Evaluation of Serum Bactericidal Antibody Assays for Haemophilus influenzae Serotype a

Nadine G. Rouphael,1,2 Sarah Satola,1,4 Monica M. Farley,1,4 Karen Rudolph,3 Daniel S. Schmidt,2 Patricia Gomez-de-León,5 John B. Robbins,6 Rachel Schneer,6 George M. Carlone,2 and Sandra Romero-Steiner2*

Division of Infectious Diseases, Emory University School of Medicine, 69 Jesse Hill Jr. Drive, Atlanta, Georgia 30303; Centers for Disease Control and Prevention, 1600 Clifton Road, N.E., Atlanta, Georgia 30333; Centers for Disease Control and Prevention, Arctic Investigations Program, 4055 Tudor Centre Drive, Anchorage, Alaska 99508; Atlanta Veterans Affairs Medical Center, 1670 Clairmont Road, Decatur, Georgia 30033; Facultad de Medicina, Departamento de Salud Pública, Universidad Nacional Autónoma de México, Mexico D.F., México; and National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland

Received 24 May 2010/Returned for modification 26 July 2010/Accepted 9 December 2010

Haemophilus influenzae type a (Hia) is an important pathogen for some American Indian, Alaskan native, and Northern Canada aboriginal populations. Assays to measure serum bactericidal activity (SBA) to Hia have not been developed or validated. Here, we describe two methods for the measurement of SBA: SBA with a viability endpoint (CFU counts) and SBA with a fluorometric endpoint using alamarBlue as the metabolic indicator. Both SBA assays measure Hia-specific functional antibody and correlate with anti-Hia IgG enzyme-linked immunosorbent assay (ELISA) concentration of naturally acquired antibodies.

MATERIALS AND METHODS

Serum samples. A total of 19 serum samples and one immune globulin preparation were used in the different assays. Sera were obtained after informed consent and protocol exemption by the CDC. Seven sera from normal, healthy, adult donors (age range, 24 to 60 years) were obtained through Emory Donor Services, Atlanta, GA; 12 unlinked cord blood sera were provided by Alberto Villaseñor Sierra (CIBO, IMSS, Jalisco, Mexico) and contained only IgG antibodies. These 12 sera were selected from a larger bank of 69 cord blood sera collected from healthy mothers. Sera were selected randomly if enough volume was available and if ELISA was positive (cut-off level of 0.09). An immune globulin preparation was also used (Gamunex 10%; Talecris Biotherapeutics, Inc., Clayton, NC). Sera were stored frozen at −70°C in 500-μl aliquots until use.

Complement source. The complement source was sterile serum from 3- to 4-week-old baby rabbits (Pel-Freez, Brown Deer, WI) previously used in the Hib SBA (15). Although the concentration of cross-reactive antibodies was not measured in the complement lots, three different complement lots were qualified for use on both assays by testing active and heat-inactivated complements. The lot chosen (8028) had 14% killing (level of acceptability is less than 25% killing) compared to time zero inoculum, where the inactivated complement had no killing.

Bacterial strains. Twelve bacterial strains were used in the SBA assays (Table 1). Six strains were obtained from the CDC Active Bacterial Core surveillance (ABCs) from Georgia (1989 to 2007) (GA41512, GA41513, GA18491, GA11151, GA44497) (12) and Oregon (M8881). Five strains were obtained from the CDC Arctic Investigations Program (AIP) (2000 to 2007) (AK1435C3, AK137339, AK137341, AK137342, AK137865). The last strain (ATCC 9006) was obtained from the American Type Culture Collection (ATCC). Seven isolates were obtained from sterile sites and caused invasive disease, and five were obtained from the colonized nasopharynx. Three isolates (GA41512, GA41513) [12], and M8881 [Satola personal communication] had the partial IS1016-bexA deletion, a mutation possibly associated with increased virulence in Hia (1).

