A competitive ELISA method is described for the measurement of total antibodies to the capsular polysaccharide of *Haemophilus influenzae* type b (HibCPS) in human sera. The competitive method showed an excellent correlation to the radioantigen binding assay (RABA, or Farr assay) and improved correlation of sera with low titers with respect to the more conventional noncompetitive method. Overestimation of samples in the low concentration range was no longer observed with the competitive ELISA method. The free HibCPS competition allowed us to eliminate the day-to-day background variation typical of some sera; thus, only values representing the true anti-HibCPS response were determined. The use of precoated microplates, which could be stored up to 8 months, greatly improved the speed of the procedure. An overall correlation coefficient of 0.9660 was found when 407 serum samples with a wide variety of anti-HibCPS antibody levels were tested with the competitive ELISA and RABA. The regression line was very close to the ideal line, with a slope of 1.0045 and an intercept of −0.1996. A subset of 96 serum samples representative of all pre- and postimmunization samples was used to compare the competitive ELISA with a previously described ELISA method. The competitive method performed in two laboratories in different countries showed a better correlation with the RABA. The correlation factors were 0.9770 and 0.9816, respectively, while a factor of 0.9547 was found with the previously described noncompetitive procedure, which was better for this method than previously reported (r = 0.917). Therefore, the competitive ELISA is proposed for the assay of anti-HibCPS titers in sera from vaccinated subjects.

*Haemophilus influenzae* type b (Hib) has been a leading cause of bacterial meningitis among infants and young children worldwide. The organism also causes other invasive infections, including epiglottitis, cellulitis, pneumonia, pericarditis, arthritis, bactereemia, empyema, and osteomyelitis (5, 19). Antibodies to the capsular polysaccharide of Hib (HibCPS) protect against invasive disease from this organism (15, 18).

Serum antibodies to HibCPS have been quantitatively determined by using the radioantigen binding assay (RABA) technique described by Farr in 1958 (6), modified for specificity and labelling (2, 5, 12). The concentration of serum anti-HibCPS antibody sufficient to confer protection is not known (2). Estimates have varied from concentrations of 0.1 μg/ml to concentrations of 1.0 μg/ml (1, 11, 14, 17). Because of qualitative differences in antibody functions attributable to a combination of differences in isotype and avidity (1, 11), precise estimates are probably not possible. However, vaccinated subjects are considered protected when a level of 1.0 μg of anti-HibCPS antibodies per ml is found (1, 11), although the use of conjugate Hib vaccines able to elicit a T-cell-dependent immune response may lower this limit in the future because of their ability to prime for memory serum antibody responses, as recently suggested by Kayhty (10).

In 1990, Phipps et al. proposed an enzyme-linked immunosorbent assay (ELISA) measurement of total immunoglobulin (Ig) to HibCPS that correlated well with RABA results (16). This ELISA procedure, although unable to resolve the dependence of the assay on antibody avidity (3), was an improvement in terms of the feasibility of assaying large numbers of serum samples, while avoiding the use of radioisotopes. However, in our hands, this assay showed a high variability in serum antibody background levels. Therefore, we developed and standardized an improved ELISA measuring total specific Ig levels with a competitive assay, in which the specific binding to HibCPS was measured in each sample by subtraction of the uninhibited fraction after addition of a saturating amount of soluble HibCPS. Here, we describe the competitive ELISA method for quantitative measurement of serum antibodies to HibCPS.

