A Novel Luminescence-Based Serum Bactericidal Assay for *Vibrio cholerae* Reduces Assay Variation, Is Time- and Cost-Effective, and Directly Measures Continuous Titer Values

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Abstract. Cholera remains a significant public health burden worldwide, and better methods for monitoring cholera incidence would enhance the effectiveness of public health interventions. The serum bactericidal assay (SBA) has been used extensively for *Vibrio cholerae* vaccine assessments and serosurveillance. Current SBA approaches for *V. cholerae* rely on colony enumeration or optical density (OD_{600nm}) readings to measure viable bacteria following complement-mediated lysis. These methods provide titer values that are constrained to discrete dilution values and rely on bacterial outgrowth, which is time consuming and prone to variation. Detection of bacterial proteins following complement-mediated lysis presents a faster and potentially less variable alternative approach independent of bacterial outgrowth. Here, we present an SBA that measures luciferase luminescence driven by lysis-released adenylate kinase. This approach is faster and less variable than growth-dependent SBAs and directly measures continuous titer values. This novel SBA method can potentially be applied to other bacteria of interest.

INTRODUCTION

The serum bactericidal assay (SBA) is an effective tool for determining levels of functional antibodies in serum. The SBA is traditionally performed by generating dilutions of heat-inactivated serum from the study subject, which are then incubated with exogenous complement. The amount of cell lysis induced by antibody and complement can then be measured by enumeration of viable colonies, bacterial outgrowth, or measurement of bacterial lysis products. Colony enumeration SBAs have been developed for a variety of pathogens, but require overnight incubations and time-consuming plating and enumeration.^{1–6} Incorporation of automated plating and counting technologies can increase throughput, but overnight incubations are often still required.^{7–11} Plating and overnight incubations can be avoided through the use of the microtiter plate SBA, which measures bacterial outgrowth directly in the microtiter plate used for serum dilutions. Bacterial growth can be measured by optical density (OD_{600nm}) or through colorimetric metabolic indicators.^{12–17} Determining titer through OD_{600nm} or metabolic indicator reduces total assay time, but variations in bacterial outgrowth can impact titer determination.

Measuring bacterial lysis products offers an alternative approach that does not require overnight incubation and is not impacted by variation in bacterial outgrowth. Serum bactericidal assays measuring extracellular adenosine triphosphate (ATP) as a proxy for bacterial lysis have been developed for several pathogens.^{18–21} In addition to ATP, adenylate kinase activity, which catalyzes the reaction 2 adenosine diphosphate (ADP) $\longleftrightarrow$ ATP + adenosine monophosphate (AMP), can also be used as a proxy for cell lysis.^{22,23} Levels of extracellular adenylate kinase released during cell lysis can be measured through the addition of exogenous ADP, luciferin, and luciferase (Figure 1). Lysis-released adenylate kinase converts the added ADP to ATP, which then drives quantifiable luciferase luminescence.

Cholera remains a significant public health burden worldwide, causing millions of cases and tens of thousands of deaths annually.²⁴ Despite their shortcomings, colony enumeration and microtiter plate SBAs remain standard assays for use in *Vibrio cholerae* vaccine assessment and serosurveillance and are the only assays available to assess correlate of protection against *V. cholerae* infection.^{6,25–29} To address the inter- and intra-assay variability and low throughput of the microtiter plate SBA method, our goal was to develop an alternative approach, through the measurement of lysis-released adenylate kinase. This luminescence-based approach reduces assay time, cost, and variability, while increasing throughput and directly measures continuous titer values.

MATERIALS AND METHODS

Bacterial strains, growth, and storage. *Vibrio cholerae* Ogawa O1 (strain X-25049) was used in all experiments. *Vibrio cholerae* Ogawa O1 stocks were stored at -80°C in Luria Bertani (LB) broth with 20% glycerol. Overnight cultures were grown in LB broth inoculated directly from glycerol stocks and incubated at 37°C with shaking (180 rpm).

Preparation of serum. Healthy donor serum was purchased from a commercial source (Sigma-Aldrich, St. Louis, MO). Deidentified clinic-collected serum samples were collected from healthy adult volunteers at the University of Utah. Deidentified field-collected samples were collected as part of a study of cholera patients in Haiti (gift from Dr. J. Glenn Morris, University of Florida, Gainesville, FL). Deidentified pre- and post-vaccine samples (gift from Dr. David Sack, Johns Hopkins Bloomberg School of Public Health Baltimore, Baltimore, MD) were collected prior to, and at 8 and 14 days after, vaccination of a healthy adult volunteer with a live oral cholera vaccine. Healthy donor serum and clinic-collected sera were filtered with a $0.22\text{-}\mu\text{m}$ filter. Clinic-collected sera were used without dilution following filter sterilization. Field-collected and pre- and postvaccine sera were diluted 1:10 in saline and were not filter sterilized. All serum samples were incubated at 56°C for 30 minutes to inactivate endogenous complement. Monoclonal anti-O-specific polysaccharide antibody used in all spiked sera experiments was provided as a gift by Dr. Jason

