Standardization of *Neisseria meningitidis* Serogroup B Colorimetric Serum Bactericidal Assay

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The correlate of protection for serogroup B meningococci is not currently known, but for serogroup C it is believed to be the serum bactericidal assay (SBA). The current SBAs are labor intensive and the variations in protocols among different laboratories make interpretation of results difficult. A colorimetric SBA (cSBA), based on the ability of *Neisseria meningitidis* serogroup B to consume glucose, leading to acid production, was standardized by using group B strain Cu385-83 as the target. The cSBA results were compared to those obtained for a traditional colony-counting microassay (mSBA). Glucose and bromocresol purple pH indicator were added to the medium in order to estimate growth of cSBA target cell survivors through color change. Different variants of the assay parameters were optimized: growth of target cells (Mueller Hinton agar plates), target cell number (100 CFU/well), and human complement source used at a final concentration of 25%. After the optimization, three other group B strains (H44/76, 490/91, and 511/91) were used as targets for the cSBA. The selection of the assay parameters and the standardization of cSBA were done with 13 sera from vaccinated volunteers. The titers were determined as the higher serum dilution that totally inhibited the bacterial growth marked by the color invariability of the pH indicator. This was detected visually as well as spectrophotometrically and was closely related to a significant difference in the growth of target cell survivors determined using Student’s *t* test. Intralaboratory reproducibility was ±1 dilution. The correlation between bactericidal median titers and specific immunoglobulin G serum concentration by enzyme immunoassay was high (*r* = 0.910, *P* < 0.01). The bactericidal titers generated by the cSBA and the mSBA were nearly identical, and there was a high correlation between the two assays (*r* = 0.974, *P* < 0.01). The standardized cSBA allows easy, fast, and efficient evaluation of samples.

Serogroup B strains of *Neisseria meningitidis* are an important cause of meningitis, an epidemic disease that is a major health problem in various parts of the world (10, 24). The role of circulating antibody and complement in protection from meningococcal disease was demonstrated in 1918 (14, 16). In the 1960s, classic studies by Goldschneider et al. provided evidence that protection of humans from meningococcal disease correlated with the presence of serum bactericidal activity (12, 14).

The serum bactericidal assay (SBA) is a functional measure of the ability of antibodies in conjunction with complement to kill bacteria and is considered the assay of choice for measurement of functional antimeningococcal antibodies in vitro. Different protocols have been developed to demonstrate the presence of bactericidal antibodies, but all of them have three main elements: bacteria, antibody, and complement. Available SBAs differ in the number of CFU per well (1, 9, 18), assay buffer (9, 18, 26), growth of the target strain (9, 12, 18), assay incubation time (18, 19, 26), complement source (5, 12, 15, 18, 12, 28, 29), complement concentration (11, 15, 26), and starting serum dilution (5, 25, 26).

The minimum level of protection by antibodies was established by Golschneider et al. (12) for serogroup C using human complement at a titer of ≥4. Recently, Borrow et al. (2) reestablished these correlates with baby rabbit complement. The potential effectiveness of polysaccharide vaccines is evaluated through detection of the induction of bactericidal antibodies. SBA is a well-established correlate for protection from serogroup A and C meningococcal disease (13). This criterion has been extended to nonpolysaccharide vaccines like those developed against serogroup B. Several studies support a relationship between SBA and clinical protection from serogroup B meningococcal disease (3, 6, 15, 20, 29). However, data from one recent study suggest that SBA may underestimate the clinical efficacy of serogroup B vaccine (22).

The traditional SBA is considered labor intensive and not workable for large numbers of samples. The major problem with traditional SBAs lies with the techniques, which involve plating and counting of target bacteria. New protocols have been developed to replace the traditional SBAs; for example, Kriz et al. described a modification of the bactericidal microassay using triphenyltetrazolium chloride solution (TTCmSBA) as a germination indicator for visualizing the results (17). Recently, Mountzouros and Howell described a fluorescence-based SBA (ISBA) for serogroup B *N. meningitidis* (21). More investigation is needed to standardize a universally accepted SBA for the detection of serogroup B *N. meningitidis*.

The purpose of this study was to select assay parameters (target strain preparation, number of CFU per well, and complement concentration) that led us to standardize a colorimetric SBA (cSBA) based on the ability of *N. meningitidis* serogroup B to consume glucose, leading to acid production. We added glucose and a pH indicator to the medium in order to
estimate growth of SBA target cell survivors through color change. Thereafter, we optimized the assay to obtain intralaboratory reproducible titers with a variety of sera from immunized adult volunteers and compared the results generated by the cSBA with those generated by a traditional colony-counting microassay (mSBA).