Each Hia strain was plated on chocolate II solid medium overnight. Few colonies were selected from the medium and added to brain heart infusion (BHI) broth, supplemented with 2% Floods enrichment (BBL, Becton Dickinson and Co., Sparks, MD), and then incubated at 37°C and 5% CO2 until optical density at 600 nm reached an average of 0.4. Aliquots were flash frozen and stored at

* Corresponding author. Mailing address: Vaccinology Laboratories, Division of Bacterial Diseases, Bldg. 18, Room B-105, Mailstop A-36, CDC, 1600 Clifton Road, N.E., Atlanta, GA 30333. Phone: (404) 639-2473, Fax: (404) 639-4518. E-mail: SSteiner@cdc.gov.

Published ahead of print on 22 December 2010.
were tested with a starting dilution of 1:8. Two-fold serum serial dilutions were performed as described by the manufacturer of the antiserum (Becto-Difco Diagnostic Systems), and PCR molecular capsule typing was performed as described by Falla et al. (5).

SBA assays. SBA assays (with a viability endpoint [CFU counts] and with a fluorometric endpoint) were performed as described by Romero-Steiner et al. (15) for Hib, which is a modification of the method described by Schlesinger and Granoff (17).

SBA assays were performed on four separate days for each strain by two independent researchers blinded to the assay results using either method.

Ten negative-control sera with negative SBA titers using the reference strain AK1435C3 were used but were not included in the comparison of strains or the final analysis.

SBA assays. (i) SBA assay with a viability count endpoint. Serum samples were tested with a starting dilution of 1:8. Two-fold serum serial dilutions were made in 10 µl of Hanks buffer with Ca2+ and Mg2+ (Life Technologies, Grand Island, NY) supplemented with 2% Fildes enrichment. Each bacterial strain was diluted to yield 1,000 bacteria in 20 µl inoculum per well. After 15 min of incubation of the serum and the bacterial strain at 37°C and in 5% CO2, 20 µl of baby rabbit complement was added to each well. An additional 30 µl of the dilution buffer was added to each well to bring the total volume of the reaction to 80 µl. After an incubation of 60 min at 37°C in 5% CO2, 5 µl from each well was plated onto chocolate agar plates using a tilt method for better definition of the CFU (ChocII; BBL, Becton Dickinson and Co., Sparks, MD). After 16 h of incubation at 37°C in 5% CO2, viability counts were performed and SBA titers were determined. SBA titers were defined as the reciprocal of the serum dilution that resulted in more than 50% killing compared to the growth in the complement controls.

(ii) SBA assay with a fluorometric endpoint. alamarBlue (Trek Diagnostics, Westlake, OH) is a commercially available metabolic indicator. In the presence of viable bacteria, alamarBlue is reduced and a color change from blue to pink occurs. The reduced compound is also fluorescent with an emission wavelength of 590 nm if excited by a UV light source at a wavelength of 530 nm. Therefore, the same protocol for the SBA assay using viabilities counts as endpoints applies except that 30 µl of alamarBlue buffer was used once 20 µl of the complement was added to each well. The alamarBlue buffer consisted of 16% alamarBlue, 64% Hanks buffer (containing Ca2+ and Mg2+, and 2% Fildes enrichment), and 20% BHI broth (BBL). alamarBlue was found to be stable under SBA assay conditions and did not result in bacterial death in the absence of antibodies (15). After a 6-h incubation period at 37°C in 5% CO2, assay plates were read in a fluorometer (model FL 600 synergy; BIO-TEK Instruments Inc., Winooski, VT). Reagent blanks (without bacteria) were used in each plate. SBA titers were defined as the reciprocal of the serum dilution with 50% of the fluorescent units (FU) detected in the complement controls.

