Measurement of Serum Bactericidal Activity Specific for *Haemophilus influenzae* Type b by Using a Chromogenic and Fluorescent Metabolic Indicator

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We evaluated alamarBlue as a metabolic indicator in a standardized assay for the measurement of serum bactericidal activity (SBA) to *Haemophilus influenzae* type b (Hib) using sera containing natural and vaccine-induced antcapsular (polyribosylribitol phosphate) antibodies. SBA assays with a colorimetric and a fluorometric end point in the presence of alamarBlue were developed and compared to a standard SBA assay, where colony counts are performed to determine the titer (12). A colorimetric end point required a spectrophotometer, whereas a fluorometric end point required a fluorometer. Prevaccination sera (*n* = 27) and postvaccination sera (*n* = 13) were tested by all three methodologies, and the SBA titers obtained in the presence of alamarBlue were compared to those from the standard method. Both the colorimetric and the fluorometric SBA titers were significantly correlated (*r* = 0.87 and *r* = 0.95, respectively) with those of the standard assay (50% killing as the SBA titer end point), and titers were not significantly different when compared to those of the standard assay (*P* > 0.68). However, the fluorometric end point had superior performance and ease of titer determination compared to the colorimetric end point (95 versus 87% of SBA titers were within 2 dilutions of the standard titer). Hib SBA assays with alamarBlue are reproducible, faster (same-day assay), and easier to perform than the standardized assay, which requires manual or automated colony counts. These semiautomated methodologies result in increased sample throughput and collection of data in digital formats that can be exported to data analysis programs for determination of SBA titers.

*Haemophilus influenzae* type b (Hib) is a respiratory pathogen which causes a variety of clinical manifestations, including meningitis, bacteremic pneumonia, epiglottitis, septicemia, cellulitis, and osteoarticular infections (2). The disease primarily affects young children of <5 years of age, causing up to 500,000 deaths each year worldwide (10). However, with the use of polyribosylribitol phosphate-protein conjugate vaccines, the incidence of invasive Hib in the United States has declined dramatically from 20,000 cases in 1987 to 227 cases in 2000 (4). These successful formulations have also been introduced as part of the routine vaccination regimens of other countries as a part of the Expanded Program for Immunizations. Currently, there is a concerted effort towards further reduction of Hib disease in the United States and in the Americas. In the United States there are a few populations that have reported a higher incidence of invasive Hib disease, despite the introduction of successful Hib conjugate vaccines (3, 4, 6). Unlike the United States, introduction of this type of vaccine has been delayed in many countries due to difficulties in recognition of disease burden and concerns about vaccine cost. In addition, active surveillance through both epidemiologic and immunogenicity studies is helpful in monitoring progress in Hib disease reduction after introduction of the vaccine.

Immunological evaluation of vaccine-induced protection includes the measurement of vaccine-induced antibodies by enzyme-linked immunosorbent assay (ELISA). Minimal levels for short-term protection (as determined for unconjugated polysaccharide vaccines) are ≥0.15 μg/ml, and for long-term protection they are ≥1.0 μg/ml (5, 8). Antibody function is measured by serum bactericidal activity (SBA) rather than by ELISA. The current SBA assay relies on the measurement of Hib viability after addition of active complement and serum antibodies (12). This method is highly reproducible, but it requires overnight incubation to allow for the formation of CFU, it has a lower sample throughput, and it requires either manual or automated counting of CFU for the determination of SBA titers. In addition, data analysis is time-consuming, as the interface with data analysis programs is not automated. In this study, we evaluated a metabolic indicator (alarBlue) to develop both a colorimetric and a fluorometric SBA assay for Hib, which is a highly fastidious organism. Mountzouros and Howell used this type of indicator for SBA assays specific for *Neisseria meningitidis* serogroup B (11). In this study, we determined that alamarBlue is suitable for same-day SBA titer determinations (fluorometric end point), it is highly reproducible, and it allows for automated data collection.
MATERIALS AND METHODS

Serum samples. A total of 40 serum samples were tested by the standardized SBA assay and the alamarBlue SBA assays (colorimetric and fluorometric end points). Twelve normal healthy donor sera (age range = 24 to 62 years) were obtained through Emory Donor Services, Atlanta, Ga. We also tested 15 pre-vaccination sera and 13 postvaccination sera from study participants (age range = 6 to 73 years) who received a single dose of HibTITER vaccine (HBOC-Wyeth-Lederle Vaccines and Pediatrics, West Henrietta, N.Y.). These sera were collected before vaccination and 8 weeks postvaccination as part of a colonization intervention study in Alaska. All participants or their legal guardians provided informed consent. Participants had no history of Hib disease, although they had history of Hib oropharyngeal colonization. Participants of age 22 to 73 (n = 9) had no previous history of Hib vaccination. Sera were stored frozen at −70°C in 500-μl aliquots until use. The quality control serum used was PSAB-90, kindly donated by Claudette Thompson, Dana Farber Cancer Institute, Boston, Mass.