MATERIALS AND METHODS

Bacteria. The Cuban vaccine N. meningitidis serogroup B strain Cu385-83 (B:4:P1.19,15I:3,7,9) was used as the target strain in the standardization of the bactericidal assay. Three other strains of serogroup B were used as target strains in the standardized cSBA. They were 511/91 (B:2b:P1.10), isolated in Argentina; 490/91 (B:14:P1.7), isolated in Uruguay; and 44/76 (B:15:P1.7.16), isolated in Norway. These strains were stored in skim milk (Oxoid Ltd., Basingstoke, Hampshire, England) at −70°C in multiple aliquots. A fresh aliquot was obtained for each experiment to eliminate differences in the passage history of the strain.

Sample sera. Sera obtained from 13 young adults (18 to 23 years old) immunized 7 years before with two doses of VA-MENGOC-BC, the Cuban antimeningococcal vaccine against serogroups B and C of N. meningitidis, were evaluated by a standardized meningococcal enzyme-linked immunosorbent assay (ELISA) and Western blot. The serum with the higher titer of immunoglobulin G (IgG) antibodies by ELISA that also reacted against main proteins in the Western blot (proteins present in the outer membrane vesicles [OMV] of VA-MENGOC-BC) was selected as the positive control. This serum was used for the evaluation of all the assays performed to standardize the test in which the other 12 sera were used.

Normal human serum (NHS) from two adults who had never been immunized with any meningococcal vaccine and had never had meningococcal disease were evaluated by ELISA and Western blot to ensure that they did not respond against OMV and were also validated by use in the standard SBA at 25 and 50% concentrations. Those sera were processed (quick-frozen with dry ice and etha-nol) to preserve hemolytic complement activity and stored at −70°C.

Intralaboratory evaluation. The standardized assay described above was used to test 12 sera and one positive control serum from vaccinated volunteers for bactericidal activities to serogroup B strain Cu385-83. Each serum sample was tested in duplicate on four different days. After visual determination of the titers, the absorbance was measured at 405 nm to confirm the titers by quantitative determination. For each evaluated serum, titration curves were drawn using the OD obtained at each serum dilution.

Specificity of cSBA. The numbers of CFU in blue and green wells were counted in all experiments to demonstrate that the color change of the pH indicator was specifically related to the growth of cSBA target cell survivors. Samples of 10 μl from some wells before and after the color change from different dilutions of seven positive sera as well as from six negative sera, the suspension control wells, and the complement-dependent control wells were removed at the end point of the cSBA, after 20 h of incubation at 37°C with 5% CO2. They were conveniently diluted, added as droplets to MH-FBS agar, and incubated as described above.

ELISA. A standardized ELISA was performed in triplicate in microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) with a peroxidase-conjugated anti-human IgG detection system (Sigma, St. Louis, Mo.), OMV (20 μg/ml) from group B strain Cu385-83 was used as the antigen. As an internal antibody standard, a twofold dilution series of a positive postvaccination serum sample was used in all experiments. The mean value of the observed OD was transformed to arbitrary units per milliliter by a sigmoidal standard curve. Initially, all serum samples were analyzed at a 1:200 dilution. Samples with OD values of ≥90% of the maximum OD of the standard were further diluted and analyzed again.

mSBA. A traditional serum bactericidal microassay was performed in a laboratory with broad experience in performing mSBA as previously described (4, 19). Briefly, 25 μl of Hanks’ solution from the 2nd to 11th columns of wells in a 96-well microtiter plate and 25 μl of serum samples in the first well of each line were added, making a twofold dilution. Then, 12.5 μl of bacterial suspension (containing around 50 CFU) and 12.5 μl of human serum as complement source were added. The plate was incubated at 37°C without CO2 for 30 min. After incubation, Mueller-Hinton agar (150 μl/well) was added to the whole plate. The plate was incubated overnight at 37°C with 5% CO2, and the colonies were counted in all wells with a stereoscopic microscope. The results were expressed as the reciprocal serum dilution yielding ≥90% killing, that is, total inhibition of bacterial growth marked by the color irreversibility of the pH indicator, and corresponds to an OD in a range of 0.1 to 0.4 at 405 nm A.