TABLE 1. Haemophilus influenzae type strains used for SBA evaluation

| Hia strain     | Site(s)             | Geographic origin |
|---------------|---------------------|-------------------|
| GA41512a      | Blood               | Georgia           |
| GA41513a      | Blood and cerebrospinal fluid | Georgia |
| M8831a        | Blood               | Oregon            |
| GA18491       | Blood               | Georgia           |
| GA11151       | Blood               | Georgia           |
| GA44497       | Blood               | Georgia           |
| AK1435C3      | Blood               | Alaska            |
| AK137339      | Nasopharynx         | Alaska            |
| AK137341      | Nasopharynx         | Alaska            |
| AK137342      | Nasopharynx         | Alaska            |
| AK137865      | Nasopharynx         | Alaska            |
| ATCC 9006     | Nasopharynx         | ATCC              |

a Hia strain with a partial IS1016-bexA deletion.

Addition of heterologous Hib polysaccharide (100 µg/ml protein) Hia-specific capsular polysaccharide (11) (and comparing the results with SBA titers obtained in the absence of added polysaccharide). For this evaluation, we used five selected bacterial strains (GA41512, GA41513, GA18491, GA11151, AK1435C3) and five selected healthy adult donor sera. We also evaluated the effect of heterologous Hib polysaccharide (provided by Moon Nahm, University of Alabama at Birmingham) in the Hia SBA assay. For this evaluation, we used the reference strain AK1435C3 and the same adult sera. Hib polysaccharide was added also at 100 µg/ml.

ELISA. Anti-Hia polysaccharide IgG concentrations ([µg/ml] were determined on cord blood sera using an Hia ELISA. The Hia ELISA is based on an anti-Hib polysaccharide IgG ELISA method previously described (6, 16). This optimized ELISA used a standard reference material which was assigned an anti-Hia polysaccharide IgG concentration (4.13 µg/ml). This anti-Hia IgG concentration was determined through a heterologous ELISA using the Hib standard reference material (FDA1983) as a calibrator serum (Schmidt, unpublished) based on the cross-standardization method described by Concepcion and Frasch (4).

Sera with negative ELISA (levels less than 0.01) were used as negative controls but were not included in the analysis.

TABLE 2. Specificity of the Haemophilus influenzae type a SBA

| Haemophilus influenzae type a strain | GMT of SBA titers in the presence of Hia polysaccharideb | GMT of SBA titers in the absence of Hia polysaccharide | Avg % decreasea of SBA titers in the presence of Hia polysaccharideb |
|------------------------------------|--------------------------------------------------------|-------------------------------------------------------|-----------------------------------------------------------------|
| AK1435C3                           | 8                                                      | 1,176.36                                              | 99.3                                                            |
| GA41512                            | 8                                                      | 1,176.35                                              | 99.3                                                            |
| GA41513                            | 8                                                      | 1,176.35                                              | 99.3                                                            |
| GA18491                            | 8                                                      | 1,351.2                                               | 99.4                                                            |
| GA11151                            | 8                                                      | 1,024                                                 | 99.2                                                            |
| All 5 strains                      | 8                                                      | 1,180.8                                               | 99.3                                                            |

a Average percentage decrease for 5 sera from adult donors.

b Competitive inhibition with 100 µg/ml of purified Hib polysaccharide.

c Addition of heterologous Hib polysaccharide (100 µg/ml) resulted in no reduction of this GMT.
Statistical analysis. Correlations between SBA assays were determined by Pearson’s product moment correlation coefficient by use of Sigma Plot and Sigma Stat software, version 2.0 (SPSS, Inc., Chicago, IL). Significant differences among assays were determined by Student’s t test or the Mann-Whitney rank sum test for data not normally distributed. The significance level was set at a P value of less than 0.05. The concordance correlation coefficients (CCC) were calculated to estimate the degree of agreement between pairs of log₂-transformed SBA titers. Paired comparisons were made for each strain and AK1435C3 (CDC reference strain).

