 °C until used since the year 2005. Among the serum samples, 25 samples from vaccinated group and 5 samples from placebo group were chosen and given arbitrary identification numbers from 1 to 30. Complements in the sera were inactivated by heating them at 56 °C for 30 min before the

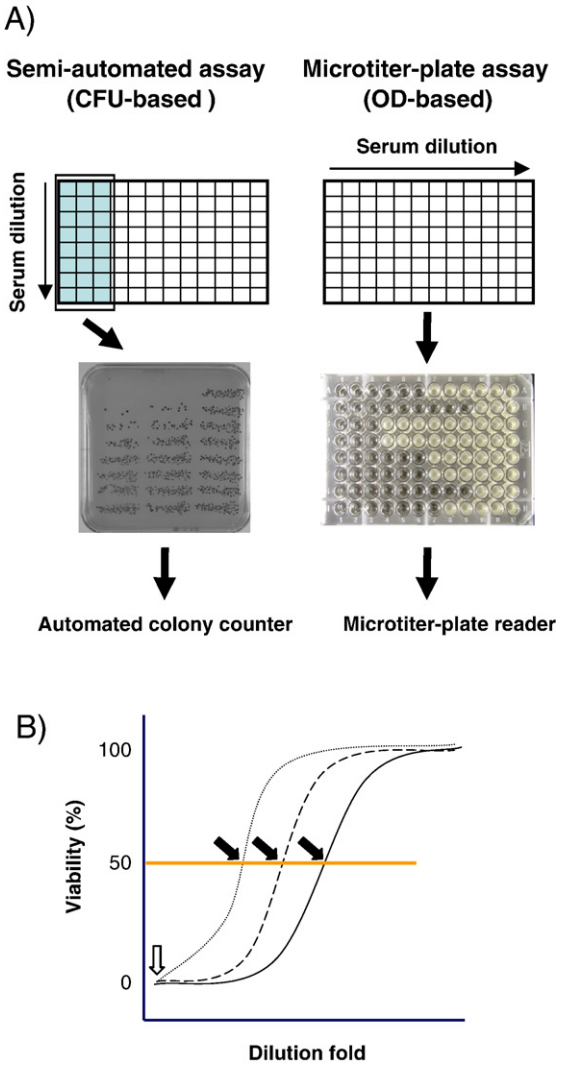

Fig. 1. Illustration of the newly-developed assay using an automated colony counting system (semi-automated assay) and the traditional vibriocidal assay (microtiter-plate assay) (A) and definitions of vibriocidal antibody titers at each assay (B). Open arrow indicates the end-point titer (100%-killing) used for the microtiter-plate assay and closed arrow indicates the median titer (50%-killing) used for the semi-automated assay.

Table 1
Comparison of the vibriocidal antibody titers between microtiter-plate assay and semi-automated assays

Group	Serum no.	Microtiter-plate assay			Semi-automated assay		
		Pre-vaccinated	Post-vaccinated	Fold increase	Pre-vaccinated	Post-vaccinated	Fold increase
Vaccinees	1	2.5	640	256	0.8	1646	2057.5
	2	2.5	160	64	2	330	165.0
	3	2.5	80	32	15	326	21.7
	4	10	1280	128	83	11,731	141.3
	5	10	1280	128	52	3563	68.5
	6	20	80	4	78	495	6.3
	7	20	80	4	41	297	7.2
	8	40	1280	32	161	6896	42.8
	9	40	160	4	155	1271	8.2
	10	40	1280	32	266	8328	31.3
	11	80	640	8	98	1548	15.8
	12	80	1280	16	515	18,632	36.2
	13	80	1280	16	279	15,517	55.6
	14	80	320	4	263	1522	5.8
	15	80	320	4	141	1122	8.0
	16	160	320	2	922	2455	2.7
	17	160	320	2	779	2821	3.6
	18	320	1280	4	700	10,653	15.2
	19	320	2560	8	2015	17,676	8.8
	20	320	2560	8	1474	16,551	11.2
	21	320	320	1	606	1790	3.0
	22	640	1280	2	2296	10,809	4.7
	23	640	1280	2	1604	5955	3.7
	24	1280	2560	2	8271	21,752	2.6
	25	2560	5120	2	4222	25,569	6.1
Placebo	26	2.5	2.5	1	5	2	0.4
	27	10	10	1	59	61	1.0
	28	80	80	1	152	167	1.1
	29	160	160	1	621	820	1.3
	30	160	320	2	1318	1713	1.3

assay. Quality control (QC) sera were prepared by pooling convalescent sera from patients with high vibriocidal titers. The QC sera were used when every assay was performed, in order to confirm the assay consistency. The use of all serum samples was approved by the institutional review board.