Standardization of assay components. The assay components (target strain preparation, number of CFU per well, and complement) described above were evaluated on the basis of reported methods and the particular characteristics of the SBA. The different variants were compared and optimized with sera from immunized volunteers. The results were obtained from at least three independent experiments run on separate days.

(i) Target strain preparation. The influence on the cSBA titers of growth (broth or agar plate growth) of the working cell culture and the number of CFU per well were evaluated. Target strain Cu385-83 was grown to log phase (2 to 3 h) in MHB at 37°C in a shaker at 150 rpm until the OD was 0.25 to 0.3 at 620 nm. The titers of five sera obtained with these log-phase cultures were compared with the titers obtained with the same strain grown for 4 h on MH-FBS agar plates. Each culture was diluted to give 100 CFU per well at T0. The effect of different CFU numbers on the cSBA titers in relation to the incubation time for visualizing the color change was evaluated. The strain was diluted to give 50, 1 × 102, 5 × 102, 1 × 103, and 1 × 104 CFU per well at T0. Four sera were run against each target cell number.

(ii) Complement. Sera from two adults that lacked bactericidal activity against the serogroup B target strain were tested to determine its suitability (the CFU per well in the complement-dependent control well was ≥80% of the CFU per well at T0 and color change of the pH indicator in this well) as a complement source. The strain was grown and diluted in HBSS-BSA as described above. Eight replicates of complement-dependent control wells were prepared, and the microtiter plate was incubated for 30 min as described above. The minimal concentration of the NHS selected as the complement source needed for efficient bactericidal killing was estimated by using target strain Cu385-83. The bactericidal titer of the positive-control serum was measured with various concentrations of the complement source (25, 20, 16.8, 13.5, 11.3, 8.8, and 6.3%).
and log₂-transformed ELISA antibody concentration for each serum sample. It was also calculated by using the log₂-transformed cSBA titer and the log₂-transformed mSBA titer for each serum sample (27). Student’s t test was used to examine the significance level of differences between the number of CFU per well corresponding to each color of the pH indicator, blue (bactericidal activity zone) and green (growth zone).

RESULTS

Standardization of assay components. (i) Target strain preparation. There were no differences between broth- and agar plate-grown serogroup B target strain Cu385-83. The effect of the CFU concentration per well on cSBA titer and its relation to incubation time for visualizing the color change was evaluated. The highest evaluated concentration of CFU per well (10^6) resulted in the lowest cSBA titer after 8 h of incubation. Little or no difference in titers of evaluated sera with 100 and 50 CFU per well were found; the color change was visualized after 20 h (Fig. 1). The target of CFU per well selected for the cSBA was 100 CFU/12.5 μL.

(ii) Complement source. Serogroup B target strain Cu385-83 was tested for sensitivity to two NHS lacking bactericidal activity against this strain. The strain was sensitive to one of the two evaluated sera as a possible source of complement. This serum reduced the cell count by at least 90% from the CFU per well at T₀. The strain had acceptable cell growth (≥80% of CFU per well at T₀) with the other evaluated serum, and it was selected as the complement source.

Various complement concentrations were tested with the target strain to ensure that complement was not limited in the cSBA. Complement concentrations between 25 and 16.3% were optimal, with no difference in cSBA titers. Reductions of 2 dilutions in titer were observed with complement concentrations of 13.8 and 11.3% and of 3 dilutions with 8.8 to 6.3% complement concentrations.

Intralaboratory evaluation. The standardized assay described above was used to test 12 sera from immunized adults for bactericidal activity against serogroup B (strain Cu385-83). In the cSBA, blue and green meant serum bactericidal activity and bacterial growth, respectively. The OD in all dilutions of the negative sera as well as in complement-dependent and suspension control wells were in the range of 0.5 to 0.9. However, lower OD values in the area of bactericidal activity of the positive sera were observed, in the range of 0.1 to 0.4, followed by an abrupt increase in OD corresponding to serum dilutions where there was growth of target cell survivors (Fig. 2A). The same titer to each serum was found in 78% of 10 replicates of five positive sera and ±1 dilution in the remaining 22% of replicates. The negative sera always had the same titer (<2).