RESULTS

Reproducibility of SBA assays. The reproducibility of the SBA assays was evaluated using the immune globulin preparation with different bacterial strains in 46 different independent assay runs. The geometric mean titer (GMT) was 237.4, with 97.8% of SBA titers within 1 dilution of the GMT.

Correlation between SBA assays. SBA titers obtained by both methods were highly correlated (r = 0.96) (Fig. 1). No statistically significant difference was observed for the two assays when using healthy adult sera (P = 0.68) or cord blood sera (P = 0.94) among the 12 strains. SBA titers measured by both methods using all sera were within 1 and 2 dilutions for 96.6% and 98.3% of the titers, respectively (Fig. 1).

SBA assay specificity for Hia polysaccharide. The specificity of the SBA assays was assessed by competitive inhibition in which the SBA assay activity was evaluated after the addition of purified Hia polysaccharide. The relative decrease in SBA titers for the five different strains tested varied between 99.2% and 99.4%, with an average of 99.3% (Table 2). No effect or reduction in SBA titers was observed by the addition of Hib polysaccharide in the panel of five sera evaluated with AK1435C3.

Correlation between SBA assay titers and IgG antibody concentrations. The correlation coefficients (r value) for SBA titers with fluorometric endpoint and IgG antibody concentrations as determined by ELISA varied between 0.77 and 0.89 depending on the strain (Table 3). SBA assay performed with the reference strain AK1435C3 had a moderate correlation (r = 0.89) with ELISA (Fig. 2).

DISCUSSION

Although Hib and Hia capsular polysaccharides are similar, they are not cross-reactive; Hia contains glucose in a 4-β-linkage, and Hib contains ribose in a 3-β-linkage (11). Fully vaccinated infants with Hib conjugate vaccine are not protected against Hia invasive disease (9).

Bactericidal antibodies against H. influenzae type b capsular polysaccharide confer immunity to this pathogen (14, 18). SBA assay has been extensively used for decades as a correlate of protection against invasive Hib as well as meningococcal diseases (7, 8). The many similarities between H. influenzae type a and type b, including their epidemiology, capsular polysaccharides (type-specific or serotype antigens), and the pathology of the systemic infections they cause, are the basis for our

| Serum sample (IgG concn [μg/ml]) | AK1435C3 | GA41512 | GA41513 | M8881 | GA18491 |
|----------------------------------|----------|---------|---------|-------|---------|
| 1 (1.16)                         | 512      | 128     | 64      | 128   | 128     |
| 2 (1.24)                         | 512      | 128     | 256     | 64    | 128     |
| 3 (0.19)                         | 32       | 32      | 64      | 32    | 32      |
| 4 (0.20)                         | 128      | 64      | 64      | ND    | 64      |
| 5 (0.26)                         | 32       | 32      | 16      | 8     | 4       |
| 6 (2.07)                         | 256      | 256     | 512     | 256   | 256     |
| 7 (0.09)                         | 32       | 32      | 64      | 32    | 64      |
| 8 (1.68)                         | 512      | 256     | 1,024   | 256   | 512     |
| 9 (4.02)                         | 1,024    | 1,024   | 1,024   | 2,048 | 1,024   |
| 10 (0.66)                        | 64       | 64      | 64      | 32    | 64      |
| 11 (0.78)                        | 128      | 128     | 128     | 64    | 128     |
| 12 (2.19)                        | 256      | 256     | 256     | 128   | 512     |
| Gamunex (4.13)                   | 256      | 256     | 512     | 256   | 512     |

CCC (95% LCI-95% UCI) | 1.0 0.821 (0.55-0.94) 0.776 (0.42-0.93) 0.707 (0.33-0.89) 0.769 (0.44-0.92)

a CCC, concordance correlation coefficient (and 95% lower [LCI] and upper confidence intervals [UCI]) for the estimation of the agreement between log₂-transformed SBA titers for each of the Hia strains against AK1435C3 (reference strain). The closer the CCC is to 1.0, the higher the agreement.