2.3. Microtiter-plate-based vibriocidal assay

The serum samples were diluted in 0.85% saline at 1 in 2.5 and then serially diluted two-fold until reaching 1:1,280 dilution or more if necessary for routine assay. Each well of a 96-well plate (Nunc, Roskilde, Denmark) was filled with 25 μl of sample. Single colony of *V. cholerae* O1 (strain T19479) grown on a BHI agar plate was inoculated and cultured in 20 ml of BHI broth for 2-3 h at 37 °C, with shaking at 160 rpm. The bacteria were harvested by centrifugation, resuspended in saline, and diluted into saline containing 10% guinea pig complements to a final bacterial concentration of 1×10^6 cells/ml. An equal volume of the reaction mixture was added to the microtiter-plate which contained serially-diluted serum samples. After incubation at 37 °C for 1 h, 150 μl of fresh BHI media was added to each well, and the plate was incubated for an additional 4 h, when the optical densities of the plate were read at 600 nm with a microtiter-plate reader (Spectramax 190, Molecular Device, Sunnyvale, CA, USA). The vibriocidal antibody titer by the microtiter-plate assay was defined as the reciprocal of the highest serum-dilution completely inhibiting bacterial growth. Titers below 2.5 were considered as 2.5 for statistical analysis. A four-fold or greater increase in titer between pre- and post-vaccination sera was used to indicate seroconversion (Anh et al., 2007).

2.4. Improved vibriocidal assay using an automated colony counting system

The initial procedures of the semi-automated assay are the same as those of the microtiter-plate assay as mentioned above with only minor modifications in bacterial number (5×10^4 cells/ml), content of the complements (2.5%) and dilution buffer (0.2% gelatin and 2.5% FBS in PBS). At the end of incubation, 5 μl each of the reaction mixtures was taken from each well and plated in 8 by 3 (24 samples in total) on a square BHI agar plate using an 8-channel micropipette as described (Fig. 1A). Once the mixtures were absorbed into the bottom agar, the agar plate was overlaid with a top agar (BHI medium containing 2% agar and 100 μg/ml of TTC dye). The plates were then incubated at 37 °C over night. Bacterial colonies on the plates were counted using an automated colony counting system (FluorChem™ IS-9900, Alpha Innotech Co., San Leandro, CA, USA). Eventually, single plate (90 mm × 90 mm) was occupied by 24 BHI squares with 25 mm × 5 mm each where 100–200 microcolonies were optimal for accurate enumeration using the