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FIGURE 1. Overview of luminescence-based serum bactericidal assay. (A) Up to eight serum samples are diluted in 2-fold dilutions across a micro-titer plate and incubated with *Vibrio cholerae* cells and guinea pig complement. (B) Anti-cholera antibodies attach to bacterial antigen and membrane attack complex from guinea pig complement lyses bacterial cells. (C) Lonza ToxiLight reagent containing adenosine diphosphate (ADP), luciferin, and luciferase is added to wells. Lysis-released adenylate kinase converts 2ADP to adenosine monophosphate (AMP) + adenosine triphosphate (ATP), which drives luciferase luminescence. This figure appears in color at www.ajtmh.org.

Harris, Massachusetts General Hospital (Boston, MA).³⁰ For titer drift experiments, monoclonal anti-OSP antibody was added in four different concentrations (serum A–D) to pooled healthy donor serum. For comparison of OD_{600nm} and luminescence assay experiments, monoclonal anti-OSP antibody was added to pooled healthy donor serum and three dilutions (1:4, 1:8, 1:16) were performed in healthy donor serum. Stocks of each dilution of spiked serum were aliquoted for use in optical density and luminescent SBAs, stored at –20°C, and used immediately after thawing. Clinic-collected, field-collected, and pre- and postvaccine deidentified serum samples were used for unspiked serum experiments.

Optical density-based serum bactericidal assay. Luria Bertani broth was inoculated 1:100 with overnight *V. cholerae* Ogawa O1 culture and incubated for 3 hours at 37°C with shaking (180 rpm). Cells were collected by centrifugation and washed twice with 0.9% saline. Cell pellets were resuspended in 0.9% saline to OD_{600nm} = 0.3. Growth indicator solution was prepared in 0.9% saline with 10% guinea pig complement (Sigma Aldrich) and 5% OD_{600nm} = 0.3 bacterial suspension. Transparent 96-well plates were prepared by serially diluting serum by 2-fold dilutions in 0.9% saline. Growth indicator solution was added 1:1 to wells with serum dilutions and positive growth controls containing 0.9% saline.

Plates were incubated at 37°C for 1 hour with shaking (60 rpm). Luria Bertani media was added 3:1 to all wells, and plates were incubated at 37°C with shaking (60 rpm). Optical density at 600 nm was read every 10 minutes after 1 hour and 40 minutes of incubation, until the average of the positive growth controls was above OD_{600nm} = 0.2. Titers were determined to be the reciprocal of the serum dilution with the OD_{600nm} closest to half of the average of the positive growth control OD_{600nm}.

Luminescence-based serum bactericidal assay. Luria Bertani broth was inoculated 1:100 with overnight *V. cholerae* Ogawa O1 culture and incubated for 3 hours at 37°C with shaking (180 rpm). Cells were collected by centrifugation and washed twice with 0.9% saline. Cell pellets were resuspended in 0.9% saline to OD_{600nm} = 0.3. Growth indicator solution was prepared in 0.9% saline with 10% guinea pig complement (Sigma Aldrich) and 5% OD_{600nm} = 0.3 bacterial suspension. White-walled 96-well plates were prepared by serially diluting serum by 2-fold dilutions in 0.9% saline. Growth indicator solution was added 1:1 to wells with serum dilutions. Plates were incubated at 37°C for 1 hour with shaking (60 rpm). ToxiLight BioAssay reagent (Lonza, Morristown, NJ) was prepared according to manufacturer protocol and added 2:1 to all wells. Plates were developed for 30 minutes and luminescence was read on a Biotek Synergy H1M plate reader (Winoski, VT). For Max/2 titer calculations, titers were determined to be the reciprocal of the serum dilution closest to half of the maximal luminescence observed. For lethal dose 50% (LD₅₀) titer calculations, percent maximal luminescence was calculated by dividing all luminescence values by the maximal luminescence value. Values under 15% were excluded, and remaining values were plotted on a log₂ scale. An exponential line was fit to the data and the LD₅₀ titer was calculated using the equation of the line, with the y value set to 50. Max/2 and LD₅₀ titer calculation examples are provided in the supplemental protocol.

Calculation of assay cost. The cost of all reagents and consumables was obtained from manufacturer websites and adjusted to the cost per assay. Cost was calculated assuming the OD_{600nm} SBA was performed in technical duplicate with three patient samples per plate, and the luminescence SBA was performed with eight patients per plate. Labor cost was calculated assuming a technician is paid \$15/hr and can prepare six plates simultaneously.