**MATERIALS AND METHODS**

Preparation of conjugated HibCPS. A procedure to prepare human serum albumin-HibCPS conjugate (HSA-Hib) was developed and standardized to provide antigen to coat microtiter plates. Twenty percent (wt/vol) HSA (Sclavo S.p.A., Siena, Italy) was first characterized for protein content according to the method of Lowry et al. (13) and then was characterized for amino group content (8). Fifty milliliters of an aqueous solution of 500 mg of HibCPS (40% [wt/wt] ribose content; lot 12/89; CHIRON S.p.A., Siena, Italy), corresponding to 1,335 μmol of ribose was added to 0.4 M NaIO₄ at a ribose/NaIO₄ molar ratio of 8. The mixture was maintained for 20 min in the dark at room temperature and then stored at 4°C. The content of ribose and the aldehyde groups was determined according to conventional colorimetric assays (4, 9). A volume containing 100 μmol of the aldehyde groups was added, while being stirred, to a volume of the HSA solution equivalent to 50 μmol of amino groups (about 50 to 60 μg of protein) and a volume of a 1 M pyridineborane (PyBH₃) solution in methanol corresponding to 1,310 μmol (molar ratio: CHO-NH₂ = 2, PyBH₃-CHO = 13, and PyBH₃-NH₂ = 26). The mixture was continuously stirred overnight at room Temperature.
temperature, \((\text{NH}_4)_2\text{SO}_4\) was added to a final concentration of 22.5\% (wt/vol), and chromatography was performed with a phenyl-Sepharose (26 by 10 mm) column previously equilibrated with 10 mM phosphate buffer (pH 7.2) containing 22.5\% (wt/vol) \((\text{NH}_4)_2\text{SO}_4\). The column was washed with about 10 volumes of equilibration buffer, then the conjugate was eluted with 100 \(\mu\)l of 6 M NaOH, and the plates were read at \(A_{405}\) with a reference filter at 595 nm.

The analysis of the positive values (\(\geq 0.19\) \(\mu\)g/ml) for sera from children (86.8\% of the samples) presented a median for specific binding after inhibition of 91.2\% with a minimum value of 28.6\%, while for sera from adults (13.2\% of the samples), the analysis presented a median of 83.55\% with a minimum value of 23.26\%.

The percentage of inhibition was calculated, assuming the optical density (OD) reading in the noninhibited well as 100\% of binding, according to the following equation:

\[
\text{percentage of inhibition} = \left(1 - \frac{\text{OD inhibited}}{\text{OD noninhibited}}\right) \times 100\%.
\]

The higher the percentage was, the more specific the calculated result was. Therefore, as a precaution, low-inhibition samples were considered negative, regardless of their absorbance, when the percentage of specific binding after inhibition obtained by comparison of inhibited and noninhibited wells was lower than 20\%, since noninhibitable binding cannot be considered specific.

**ELISA specificity.** Competition with purified capsular polysaccharide of *Neisseria meningitidis* serotype C (MenCPS) was carried out to confirm the specificity of Hib CPS inhibition. Six serum samples representative of a wide range of serum titers were serially diluted from 0.25 to 0.0019 \(\mu\)g/ml. The binding was then inhibited by addition of a constant excess of purified Hib CPS or MenCPS (100 \(\mu\)g/ml, final dilution). The first dilutions of the sera were as follows: A110 (90 \(\mu\)g/ml) was diluted 1:360, 14/2 (55.42 \(\mu\)g/ml); A148 (6.8 \(\mu\)g/ml) was diluted 1:40, 25/2 (2.81 \(\mu\)g/ml); A141 (1.7 \(\mu\)g/ml) was diluted 1:800, 5/2 (0.21 \(\mu\)g/ml); A131 (0.875 \(\mu\)g/ml) was diluted 1:5. It should be underlined that these samples at dilutions lower than the first serum dilution in the assay (1:50) to obtain the inhibition curve.

**Data selection and statistical analysis.** A titration curve was obtained for each serum sample by plotting the absorbance values as a function of the logarithm of the reciprocal of the serum dilution. Sample concentrations were determined by using only absorbance values in the linear portion of the standard curve (OD of 0.050 to the reading plateau of an OD of about 2.600). To calculate serum anti-Hib CPS antibody concentrations, absorbance values in wells containing serum dilutions incubated with soluble Hib CPS were subtracted as background from the corresponding values of the wells in which sera were diluted with buffer alone. Only sera with absorbance values which were inhibited at least 20\% by soluble Hib CPS and giving a difference between noninhibited and inhibited \(A_{405}\) values of \(P<0.05\) were considered positive. Antibody concentrations in sera were calculated from the standard curve and were expressed in micrograms per milliliter.