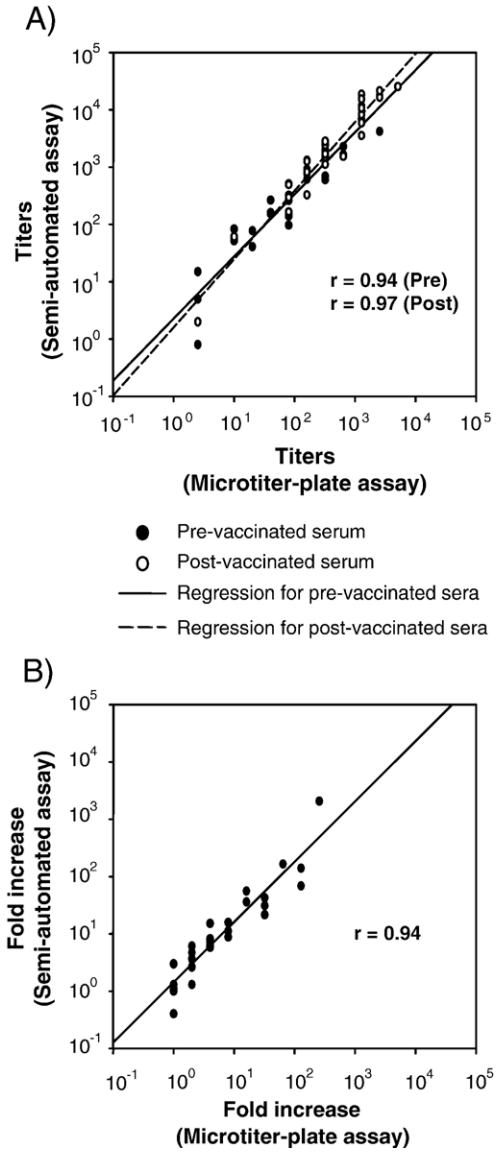

Fig. 2. Comparison of vibriocidal titers (A) and fold increase (B) between the microtiter-plate assay and the semi-automated assay. Thirty pairs (pre- and post-vaccination) of vaccinees' sera including placebo were analyzed for the vibriocidal titers using the semi-automated assay and the microtiter-plate assay. The Pearson coefficient and Regression coefficient were obtained to compare the assays as described in the Materials and Methods section.

colony counting system as compared by manual counting. The vibriocidal titer by the semi-automated assay was defined as a reciprocal of serum dilution fold exhibiting 50% bacterial killing (median vibriocidal antibody titer) to the complement control (no antibody was added) as defined in the opsonophagocytic-killing assay for the evaluation of pneumococcal vaccines (Burton and Nahm, 2006; Kim et al., 2003).

Table 2
Summary of results

Fold increase in titer	Number of individuals with indicated titer increase	
	Microtiter-plate assay	Semi-automated assay
<4	12	10
≥4	18	20

2.5. Statistical analysis

To compare the performances of the semi-automated assay and the microtiter-plate assay, the results of each serum sample obtained by two methods were plotted against each other. The Pearson correlation coefficient (r) was obtained, and regression lines were fitted for each fold increase in titers between pre- and post-vaccination to determine the Regression coefficient (β).

3. Results

3.1. Comparison of the semi-automated assay and the microtiter-plate assay

To improve the vibriocidal antibody titer assay, we developed an improved agar plate assay combined with an automated colony counting system as illustrated in Fig. 1A. Theoretically, sera with the same vibriocidal titer by the microtiter-plate assay would have different titers by using the semi-automated assay (Fig. 1B). In order to compare the performance of two assays, we analyzed 30 pairs (pre- and post-vaccination) of placebo and vaccinees' sera with low, medium, and high vibriocidal antibody titers which had been examined in the earlier study (Anh et al., 2007). As shown in Table 1 and Fig. 2, both assays produced similar results with high correlations. Titers between two methods have $r=0.94$ for pre-vaccinated samples and $r=0.97$ for post-vaccinated samples. Regression coefficients between the methods are $\beta=1.084$ for pre-vaccinated samples and $\beta=1.266$ for post-vaccinated samples (Fig. 2A). Fold increases in the titers between the assays have $r=0.94$ (Fig. 2B).

Although these two assays appear to be highly correlated in the vibriocidal titers, critical differences are also observed. As

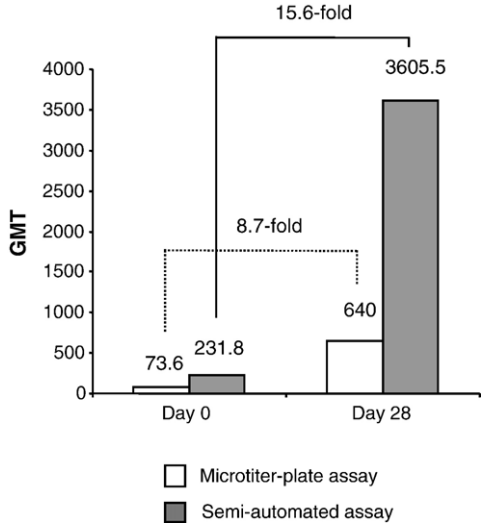

Fig. 3. Geometric mean titer (GMT) of the pre- and post-vaccinated sera between the microtiter-plate assay and the semi-automated assay. Thirty pairs (pre- and post-vaccination) of vaccinees' sera including placebo were analyzed for the vibriocidal titers using the semi-automated assay and the microtiter-plate assay. GMT and fold increases were calculated from the results.