Specificity of cSBA. The count of CFU of each color showed that the change from blue to green in all evaluated positive sera corresponded to an abrupt increase in CFU. The average CFU in blue wells of evaluated positive sera was 2.87 × 10⁷, while the average CFU in green wells was 4.93 × 10⁸. In all negative sera, the color of the pH indicator changed from blue to green at all dilutions, and the average CFU count was 6.4 × 10⁸. In complement-dependent and suspension control wells,
the number of CFU was approximately the same as in green wells for all positive and negative sera (Fig. 2B). The average CFU in blue and green wells was calculated, and significant differences ($P < 0.01$) between them were found.

Use of different target strains on standardized cSBA. After the assay parameters were optimized, growth of target cells (MH-FBS agar plates), target cell number (100 CFU/well), human complement source used at a final concentration of 25%, and assay incubation time (20 h), three disease strain isolates were used as the target for the cSBA. Three positive sera and the positive control serum were titrated. Titers varied by 6 dilutions (Fig. 3). Titer differences were serum dependent. Strain 511-91 produced titers consistently lower than those of the other strains evaluated, but for the evaluated sera, the highest titers were produced against the vaccine strain Cu385-83. Each strain gave consistent ($\pm 1$ dilution) intrastrain titers with the evaluated sera.

cSBA titers correlate to titers obtained in an mSBA and to ELISA IgG concentrations. The 13 human sera used in the standardization of the cSBA and the serum used as the source of complement were evaluated by ELISA and by mSBA. The results of these assays were compared with the cSBA titers in order to determine the relationship between them. There was high correlation between ELISA and cSBA titers ($r = 0.910$, $P < 0.01$). The bactericidal titers generated by the two assays were nearly identical; 50% of evaluated sera generated the same titer in both assays, and in the other 50% the cSBA titers were in a range of $\pm 1$ dilution of the titers obtained for the mSBA. A direct correlation ($r = 0.974$, $P < 0.01$) between the cSBA and mSBA titers was demonstrated.

DISCUSSION

The bactericidal assay is a good laboratory surrogate for predicting protective titers for meningococcal vaccines (3, 6, 10). The main drawback with traditional SBAs is that the colony counts cannot be totally automated (1, 9, 15, 18), and that is why they have been described as difficult to standardize, labor-intensive, and cumbersome (21). In this study, we optimized the parameters for intralaboratory reproducibility of a serogroup B *N. meningitidis* cSBA and compared it to a traditional mSBA.

A number of bactericidal buffers have been used by different workers, but HBSS-BSA was selected for this cSBA because this has been the most widely used for serogroup B SBAs (9, 15). In order to avoid a pH increase in HBSS-BSA with storage, we used it for only 5 days after preparation. Maslanka et al. (19) reported that different lots of BSA gave different results; in our study, we used the same source of BSA in all assays. Further studies will show the influence of BSA in the cSBA buffer and the possibility of using HBSS without BSA, as was suggested by Maslanka et al. (19).

An incubation time that gives maximum titers with a minimum reduction in the number of CFU in control wells must be selected for each strain. Many researchers have shown that the optimal incubation time of target strains with complement in SBA is strain dependent (1, 18, 19, 26). We used a 30-min incubation time, as reported by Milagres et al. (20), who used the same target strain (Cu385-83).

Although various researchers reported different methods for growing target cells prior to use, most agree that cells should be in log phase. Frasch and Robbins (9) have stated that serogroup B strains grown on agar plates for 4 h are in log phase. Our data showed that growth of the serogroup B target strain Cu385-83 to log phase in broth gave titers that were more or less equivalent to those for the same strain grown for 3 to 5 h on agar plates, suggesting that the organisms were in the same metabolic state. Goldschneider et al. (12) reported that assay results are dependent on the lot of Mueller-Hinton agar used to grow target strains. We did not observe any noticeable difference in assay titers when we used different lots of MH-FBS agar plates.

The number of CFU per well is a very important parameter on cSBA because it is closely related to assay sensitivity and time to visualization of the results. This assay uses 100 CFU per well, which allowed color change to be observed after 20 h of incubation of the plate. When 10^6 CFU per well was evaluated, the incubation time was reduced to 8 h, but lower titers were detected. The cell number used in previously reported SBAs varies between the different growth methods of target cell survivors of each technique. In bactericidal assays, which are carried out in microtiter plates on solid medium, 40 or 50 to 130 CFU per well is regarded as acceptable to facilitate the counting of target cells in microtiter plates (4, 9, 15). In contrast, in bactericidal assays in which colony counting is done in large agar plates, the cell number is higher (1, 18).