b ND, not determined.
The moderate correlation between ELISA and SBA assay results can be characterized with viability, fluorometric, or colorimetric endpoints. Previous work in Hib demonstrated that the best correlation for Hib SBA assay was obtained between the viability count endpoint and the fluorometric endpoint for titer determination (15). Therefore, we chose to compare only SBA assay with viability colony counts and fluorometric endpoints and not to assess the colorimetric endpoint. SBA assay using viability counts as endpoints is highly reproducible but time-consuming, with a low sample throughput and overnight incubation for CFU growth, and since Hia and Hib are grown on chocolate agar, the use of the available automated programs for data acquisition is quite difficult due to lack of contrast from the opaque medium. SBA assay using viability counts as endpoints is highly reproducible, easy to use, with higher throughput, automated and fluorometric endpoints and not to assess the colorimetric endpoint. Previous work in Hib demonstrated that the best correlation for Hib SBA assay was obtained between the viability count endpoint and the fluorometric endpoint for titer determination (15). Therefore, we chose to compare only SBA assay with viability colony counts and fluorometric endpoints and not to assess the colorimetric endpoint. SBA assay using viability counts as endpoints is highly reproducible but time-consuming, with a low sample throughput and overnight incubation for CFU growth, and since Hia and Hib are grown on chocolate agar, the use of the available automated programs for data acquisition is quite difficult due to lack of contrast from the opaque medium. SBA assay using viability counts as endpoints is highly reproducible, easy to use, with higher throughput, automated and should be considered the preferred method for measuring Hia SBA.

Conclusion. We conclude that the viability colony counts and fluorometric SBA assays are reproducible, measure Hia-specific antibody function, and correlate with anti-Hia IgG antibody concentrations (µg/ml). In addition, SBA assay with a fluorometric endpoint is fast, easy to use, and highly reproducible and should be considered the preferred method for measuring Hia SBA.

TABLE 3—Continued

|           | GA44497 | GA11151 | ATCC 9006 | AK137339 | AK137341 | AK137342 | AK137865 |
|-----------|---------|---------|-----------|-----------|-----------|-----------|-----------|
| 128       | 128     | 64      | 128       | 128       | 128       | 128       | 128       |
| 64        | 64      | 64      | 64        | 64        | 64        | 64        | 64        |
| 8         | 32      | 32      | 16        | 16        | 16        | 16        | 16        |
| 32        | 64      | ND      | 64        | 64        | 64        | 64        | 64        |
| 8         | 4       | 8       | 8         | 8         | 8         | 8         | 8         |
| 256       | 512     | 256     | 256       | 256       | 256       | 256       | 256       |
| 32        | 32      | 32      | 64        | 16        | 32        | 32        | 32        |
| 512       | 512     | 256     | 128       | 128       | 128       | 128       | 128       |
| 1,024     | 1,024   | 1,024   | 2,048     | 512       | 512       | 1,024     |           |
| 64        | 32      | 32      | 64        | 64        | 64        | 64        | 64        |
| 128       | 64      | 64      | 64        | 64        | 64        | 64        |           |
| 256       | 128     | 128     | 128       | 128       | 128       | 128       |           |
| 256       | 256     | 256     | 256       | 128       |           |           |           |
| 0.749 (0.44–0.90) | 0.729 (0.40–0.89) | 0.697 (0.33–0.88) | 0.683 (0.38–0.85) | 0.782 (0.50–0.94) | 0.711 (0.40–0.87) | 0.735 (0.45–0.88) |