shown in Table 2, 18 sera out of 25 vaccinees' sera (72%) exhibited greater than 4-fold increase in the titers between pre- and post-vaccination through the microtiter-plate assay, while 20 out of 25 (80%) gave similar results using the semi-automated assay. It is notable that a 4-fold or higher increase in the vibriocidal titers between pre- and post-vaccinated sera is considered to be protective against cholera infection (Anh et al., 2007). In addition, Fig. 3 shows that these two assays differ in the geometric mean titer (GMT) values calculated as previously described (Anh et al., 2007). In the semi-automated assay, GMT values were 231.8 and 3605.5 for pre- and post-vaccinated sera, respectively, with approximately 15.6-fold between the values. In the microtiter-plate assay, GMT values were 73.6 and 640.0 for pre- and post-vaccinated sera, respectively, with approximately 8.7-fold between the values (Fig. 3). As expected, sera obtained from placebo showed minimal changes by both assays.

3.2. Improved resolution in the measurement of vibriocidal antibody titers and specificity of the semi-automated assay

Although both assays have good correlations in the vibriocidal titers and fold increases between pre- and post-vaccination, most serum samples with the same titers by the microtiter-plate assay turned out to have the distinctive titers by using the semi-automated assay (Table 1). Indeed, for example, titers of the serum samples, 21 (pre), 15 (post), and 17 (post), were all 320 in the microtiter-plate assay, while titers obtained through the semi-automated assay were 606, 1122, and 2821, respectively (Fig. 4A). The improved resolution was made by taking median titers (50%-killing point) from the different vibriocidal kinetics rather than end-point titers (100%-killing point). In addition, specificity of the semi-automated assay was examined by testing irrelevant enteric bacteria *Escherichia coli* BL21 and *Salmonella typhi* Ty21a together with *V. cholerae* O1 El Tor Inaba (Fig. 4B). Growth of the only *V. cholerae* was inhibited in proportion to the vaccinated serum while those of the irrelevant bacteria were not affected under the same condition.

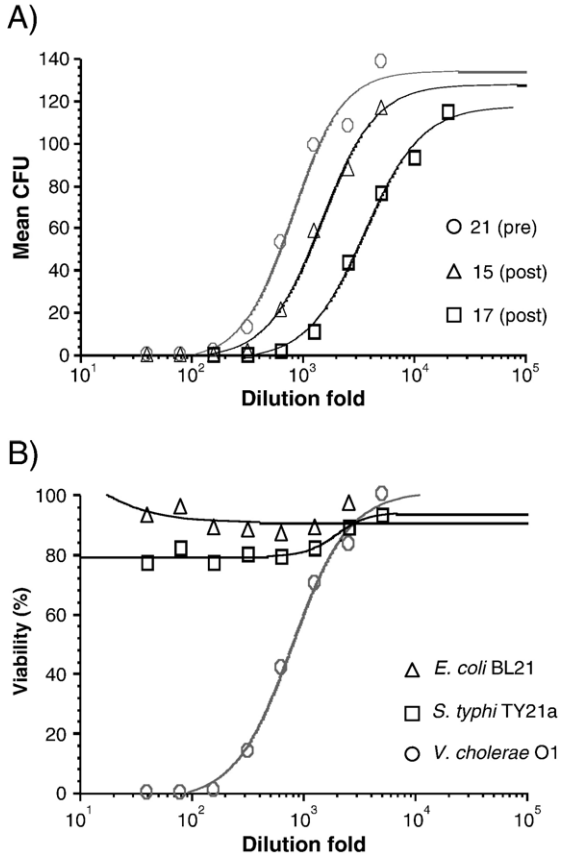

Fig. 4. Sensitivity and specificity of the semi-automated vibriocidal assay. Three human sera with the same end-point vibriocidal titers by the microtiter-plate assay were tested by the semi-automated assay (A). The semi-automated vibriocidal assay was performed using a vaccinee's serum against *E. coli* BL21, *S. typhi* Ty21a, or *V. cholerae* O1 (B).