The SBA titer of either 2,048 or 4,096 for PSAB-90 was determined by testing (n = 7) in parallel with the control preparation, bacterial polysaccharide immune globulin (SBA titor, 16,384) previously used as the quality control preparation for the standard SBA assay (12). The immunoglobulin G ELISA concentration of PSAB-90 (mean ± standard deviation [SD]) was determined to be 60.3 ± 9.45 μg/ml after 35 independent measurements against the standard reference serum, lot 1983 (provided by Carl Frasch, Center for Biological Evaluation and Review, Food and Drug Administration, Bethesda, Md.).

Standard SBA assay. We performed the standardized SBA assay, which measures the direct bactericidal activity of serum antibodies as previously described by Poiesz et al. (12), which is based on the method developed by Schlesinger et al. (13). This assay was used as the standard method for comparison of SBA titers generated by the assays containing alamarBlue. Specifically, serum samples were tested in duplicate at a starting dilution of 1:8.

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Results

aluMARBlue SBA optimization. Multiple assay conditions were tested with the control serum, PSAB-90, to optimize the assay conditions that would allow for both colorimetric and fluorometric end points from the same assay plate. The metabolic activity (reduction of alamarBlue) of Hib was dependent on the type of buffer used at the time of addition of alamarBlue to the assay plate. We evaluated four different buffers containing the same amount of alamarBlue but various amounts of BHI broth (84, 20, 10, and 0%). The optimal buffer was found to contain 20% BHI broth, 64% Hanks buffer (containing Ca2+ and Mg2+ and 2% Fildes enrichment), and 16% alamarBlue. Incubation times tested were 0, 2, 4, 6, 7, 8, 19, 22, and 24 h of incubation at 37°C in 5% CO2. The optimal time for the fluorometric assay was 6 h, and for the colorimetric assay it was 19 to 22 h. Reduction of alamarBlue by Hib was directly proportional to the number of bacteria present in the assay wells. Bacterial inocula of 1,000, 5,000, and 10,000 bacteria per well were tested in parallel. Although 10,000 bacteria per well allowed for a more rapid reduction of alamarBlue under most media conditions, the use of 1,000 bacteria per well allowed for both a fluorometric (6 h) and a colorimetric (19 to 22 h) readout of the same assay plate when the alamarBlue buffer was supplemented with 20% BHI broth. The higher bacterial inoculum resulted in a 4- to 5-h fluorometric readout and 10- to 12-h colorimetric readouts. The optimal assay conditions for both colorimetric and fluorometric end points are described in Materials and Methods. Once the optimal assay conditions were defined, a panel of 40 human sera and a quality control serum (PSAB-90) were tested for evaluation of the assay performance in comparison to the standard SBA assay.

Reproducibility of Hib SBA assays containing alamarBlue. The reproducibility of both the colorimetric and the fluor-
metric assays was evaluated using the quality control serum PSAB-90, as shown in Fig. 1. For this control serum, the range (mean ± SD) of absorbance measured at 490 nm was 0.4 ± 0.14 to 0.07 ± 0.05 (Fig. 1A). The overall mean OD ± SD values for reagent blanks, complement control, and blanked complement control wells were 0.20 ± 0.07, 0.58 ± 0.08, and 0.37 ± 0.08, respectively. The coefficients of variance for reagent blanks, complement control, and blanked complement control wells were 35, 13.8, and 22%, respectively. The colorimetric SBA assay had a very restricted curve range. This narrow range made the interpretation of the SBA titers more difficult than in the fluorometric SBA assay. The range (mean ± SD) of the fluorometric SBA assay measured at 6 h under the conditions described was from 19,985 ± 3,221 FU for complement controls to 3,106 ± 525 FU for reagent blanks. The coefficients of variance for complement controls and reagent blanks were 16.1 and 16.9, respectively, with the fluorometric end point. Figure 1B gives the fluorescence signals observed for PSAB-90. In the fluorometric SBA assay, data were not blanked, since there was a high signal-to-noise ratio (6.4). In both alamarBlue SBA assays, the calculated median titer for PSAB-90 was 2,048 ± 1 dilution. This median titer was 1 dilution lower than the median titer observed with the standard SBA assay. The minimal level of quantification was a titer of 8 for the standard SBA assay as well as for the alamarBlue SBA assays. A titer lower than 8 was assigned a titer of 4 to allow for statistical analysis. Table 1 gives the standard SBA colony counts (CFU) that corresponded to the colorimetric and fluorometric SBA end points after a 6-h and a 19-h incubation period for the control serum, PSAB-90.

