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Measurement of *Haemophilus influenzae* Type a Capsular Polysaccharide Antibodies in Cord Blood Sera

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Abstract

We measured anti-Hia capsular polysaccharide serum immunoglobulin G (IgG) antibodies in cord blood sera from Mexican (n=68) and Chilean mothers (n=72) by ELISA. Measurable antibodies were found in 79.3% of samples. IgG antibodies correlated with serum bactericidal activity (r=0.66). This ELISA can be used for the evaluation of adaptive immune responses to Hia and sero-surveillance studies in populations at risk.

Keywords

Haemophilus influenzae type a; ELISA; anti-capsular antibodies

INTRODUCTION

Haemophilus influenzae type a (Hia) has been recognized as a causative agent of pneumonia, meningitis, and otitis media in Native American and Alaskan populations.^{1, 2} Most North American Hia cases have been reported in infants <1 year of age which may indicate that older children and adults develop antibodies over time in a similar fashion to natural responses observed with *Haemophilus influenzae* type b (Hib)^{1, 2}. In Utah, recent reports document an increase in invasive disease caused by Hia and other Hi serotypes in

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children and adults.³⁻⁵ Additionally Hia has been reported in countries such as Brazil, Papua New Guinea, and South Africa.⁶

Laboratory assays measuring antibodies specific to Hia capsular polysaccharide (CP) have been lacking. While we recently described an *in vitro* serum bactericidal assay to measure the functional capacity of adult and cord blood to kill Hia,⁷ the laboratory assay to quantify Hia CP-specific antibodies has not been described. In this study, we describe the development and use of a sensitive and specific enzyme linked immunosorbent assay (ELISA) for quantifying immunoglobulin G (IgG) anti-capsular polysaccharide (CP) antibodies to Hia using cord blood sera. Cord blood is available in larger quantities than infant serum for assay development and it has little or no IgM antibodies which may confound the correlation with serum bactericidal assays, and may confer passively acquired immunity.

MATERIALS AND METHODS

This study was reviewed by the CDC Human Research Protection Office and determined to be exempt from Institutional Review Board review. Cord serum samples were collected after informed consent, from 140 healthy mothers at two sites: Instituto Mexicano del Seguro Social, Jalisco, México (n=68) and Dr. Hernán A. Henríquez Hospital, Temuco, Chile (n=72). Serum samples were submitted frozen to the CDC and stored at -70°C .

The incubation periods and buffers used were similar to the described Hib ELISA.⁸ Two lots of CP were evaluated (NIH052978 and NIH1981). Both lots of capsular polysaccharide were produced from culture supernatants at the NIH as described for *Salmonella paratyphi C* capsular polysaccharide.⁹ Hia CP was stored at -70°C in 100 μl aliquots of 1mg/ml. Immulon 2 HB plates (Thermo Labsystems, Franklin, Md.) were coated with 4 $\mu\text{g}/\text{ml}$ of CP in buffer (0.01M PBS, pH 7.2) at 37°C for 90 minutes. Plates were stored at 4°C and used within two weeks. CP-coated plates were washed five times with 0.01M PBS/Tween-20, 0.01M, using an ELx405 (Bio-Tek Instruments, Winooski, Vt.), and ELISA diluent (PBS 0.01M; 0.3% Tween-20) was added. Two-fold serial dilutions of the standard and test sera were made in duplicate, including a positive cord serum control (UNAM-17), in a seven and eight dilution series, respectively. The plates were incubated at 22°C for 90 minutes, then washed, and anti-human IgG antibody (HP6043, Hybridoma Reagent Laboratory, Baltimore, Md.) conjugated to horseradish peroxidase at a 1/4000 dilution in 0.01M PBS, 0.05% Tween-20 (Invitrogen Corp., Grand Island, N.Y.) was added. After two hours incubation, the assay plates were washed and developed with TMB substrate (KPL, Gaithersburg, Md.) for 30 minutes. The reaction was stopped with 1N HCl (Fisher Scientific, Fair Lawn, N.J.) and read in an ELx808 (Bio-Tek Instruments) at 450 nm (630 nm reference). Data were analyzed using ELISA for Windows software (CDC, Atlanta, Ga.). Human IgG (Telacris Biotherapeutics, Inc., Research Triangle Park, N.C.) was used to prepare the standard by diluting it 1:5 with IgG depleted human serum (Sigma-Aldrich, St. Louis, Mo.), which acted as a cryogenic preservative and stored at -70°C . The anti-Hia CP IgG concentration of the standard was determined by cross-standardization to the Hib (FDA1983) standard.¹⁰

The specificity of the Hia ELISA was determined by a competitive inhibition ELISA. Eighty $\mu\text{g}/\text{ml}$ of Hia CP was added to diluted cord sera ($\text{OD}_{450} = 1.0$). Tubes were mixed overnight at 4°C and centrifuged for 10 minutes at $9,000 \times g$ to remove precipitate. The percent inhibition was calculated ($\text{Percent Inhibition} = (1 - (\text{OD inhibited replicate average} / \text{OD uninhibited replicate average})) * 100\%$). For the standard, we used Hia CP concentrations from 0.87 to 0.11 mg/ml in quadruplicates. Absorption with Hib CP (0.87 mg/ml) served as the negative control. To determine the level of cross-reactivity, 30 samples were tested in both Hia and Hib ELISA.