SBA is a functional measure of the ability of antibodies in conjunction with complement to kill bacteria. The age-related inverse relationship between meningococcal bactericidal activity and meningococcal disease was first demonstrated with a human complement source (12). We used as the complement source a serum from an individual who had never been immunized with any meningococcal vaccine and never had meningococcal disease. The optimal final complement concentration in the assay was 25%. Ideally, this assay would combine antibodies and complement derived from the same individual to give a complete assessment of an individual's ability to fight infection. However, it would be difficult to ensure that all sera...
were treated to preserve complement, particularly in remote areas. That is why the majority of studies of serogroup B meningococcal vaccines have used donor (nonvaccinee) human complement for the measurement of bactericidal titers. Such sera may, however, be difficult to find for some strains (18).

Some researchers have evaluated the use of other complement sources, mainly pooled baby rabbit serum (BRS). Those reports (18, 29) have noted differences in the level of bactericidal activity when rabbit serum was replaced with nonimmune human serum. Mandrell et al. (18) suggested that the difference in the level of serogroup B polysaccharide bactericidal activity between human complement and rabbit complement may be due to IgM antibody, since it appeared that there was no difference in activity between complement sources with IgG antibody. One recent study in which SBA on the same specimens using both donor human complement and the vaccinee’s own complement was measured found no difference between the bactericidal titers in tested sera using donor or the vaccinee’s own complement source. These findings support the validity of using properly screened human donor complement in SBAs against serogroup B meningococci (8).

When all assay parameters were selected, the intralaboratory evaluation was done. This study has confirmed the established intralaboratory reproducibility of ±1 dilution (12). Further study with pre- and postimmunization sera is necessary to confirm that the cSBA is a useful method to detect functional antibody produced in response to VA-MENGOC-BC or other existing and new serogroup B meningococcal vaccines.

We demonstrated that the color change of the pH indicator was directly related to the growth of target cell survivors when significant differences between the number of CFU on each color were found. That is why we can affirm that the color change allows us to differentiate unequivocally the points where there was growth inhibition due to the serum bactericidal activity from those where bacterial growth was not affected. The surviving bacteria in blue wells was minor, 8% of the number of CFU in green wells. It means that more than the 90% of target bacteria were killed in each blue well in the cSBA. That is why we expressed the titer as the highest serum dilution which caused total inhibition of bacterial growth marked by the color invariability of the pH indicator. While the Cuban vaccine strain Cu385-83 was used for assay standardization, three other serogroup B strains were used as targets for the cSBA, showing that this assays works with group B strains other than Cu 385-83.

The cSBA was compared with the mSBA. The main difference between these assays lies in how the surviving bacteria are detected. The comparison of the results generated by these methods demonstrated a high correlation despite the intervention of two different operators, the use of two different human sera as source of complement, and two different laboratories.

Various methods (agar overlay, tilt, and spot) have been used for growing the surviving cells after incubation with serum and complement. All of them, as well as the microassay compared in this study to the cSBA, employ colony-counting techniques to determine the results. Those techniques of titration are labor-intensive, and the evaluation of a large number of samples in one assay is a hard task. A recently described ISBA for group B N. meningitidis uses the reduction-oxidation (redox) indicator in Alamar Blue to detect surviving bacteria after SBA components are allowed to react in a microtiter plate (21). The cSBA, like the fSBA and the TTCmSBA, eliminates the labor-intensive steps of plating reaction mixtures and counting numbers of individual CFU, which are critical for interpretation of the results of traditional SBAs (17, 21).

The 96-well format makes both ISBA and cSBA potentially adaptable to robotic systems similar to those used for ELISA but taking appropriate safety stops because both assays use live bacteria until the end point. Although the fSBA has the advantage of quantitatively detecting viable microorganisms more rapidly than a traditional SBA, the cSBA gives the possibility of titrating by just looking at the plate, without the use of any equipment. Furthermore, the titration can be confirmed spectrophotometrically. Those new protocols allow investigators to evaluate serum bactericidal activity against serogroup B N. meningitidis without the limitations of traditional SBAs.

In conclusion, the standardized cSBA can potentially be used to screen several serum samples, allowing an extremely easy and quick determination of results. This assay promise to be a useful tool in further studies to elucidate the correlates to protection in animal models, vaccine efficacy trials, postmarketing surveillance studies, and epidemiological studies.

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