Statistical analysis. The χ^2 test was used to compare the agreement of technical replicates between OD_{600nm} and luminescence methods. Coefficient of variation (CV) was calculated between trials using titer values determined from averaged technical duplicate wells. To ensure all titer calculations could be performed without experience with more sophisticated analysis programs, all titer calculations were performed in Microsoft Excel, as shown in the supplemental protocol.

RESULTS

Impact of incubation time on titer in OD_{600nm} SBA. Titers were determined using optical density for four spiked serum samples (serums A–D) after 2, 2.5, 3, and 3.5 hours of incubation (Table 1). The titers of serum A, B, and C decreased 2-fold between 2 hours (512, 256, and 64, respectively) and 3 hours (256, 128, and 32, respectively). The titer of serum D

TABLE 1
Titers of four spiked serum samples read at four time points

	2 hours	2.5 hours	3 hours	3.5 hours
Serum A titer	512	512	256	256
Serum B titer	256	256	128	128
Serum C titer	64	64	32	32
Serum D titer	16	8	4	1
Growth control OD _{600nm}	0.213	0.253	0.246	0.247

OD = optical density.

decreased 2-fold every 0.5 hours, from 16 to 1, over the course of 1.5 hours.

The luminescence-based SBA has lower inter- and intra-assay variability and directly measures continuous titer values. Titers were determined for spiked serum and 1:4, 1:8, and 1:16 dilutions of spiked serum using OD_{600nm}, Max/2 luminescence, and LD₅₀ luminescence approaches (Table 2). The luminescence-based SBA has lower inter- and intra-assay variability and provides direct measurement of continuous titer values. Titers were determined for spiked serum and 1:4, 1:8, and 1:16 dilutions of spiked serum using OD_{600nm}, Max/2 luminescence, and LD₅₀ luminescence. Average titer values decreased with dilution for OD_{600nm} (341, 107, 43, 27), Max/2 luminescence (256, 107, 64, 32), and LD₅₀ luminescence (280, 98, 54, 32) approaches. The average of the OD_{600nm} titers for the four serum dilutions tested had greater CV percentage (43.3%, 34.6%, 43.3%, 34.6%) than the average of three of four Max/2 titers (0%, 34.6%, 0%, 0%) and all of the average LD₅₀ titers (2.5%, 1%, 7.7%, 10.2%). Variation between technical replicates was also evaluated by comparing titers of individual technical replicates. OD_{600nm} technical replicates had greater disagreement (6 of 27 replicates disagreed) than luminescence technical replicates calculated with the Max/2 approach (1 of 27 replicates disagreed). This difference was statistically significant at $P < 0.05$ without Yates correction (χ^2 statistic = 4.1, $P = 0.04$) and had a P value of 0.11 with Yates correction (χ^2 statistic = 2.6).

The luminescence SBA can be used for field-collected and pre- and postvaccine sera and is complement specific. Clinic-collected sera from two donors with previously known positive cholera SBA titers based on OD_{600nm} SBA analysis, seven field-collected sera from Haiti, and a pre- and postvaccine serum sample were tested using the OD_{600nm} SBA and the luminescence SBA (Table 3). The Max/2 luminescence approach and the LD₅₀ luminescence approach provided titer values for all serum samples tested and most values agreed with the OD_{600nm} titer or were within a 2-fold dilution. To determine background signal produced from serum and complement specificity, sera from three separate donors was tested using the luminescence SBA with and

TABLE 2

Comparison of titers calculated from OD-based vibriocidals and luminescence-based vibriocidals using healthy donor serum spiked with anti-OSP monoclonal antibody

	OD _{600nm}	Max/2 luminescence	LD ₅₀ luminescence
Spiked serum	256, 512, 256*	256, 256, 256	280, 273, 287
1:4 serum	64, 128, 128	64, 128, 128	97, 98, 99
1:8 serum	32, 64, 32	64, 64, 64	51, 59, 53
1:16 serum	16, 32, 32	32, 32, 32	28, 33, 34

LD = lethal dose; OD = optical density.

* Titer values from three independent trials

TABLE 3
Titers calculated from OD-based vibriocidals and luminescence-based vibriocidals using unspiked donor serum

Serum donor	OD _{600nm}	Max/2 luminescence	LD ₅₀ luminescence
Clinical donor 1*	16	16	39
Clinical donor 2	512	512	623
Field donor 1*	10	20	32.3
Field donor 2	40	160	185
Field donor 3	80	160	187
Field donor 4	20	40	58
Field donor 5	640	640	800
Field donor 6	640	2560	2120
Field donor 7	640	1280	2080
Prevaccine	10, 10	20, 20	34, 35
Postvaccine day 8**	40, 40	80, 80	104, 85
Postvaccine day 14	320, 320	640, 640	751, 826

* Clinic-collected samples were filter sterilized and used undiluted.