Noncompetitive ELISA results were obtained from the same data generated with competitive ELISA, but with the average absorbance of the buffer subtracted as background instead of the absorbance of the inhibited serum sample. At a 1:50 dilution of serum, the ELISA sensitivity limit in undiluted sera corresponded to 0.10 \(\mu\)g/ml. For data analysis, samples with an antibody concentration of <0.10 \(\mu\)g/ml were assigned 50\% of the minimum (0.05 \(\mu\)g/ml). Logarithmically transformed values of antibody concentrations were used for determination of the correlation coefficients between the different assay methods.

**Comparison of competitive ELISA and Phipps' ELISA.** The Phipps' ELISA was carried out as described previously (16). Two minor differences were the starting dilution of the samples, which was 1:20, and the fact that the alkaline phosphatase-conjugated polysaccharide goat anti-human Ig was purchased from Caltag (San Francisco, Calif.). The main differences between the two procedures are summarized in Table 1 and are due to the incubation temperature, in addition to the materials used. For the comparison, the panel of 96 serum samples described above was used.

**RESULTS AND DISCUSSION**

**HSA-Hib conjugate and plate coating.** Three lots of HSA-Hib conjugate were prepared. As summarized in Table 2, the results were consistent. Therefore, the lots were pooled. The activity of the pooled lot was assayed against that of a previous lot by comparing standard reference serum curves and medium-titer (6.5 \(\mu\)g/ml) serum dilution curves in an ELISA. A test showed that the difference between the mean values of the two groups was not great enough to exclude the possibility that the difference was just due to random sampling variability. No statistically significant difference between the groups was detected. In fact, the Student's t-test for two samples with an associated \(P = 0.896\) for the standard reference serum curves, and \(t = -0.019\), with an associated \(P = 0.985\) for the medium-titer serum curves. The same results were also obtained with a nonparametric analysis according to the Mann-Whitney rank-sum test.
The final lot gave a total amount of 133 mg (in protein) of HSA-Hib conjugate that was stored in aliquots at 2–20°C. The HSA-Hib conjugate was stable for at least 8 months at 4°C and could be frozen at −20°C for prolonged storage, since at least four freeze-thawing cycles did not affect antigen stability.

HSA-Hib conjugate-coated plates prepared as described in Materials and Methods were stored for up to 8 months at 4°C in sealed plastic bags. Plates were tested simultaneously after different storage times: when freshly prepared (0 months) and at 1.5, 4, 6, and 8 months. Coated plates were proved stable by the consistency of a standard curve with A110 serum in the different plates, as depicted in Fig. 1. The HSA-Hib conjugate was also successfully tested for equivalence against Phipp’s HbHOA conjugate (data not shown).

ELISA specificity. The use of competitive ELISA ensured the specificity of antibody measured for each serum sample. Competition with purified MenCCPS was carried out in parallel to that with HibCPS to confirm the specificity of HibCPS inhibition; the results are shown in Fig. 2. The curves obtained with the six diluted serum samples representative of a wide range of serum titers showed that the excess concentrations of the specific Haemophilus polysaccharide were able to completely inhibit the specific binding in all cases, while the non-correlated Neisseria polysaccharide did not interfere with the specific antibody binding to the plate wells.

ELISA results. With sera containing low (<2.0 μg/ml) antibody concentrations, day-to-day variability and background variability were observed with the conventional indirect ELISA. Therefore, a specific binding inhibition with purified HibCPS was evaluated. Figure 3 shows the correlation between competitive and noncompetitive ELISA results. The overall correlation was very good (r = 0.9714; n = 407). This corre-
lation was even better \( (r = 0.9973; n = 232) \) when calculated for samples at \( \geq 2.0 \) mg/ml, but decreased when calculated for samples at \( < 2.0 \) mg/ml \( (r = 0.7618; n = 175) \).