4. Discussion

Measurement of vaccine efficacy with high accuracy and sensitivity is pivotal in the development and evaluation of vaccines. Although the vibriocidal assay using microtiter-plate has been widely used in many clinical trials mainly due to its ease of performance, it has certain limitations. These limitations include the possibility of a change in titers in proportion to the incubation period leading to inconsistent results and indiscrimination of samples with different bacterial killing kinetics. The semi-automated assay generates consistent vibriocidal antibody titers without making any change in the titers during the incubation period because longer incubation increases only the size of colonies but not the numbers. Furthermore, it is possible to distinguish samples with different bacterial killing kinetics by obtaining titers at the dilution fold with 50% killing of the target bacteria. Therefore, the semi-automated assay is more stable, accurate and sensitive than the conventional microtiter-plate assay and would be useful for the evaluation of cholera vaccine efficacy.

Besides enhanced accuracy and sensitivity of the semi-automated assay, additional advantages could be found. First, the newly-developed assay makes high throughput possible by running 24 samples on a single plate. These results show that the new assay is 24-times more efficient than a normal agar plate assay which runs one sample per plate. Second, the colonies were counted with an automated colony counting system which is rapid, convenient and well-controlled. Third, overlay using top agar can reduce the researchers' exposure to live culture of *V. cholerae,* which is a dangerous and potentially infectious bacterium in the laboratory environment. Fourth, presence of top agar could retard bacterial growth by providing anaerobic condition, so that a microcolony forms in appropriate size countable by the automated colony counter and the time point to read the results is flexible. Fifth, the use of TTC dye, which is well known as an indicator for the viability of bacteria, makes colonies clearly visible in red, which allows the count of only live bacterial colonies (Thom et al., 1993). Sixth, the assay can reduce the time necessary to perform an assay because there is no incubation time for the second growth phase, while the incubation is an unavoidable step in the microtiter-plate assay.

Seventh, fewer bacteria and fewer amounts of complements were used in the colony counting system than in microtiter-plate assay. Thus, the semi-automated assay is not only less labor-intensive and less-time consuming but also less costly.

Such semi-automated assay system was previously introduced in a pneumococcal opsonophagocytic-killing assay (Kim et al., 2003). Since then, it has progressed to have better efficiency, shortened assay time, and lower cost. It is worthwhile to note that laboratories do not always have to possess the colony counting system. Normal digital camera or document scanners can be used for capturing the image of agar plates. The microcolonies on the scanned image are then enumerated at an institution equipped with the colony counting system (Putman et al., 2005). The other approach is multiplexing capable of testing multiple serotypes with a single assay running. It might be valuable to have the multiplexed assay especially when only limited blood samples are obtained from certain subject groups such as pediatric vaccinees. In light of the fact that the current cholera vaccines are formulated with multiple serotypes (such as O1 Inaba, O1 Ogawa and O139), a multiplexed assay would certainly improve the assay performance. With pneumococcal assays, a 4-fold multiplexed assay was introduced and consumed the same amount of serum as needed for the single-plexed assay as a result of use of antibiotic-resistant strains (Burton and Nahm, 2006).

Although this semi-automated assay provides valuable advantages, some issues should be further addressed before its application in clinical trials. First, the sources of complement may change the vibriocidal titers since the assay uses complements from guinea pigs but not humans. Deposition of complements from other species to target bacteria may differ in the bactericidal ability of the antibodies. Second, structures of the antibodies with protective efficacy should be characterized. Most of current cholera vaccines are administered orally, and secretory antibodies, such as IgA, are supposed to be a major isotype responsible for the bactericidal functions. But the vibriocidal assay tests serum prepared from blood, not from mucosal areas. Even the structure of serum IgA is known to be different from secretory IgA found in mucosal areas (Otten and van Egmond, 2004). In addition, bacterial epitopes conferring opsonization should be characterized. Third, vibriocidal assay conditions should be standardized to produce consistent results. The results may vary depending on bacterial strains and their growing conditions. In addition, reference serum and calibration serum are, so far, not available.

In conclusion, the semi-automated assay is a potential surrogate assay to evaluate efficacy of vaccines against *V. cholerae* because it provides better sensitivity, accuracy, and stability of the results but minimized efforts when compared to conventional methods used in clinical trials.

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