† Field-collected samples were used unfiltered and diluted 1:10.

‡ Pre- and postvaccine samples were used unfiltered, diluted 1:10, and tested in two independent replicates.

without complement (Supplemental Table 1). Serum wells without complement had reduced signal across all dilutions and background signal from the sera did not exceed 10% of the signal in titer-determining, complement-containing wells.

The luminescence SBA is more time- and cost-effective. Total time to perform the luminescence SBA (5 hours) was less than the time required to complete the OD_{600nm} SBA (7–8 hours) and the estimated cost per sample for the luminescence SBA (\$11.82) was less than the estimated cost per sample for the OD_{600nm} SBA (\$19.63).

DISCUSSION

Colony enumeration and optical density SBAs have been used extensively for *V. cholerae* vaccine assessment and serosurveillance,^{6,25–29} as the SBA has been shown to be the best serologic marker for protection and prior infection. Although these assays have provided valuable data, the colony enumeration approach is resource- and time-intensive, and the OD_{600nm} approach is prone to variability and drifting titer values (Table 1). Thus, our goal was to develop a method that addresses these deficiencies. By measuring levels of adenylate kinase released following complement-mediated cell lysis, the luminescence-based SBA avoids additional bacterial culturing and the time, resources, and variability associated with it. The luminescence SBA was used to calculate titers across a range of common values, and we demonstrated it could be used to test filtered sera, for situations where sterility is a concern, and unfiltered sera, for situations where serum volume is limited.

The variability in the OD_{600nm} SBA has traditionally required serum samples to be tested in technical duplicate; however, technical replicates showed greater agreement in the luminescence SBA as compared with the OD_{600nm} assay, potentially allowing for testing of samples with a single replicate test well. Although we acknowledge the value of testing in technical duplicate, the increase in throughput and decrease in cost provided by testing in singlet may be beneficial in settings with limited resources. Additionally, the OD_{600nm} SBA traditionally leaves the edge wells of the microtiter plate empty due to edge well growth variation, which limits plate capacity to three samples, if testing in duplicate. As the luminescence SBA does not rely on bacterial outgrowth, all of the wells of the microtiter plate can be used without concern for edge well

TABLE 4
Comparison of optical density SBA and luminescence SBA

	Optical density	Luminescence
Cost/sample*	\$19.63	\$11.82
Assay time	7–8 hours	5 hours
Samples/plate	3–4	8
Values provided	Discrete	Discrete or continuous

SBA = serum bactericidal assay.

* Cost per sample includes all required reagents and consumables and assumes a technician paid at \$15/hr can run six plates simultaneously. Cost was calculated with optical density plates containing three samples in technical duplicate and luminescence plates containing eight samples.

growth effects. This increases the throughput of the luminescence SBA to eight samples per plate in singlet, in comparison to three samples per plate in duplicate for the OD_{600nm} SBA. The increase in number of samples per plate and the reduction in reagent and consumable usage from elimination of technical replicates reduce the estimated cost of the luminescence SBA to \$11.82 per sample, compared with \$19.63 per sample for the OD_{600nm} SBA (Table 4), despite requiring an additional detection reagent. A potential up-front cost in performing the luminescence SBA is a plate reader equipped with a luminometer; however, many plate readers used for OD_{600nm} measurements are already equipped with a luminometer and there are a number of affordable luminometer options if a laboratory was not already equipped with one.

The luminescence SBA provides an additional advantage by producing data with increased resolution. Although traditional SBA approaches provide titer values that are constrained to discrete dilutions (i.e., interval censored), the luminescence-based approach allows for the direct measurement of continuous titer values. This approach allows for more accurate determination of titer. This is illustrated in the titer values for the 1:4 antibody dilution in Table 2. Replicates of the OD_{600nm} titer values (64, 128, 128) were either higher or lower than the LD₅₀ titer values (97, 98, 99), which could represent a more accurate measure of the true titer of the sample. Increasing the resolution of the data provided by the SBA will enhance vaccine development and serosurveillance efforts by providing more accurate titer values.

Determining titer by measuring the levels of lysis-released adenylate kinase could be applied to a variety of pathogens for which traditional SBA approaches are being used. The luminescence SBA could be particularly useful for bacteria with slow growth rates or challenging culture conditions. The reduced variability and ability to directly measure continuous titer values make this approach a more robust and accurate alternative to current approaches.

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