**Correlation with standard SBA assay.** We evaluated the performance of the alamarBlue SBA assays by direct comparison to the standard assay. Figure 2 gives the correlations between the colorimetric and standard assays (r = 0.87; Fig. 2A) and between the fluorometric and standard assays (r = 0.95; Fig. 2B). Both assays significantly (P < 0.05) correlated with the standard assay. However, the fluorometric SBA assay had a significantly higher (chi-square P = 0.01) number of SBA colony counts (CFU) that corresponded to the colorimetric and fluorometric SBA end points after a 6-h and a 19-h incubation period for the control serum, PSAB-90.

**TABLE 1. Comparison of a Hib standard SBA assay with fluorometric and colorimetric end points of an SBA assay using alamarBlue.**

| Colony count or end point for: | Standard SBA (CFU/5-μl aliquot) | Fluorometric SBA (FU/well)* | Colorimetric SBA OD<sub>490</sub> |
|-------------------------------|---------------------------------|----------------------------|---------------------------------|
| PSAB-90 dilution               |                                 |                            |                                 |
| 1:512                         | 24                              | 3,468                      | 0.162                           |
| 1:1,024                       | 27                              | 3,100                      | 0.208                           |
| 1:2,048<sup>b</sup>           | 43                              | 4,590                      | 0.327                           |
| 1:4,096                       | 65                              | 14,244                     | 0.362                           |
| 1:8,192                       | 90                              | 21,267                     | 0.410                           |
| Complement wells              | 116                             | 20,750                     | 0.367                           |
| Blank wells                   | NA<sup>c</sup>                  | 2,590                      | 0.156                           |
| Range                         | 0 to 150                       | 19,985 ± 3,221 to 3,106 ± 525 | 0.20 ± 0.07 to 0.37 ± 0.08     |
| MLQ<sup>d</sup>               | 8                               | 8                          | 8                               |
| Total assay time (h)<sup>e</sup> | 24                            | 8                          | 8                               |
| Instrumentation               | Dissecting scope or automated reader | Fluorometer | Spectrophotometer (ELISA reader) |
| Correlation (r)<sup>f</sup>   | NA                             | 0.95                       | 0.87                            |

<sup>a</sup> For the standard SBA assay, values are numbers of CFU per 5-μl aliquot; for the fluorometric SBA, values are numbers of nonbanked fluorescence units measured at 530 and 5990 nm wavelength (excitation and emission) per well; and for the colorimetric SBA, values are ODs. The PSAB-90 titer is in bold.

<sup>b</sup> NA, nonapplicable.

<sup>c</sup> MLQ, minimum level of quantification (SBA titer).

<sup>d</sup> Values are numbers of hours. Total assay time includes estimates for assay setup, SBA incubation, and titer determination and quantification.

<sup>e</sup> r values, Pearson's product moment correlation coefficient with standard assay, are listed. Absorbance values were blanked.

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**FIG. 1.** Serum bactericidal dilution curve for the serum control, PSAB-90, read under colorimetric optimal conditions (A) and under fluorometric optimal conditions (B). The arrow in panel A shows the inflection point in the SBA assay absorbance curve used to define the SBA titer compared to that in corresponding complement control wells. The dashed line in panel B indicates the 50% threshold used to define the SBA titer compared to that in complement control wells.
was 100%, and for titers of \( f/H_11022 \) was 50%. Similarly, the percent agreement for \( f/H_11022 \) colorimetric end point for titers of \( e/2/3/9/5 \)

coefficients were determined by Pearson's product moment correlation coefficient. The percent agreement between the standard SBA and the fluorometric SBA titers (log2 transformed) were compared (Student's \( t \) test) to the standard SBA titers, there were no significant differences (\( P \) range = 0.67 to 0.86) observed.

Correlation coefficients obtained by either the colorimetric SBA (A) or the fluorometric titers (B) of 40 human serum samples (Table 2). Table 2 gives the overall cumulative percent agreement between the standard SBA and the fluorometric SBA titers for 40 sera were determined by chi-square. However, when all of the colorimetric or fluorometric SBA titers (log2) were compared on the line of identity than did the colorimetric assay (7 of 40). Table 2 gives the overall cumulative percentages of SBA titers in agreement at ±1, ±2, or ≥3 dilutions from the standard SBA assay.