Antibody concentrations in low-titer samples were often overestimated when the noncompetitive assay was used, which is illustrated in Fig. 4. This is also illustrated by comparison of the geometric means. The geometric means for samples with concentrations \( \geq 2.0 \) mg/ml were 13.36 mg/ml for the competitive assay versus 14.05 mg/ml for the noncompetitive one, while for samples with concentrations \( < 2.0 \) mg/ml, they amounted to 0.16 and 0.28 mg/ml, respectively. Discrepancies in the results from low-titer sera with antibody concentrations of \( < 1.0 \) mg/ml, as determined by RABA, are greater with the noncompetitive ELISA (Fig. 4A) than with the competitive ELISA (Fig. 4B). As a result, the percentages of samples with antibody concentrations \( > 1.0 \) mg/ml were overestimated after analysis with the noncompetitive ELISA, as shown in Table 3 for preimmune sera, especially for adults. Thus, the noncompetitive ELISA might overestimate the percentage of subjects with an antibody concentration \( > 1.0 \) mg/ml.

**Reproducibility.** The standard curve, quality control sera, three negative samples, and three low-concentration samples were used to determine the consistency of the assay results from day to day and with different operators. These sera were measured by competitive ELISA on three different days by two different operators testing four replicates of each point of the standard curve, six replicates of each quality control point, and four replicates of each sample serum dilution. The results, expressed as percentages of coefficients of variation (%CVs), were calculated by the absorbances read for each point and are summarized in Tables 4 to 7.
For the standard curve (Table 4), the %CVs were calculated as the mean of the averages of the absorbances of all of the individual points before and after background (buffer) subtraction. For the quality control (Table 5), negative (Table 6), and low-titer (Table 7) sera, the %CVs were calculated as the averages of the absorbancies of the individual point both in the noninhibited and in the inhibited wells. Good reproducibility of data was observed, and the calculated %CVs were always in an acceptable ELISA range.

The reference values of the quality control sera (Table 5) were calculated by using the values obtained from 15 different experiments performed by different operators on different days. The averages of the two replicates of each quality control serum were subtracted from the averages of the corresponding inhibited wells, the resulting absorbancy values were interpolated on the corresponding standard curves, and the values obtained were multiplied by the respective dilution factors and then expressed as micrograms of specific Igs per milliliter. The calculated values were very close to those determined by RABA reported in parentheses as follows). A143 serum gave a mean (± standard deviation) value of 1.05 ± 0.41 μg/ml (1.09 ± 0.32 μg/ml), for A144 was 55.8 ± 16.8 μg/ml (51.90 ± 10.16 μg/ml), and for A146 was 10.1 ± 3.2 μg/ml (14.40 ± 2.59 μg/ml), and that for A148 was 3.57 ± 1.17 μg/ml (5.06 ± 1.36 μg/ml).

Comparison of competitive ELISA and RABA. Competitive and noncompetitive ELISA results were compared to the RABA results by analyzing a panel of 407 serum samples which belong to different groups, as shown in Table 3. In Fig. 4, the ratios of noncompetitive (Fig. 4A) and competitive (Fig. 4B) ELISA values versus RABA values are illustrated as a function of calculated RABA antibody concentrations, confirming that the specific binding inhibition allowed a decrease in low-concentration sample overestimation. The overall competitive ELISA versus RABA correlation was good, with a correlation coefficient of 0.9660 (n = 407), indicating that no significant difference between competitive ELISA and RABA was found. The regression line of competitive ELISA versus RABA is y = 1.0045x − 0.1996, which is very close to the ideal line, because it is shown by a slope very close to 1.0 and an intercept close to origin. This correlation was well conserved (r = 0.9251) when calculated for samples with RABA values ≥2.0 μg/ml (n = 232) and lightly decreased (r = 0.7910) when calculated for samples with RABA values <2.0 μg/ml (n = 175).

The reproducibility of the competitive ELISA was also demonstrated with the subset of 96 serum samples being assayed in two laboratories in different countries. This measurement revealed correlation factors of 0.9770 and 0.9816, respectively, compared to those of the RABA. The correlation between the two ELISAs yields a comparable factor of 0.9734.