REFERENCES

1. Adderson, E., et al. 2001. Invasive serotype a Haemophilus influenzae infections with a virulence genotype resembling Haemophilus influenzae type b: emerging pathogen in the vaccine era? Pediatrics 108:18.
2. Bruce, M. G., et al. 2008. Epidemiology of Haemophilus influenzae serotype a, North American Arctic, 2000–2005. Emerg. Infect. Dis. 14:48–55.
3. CDC. 2002. Progress toward elimination of Haemophilus influenzae type b invasive disease among infants and children—United States, 1998–2000. MMWR Morb. Mortal. Wkly. Rep. 51:234.
4. Concepcion, N., and C. E. Frasch. 1998. Evaluation of previously assigned antibody concentrations in pneumococcal polysaccharide reference serum 98SF by the method of cross-standardization. Clin. Diagn. Lab. Immunol. 5:199–204.
5. Falla, T. J., et al. 1994. PCR for capsular typing of Haemophilus influenzae. J. Clin. Microbiol. 32:2382–2386.
6. Fernandez, J., et al. 2000. Randomized trial of the immunogenicity of fractional-dose regimens of PRP-T Haemophilus influenzae type b conjugate vaccine. Am. J. Trop. Med. Hyg. 62:485–490.
7. Fothergill, L., and J. Wright. 1933. Influenzal meningitis: relation of age incidence to the bactericidal power of blood against the causal organism. J. Immunol. 24:273–284.
8. Goldschneider, L., E. C. Gotschlich, and M. S. Artenstein. 1969. Human immunity to the meningococcus. I. The role of humoral antibodies. J. Exp. Med. 129:1307–1326.
9. Hammit, L. L., et al. 2005. Outbreak of invasive Haemophilus influenzae serotype a, North American Arctic, 2000–2005. Emerg. Infect. Dis. 11:2933–2939.
10. Hammitt, L. L., T. W. Hennessy, S. Romero-Steiner, and J. C. Butler. 2006. Assessment of carriage of Haemophilus influenzae type a after a case of invasive disease. Clin. Infect. Dis. 43:386–387.
11. Jin, Z., S. Romero-Steiner, G. M. Carlone, J. B. Robbins, and R. Schneerson. 2007. Haemophilus influenzae type a infection and its prevention. Infect. Immun. 75:2650–2654.
12. Kapogiannis, B. G., S. Sotola, H. L. Keyserling, and M. M. Farley. 2005. Invasive infections with Haemophilus influenzae serotype a containing an IS2016-bcd partial deletion: possible association with virulence. Clin. Infect. Dis. 41:e97–e103.
13. Millar, E. V, et al. 2005. Epidemiology of invasive Haemophilus influenzae
type A disease among Navajo and White Mountain Apache children, 1988-2003. Clin. Infect. Dis. 40:823–830.

14. Robbins, J. B., J. C. Parke, Jr., R. Schneerson, and J. K. Whisnant. 1973. Quantitative measurement of “natural” and immunization-induced Haemophilus influenzae type b capsular polysaccharide antibodies. Pediatr. Res. 7:103–110.

15. Romero-Steiner, S., et al. 2004. Measurement of serum bactericidal activity specific for Haemophilus influenzae type b by using a chromogenic and fluorescent metabolic indicator. Clin. Diagn. Lab. Immunol. 11:89–93.

16. Romero-Steiner, S., et al. 2006. Evaluation of the natural immunity to Haemophilus influenzae type a (Hia) in Latin America mothers, presentation 9, p. 57–58. Prog. Abstr. Int. Conf. Women Infect. Dis., Atlanta, GA. ASM press, Washington, DC.

17. Schlesinger, Y., and D. M. Granoff. 1992. Avidity and bactericidal activity of antibody elicited by different Haemophilus influenzae type b conjugate vaccines. JAMA 267:1489–1494.

18. Sutton, A., R. Schneerson, S. Kendall-Morris, and J. B. Robbins. 1982. Differential complement resistance mediates virulence of Haemophilus influenzae type b. Infect. Immun. 35:95–104.

19. Trollfors, B., et al. 1992. Characterization of the serum antibody response to the capsular polysaccharide of Haemophilus influenzae type b in children with invasive infections. J. Infect. Dis. 166:1335–1339.