**DISCUSSION**

Serum bactericidal activity can be measured using a standard assay that requires bacterial colony counts for the determination of the serum SBA titer. This process is time-consuming, it requires special growth medium, and it is labor-intensive for use in large immunogenicity studies. Alternatively, a chromogenic oxidation-reduction (redox) compound such as alamarBlue can be used to monitor bacterial survival. AlamarBlue is a metabolic indicator formulated to quantitatively measure the proliferation of a variety of human or animal cells, bacteria, mycobacteria, and fungi (1, 7, 15, 16). It consists of an indicator that yields a colorimetric redox change and a fluorescent signal in response to metabolic activity.

AlamarBlue is blue in its oxidized form. When reduced by bacteria or tissue culture cells, it changes to a bright pink color that can be measured at 570 nm in the visible range or in the fluorometric UV range at 590 nm. The oxidized blue state can only be read at 600 to 630 nm in the visible range, and it is not fluorometric. Under the Hib SBA assay conditions (buffers are dark amber in color), the oxidized state was green instead of blue and the optimal wavelength for measurement of the reduced pink state was 490 nm. Other wavelengths tested were 405, 450, 562, and 620 nm (data not shown).

Use of alamarBlue in Hib SBA assays was found to be simple and practical for the measurement of SBA specific to Hib. However, improved assay performance was found (Fig. 2B) with the fluorometric readout (530/590 nm [excitation/emission UV wavelength]). The use of alamarBlue in Hib SBA assays offers a number of advantages over the standard SBA assay, such as (i) rapid instrument collection of results (40 to 60 s per 96-well plate), (ii) interface to computer software, (iii) 1-day assay for the fluorometric end point, (iv) potential for higher throughput of serum samples, (v) use of instruments available in most laboratory settings, and (vi) elimination of manual or automated viability counts and consequently the use of agar plates (ChocII). The main disadvantages of using alamarBlue in Hib SBA assays were observed for the colorimetric end point rather than the fluorometric end point. These disadvantages were (i) high sensitivity to buffer conditions, especially for the colorimetric end point; (ii) incubation times require close monitoring for the fluorometric assay (6 h ± 30 min); (iii) SBA titer determination in the colorimetric assay is difficult and may require plotting the SBA curve or a data analysis program to avoid subjectivity; and (iv) a narrow absorbance range for the colorimetric endpoint (blanked OD490 range, 0.07 to 0.4).

In the evaluation of alamarBlue as a metabolic indicator of SBA activity for Hib, we found this compound to be highly stable under the SBA assay conditions and under the recommended storage conditions (4°C in the dark until expiration date). This compound was not toxic to Hib and did not result in bacterial cell death in the absence of anti-polyriboylribitol phosphate antibodies. Bacterial viability counts were identical

**FIG. 2. Comparison of SBA titers** (n = 40 human serum samples) obtained by either the colorimetric SBA (A) or the fluorometric SBA (B) with the standard SBA. The dotted line represents the identity line, and the solid line represents the regression line. Correlation coefficients were determined by Pearson's product moment correlation coefficient. The percent agreement between the standard SBA and the fluorometric end point for titers of ≥8 was 97%, and for titers of ≤8 it was 50%. Similarly, the percent agreement for fluorometric titers of ≥8 was 100%, and for titers of ≤8 it was 83%.

**TABLE 2. Cumulative percentage of serum samples within a given dilution difference compared to that with the standard SBA assay**

| Dilution difference | Fluorometric SBA (n = 40) | Colorimetric SBA (n = 40) |
|---------------------|--------------------------|---------------------------|
| No. in group | Cumulative % | No. in group | Cumulative % |
| 0                   | 17 | 42.5 | 7* | 17.5 |
| ±1                  | 15 | 80.0 | 17 | 60.0 |
| ±2                  | 7  | 97.5 | 10 | 67.5 |
| ≥3                  | 1  | 100 | 6  | 100 |

* Number of sera in agreement with the standard assay was significantly lower in the colorimetric SBA assay (\( P < 0.05 \)) when compared to the fluorometric SBA. Significance between pairs was determined by chi-square. However, when all of the colorimetric or fluorometric SBA titers (log2) were compared (Student's \( t \) test) to the standard SBA titers, there were no significant differences (\( P \) range = 0.67 to 0.86) observed.
in the presence or absence of alamarBlue after a 1-h incubation period (data not shown).

Due to its superior performance, we recommend the fluorometric over the colorimetric SBA assay for the measurement of Hib SBA titers. SBA titers can be determined the same day as assays require a fluorometer, the SBA titers were easily defined and highly comparable to the standard SBA titers. We are currently evaluating the use of this metabolic indicator for meningococcal SBA (serogroups A, C, Y, and W135). The use of alamarBlue in measuring Hib SBA should facilitate broader application of this immunological assay for the evaluation of Hib vaccines.

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