To determine the correlation of IgG concentrations with functional activity cord sera (Mexican, n=36 and Chilean, n=28) were tested in the serum bactericidal assay (SBA) as described previously.⁷

The Pearson's correlation coefficient determined significant associations ($P < 0.05$). The Mann-Whitney test was used to determine differences among non-normally distributed data. Proportions were compared by Chi-square (significance level $P < 0.05$).

RESULTS

An IgG anti-Hia concentration of 4.1 µg/ml was assigned to the Hia standard (coefficient of variation <20%) and a mean SBA titer of 512. The calculated range of the standard curve was 82 ng/ml to 1.28 ng/ml based on seven two-fold dilutions with a starting dilution of 1:50. The background was 6-fold less than the highest standard dilution signal. The lowest sample concentration was 11 times higher than the lower limit of quantification (1.28 ng/ml).

Competitive inhibition of the Hia standard with Hia and Hib CPs reduced the value by 82.6 % and 5.8 %, respectively. Competitive inhibitions of five cord sera (0.46 to 3.15 µg/ml) resulted in a mean inhibition of 97.6 % (range 97 to 99%). There was no correlation ($r = 0.15$, $P = 0.44$) by Pearson's correlation coefficient between Hia and Hib IgG concentrations (n=30). A Mann-Whitney test indicated a significant difference between the data sets ($P < 0.001$).

The anti-Hia IgG concentrations for Mexican and Chilean sera are given in Table 1. Two breakpoints are presented: the lowest concentration (0.1 µg/ml) associated with an SBA (titer of 8, n = 11) and an arbitrary breakpoint of 1 µg/ml. A total of 72.1% of Mexican and 51.4% of Chilean sera had concentrations above 0.1 µg/ml. Detectable anti-Hia CP IgG was found in 111 of 140 samples (79.3%). The percentage of cord sera with 1 µg/ml was significantly lower (Table 1). The Pearson's correlations between SBA and ELISA were $r = 0.71$ and $r = 0.65$ for Mexican and Chilean sera, respectively.

DISCUSSION

In this study we present a sensitive and specific ELISA for the quantification of anti-Hia IgG antibodies in human sera. We demonstrate a high degree of agreement between IgG anti-Hia and SBA, since the cord serum had little or no detectable anti-Hia IgM (data not shown). It is possible that some of the antibodies measured were elicited by cross-reactive polysaccharides (i.e., *Streptococcus pneumoniae* serotype 6B or *Bacillus pumilus* Sh 18, among other gram positive bacteria which share common epitopes [D-ribitol-5-phosphate]).¹¹ Although the CP structures of Hib and Hia are similar, antibody cross-reactivity was not anticipated since *H. influenzae* serotyping is capsular-based. The specificity of this ELISA was high as shown by the 5% non-specific cross-reactivity between anti-Hia and anti-Hib CP IgG antibodies, the lack of correlation between anti-Hia and Hib IgG concentrations, and homologous and heterologous CP competitive inhibitions. Regardless of their origin, the presence of functional antibody in the cord blood may protect infants from infection. A limitation of our study is that we were unable to determine Hia disease history or colonization status, since the serum samples were unlinked from patient identifying information.

Due to the lack of cross-reactivity, Hib vaccination is unlikely to confer protection against Hia disease. While this assay is not intended for clinical diagnosis it has the capacity to be a serological surveillance tool to monitor disease trends in endemic areas.

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Table 1

Anti-Hia capsular polysaccharide (CP) IgG in cord blood sera from healthy Mexican and Chilean mothers and correlation to serum bactericidal activity (SBA)

Anti- Hia CP IgG ELISA Concentration	Percent of cord blood sera above each breakpoint (proportion)		Population Comparison
	Mexico (n=68)	Chile (n=72)	
GMC µg/ml (CI) [*] Range	0.12 (0.07 – 0.21) <0.0013 to 3.19	0.04 (0.02 – 0.06) <0.0013 to 4.16	P = 0.015 [†]
0.10 µg/ml	72.1% (49/68)	51.4% (37/72)	P = 0.012 [‡]
1.0 µg/ml	13.2% (9/68)	5.6% (4/72)	P = 0.118
Correlation with SBA [§]	r = 0.65 (n = 36)	r = 0.71 (n = 28)	NA

^{*} Confidence interval $\alpha = 0.05$

[†] There was a significant difference between the anti-Hia CP IgG antibody concentration of Mexican and Chilean mothers by the Mann-Whitney rank sum test.

[‡] Comparisons of proportions by the Chi-square method in 2×2 tables of the percentage of cord blood sera from Mexico and Chile above the breakpoint concentration given. Significance preset at P value = 0.05.

[§] Correlation between ELISA IgG concentration and functional serum bactericidal activity (SBA) was determined by Pearson's correlation coefficient. Cord blood sera from Mexican mothers (n=36, $P<0.001$) and Chilean mothers (n=28, $P<0.001$) used in the comparison.