Comparison of competitive ELISA and Phipps’ ELISA. With the same subset of 96 serum samples, the competitive ELISA was also compared with the Phipps’ ELISA (16). This yielded correlation factors of 0.9376 and 0.9516 as found in comparison with the competitive ELISA which was performed in the two laboratories. The correlation factor with the RABA was 0.9547, which is even better than the factor (r = 0.917) reported by Phipps (16). Although the correlation found with the Phipps’ ELISA and RABA is quite good, it is lower than the factors observed with the competitive ELISA and RABA. When the Phipps’ ELISA was performed with the incubation times, buffer, and temperature used in the competitive ELISA, the correlation with the RABA and competitive ELISA improved only slightly. The overestimation of sera in the lower concentration range was still present in the results of the

### Table 3. Comparison of RABA and ELISA results with sample sera

| Group          | No. (%) of samples (n = 407) | % of samples with titer result<sup>a</sup> |
|----------------|------------------------------|-----------------------------------------|
|                | RABA                        | ELISA                                   |
|                | >0.15 | >1.0 | GMT  | >0.15 | >1.0 | GMT  | >0.15 | >1.0 | GMT  |
| Infants        |        |      |      |        |      |      |        |      |      |
| Preimmunization| 93 (22.9) | 55.9 | 9.7 | 0.19 | 34.4 | 8.6 | 0.11 | 49.5 | 10.8 | 0.16 |
| Post-1         | 8 (2.0)   | 100  | 100 | 6.57 | 100  | 87.5| 7.00 | 100  | 87.5| 7.83 |
| Pre-3          | 21 (5.2)  | 71.4 | 42.9| 0.72 | 66.7 | 23.8| 0.42 | 90.5 | 28.6| 0.72 |
| Post-3         | 111 (27.3)| 91.0 | 82.9| 7.70 | 89.2 | 82.0| 5.65 | 94.6 | 81.1| 6.40 |
| Pre-4          | 56 (13.8) | 98.2 | 80.4| 2.91 | 92.9 | 71.4| 1.68 | 98.2 | 78.6| 2.10 |
| Post-4         | 62 (15.2) | 100  | 100 | 64.3 | 100  | 100| 34.5 | 100  | 100| 35.1 |
| Adults         |        |      |      |        |      |      |        |      |      |
| Preimmunization| 28 (6.9) | 82.1 | 42.9| 1.11 | 75.0 | 46.4| 0.81 | 100  | 64.3| 2.19 |
| Post-2         | 28 (6.9) | 100  | 92.9| 9.41 | 92.9 | 89.3| 6.29 | 100  | 96.4| 8.74 |

<sup>a</sup> >0.15, percentage of serum samples with a titer >0.15 μg of specific Igs per ml (prevaccination protection); >1.0, percentage of serum samples with a titer >1.0 μg of specific Igs per ml (postvaccination protection); GMT, geometric mean of the calculated titers.

### Table 4. Standard curve reproducibility of the ELISA used in this study with reference serum

| Run    | Operator | No. of replicates/ run | No. of points/ run | Within run %CV<sup>a</sup> | Between run %CV<sup>a</sup> |
|--------|----------|------------------------|--------------------|---------------------------|-----------------------------|
| Series 1| 1        | 1                      | 4                  | 8                         | 6.8                         |
|        |          |                        |                    |                           | 7.6                         |
|        | 2        | 7.8                    | 8.1                |                           | 4.5                         |
|        | 3        | 4.5                    | 4.8                |                           | 3.1                         |
| Series 2| 2        | 6.4                    | 6.9                |                           | 9.6                         |
|        | 2        | 10.2                   | 15.3               |                           | 13.9                        |
|        | 3        | 11.2                   | 17.7               |                           | 23.8                        |

<sup>a</sup> Average of the individual point %CVs calculated by the absorbancy of each point (overall %CV, 8.1).
Phipps’ ELISA performed with the change in incubation conditions, as illustrated in Fig. 5.

**Conclusions.** The effectiveness of vaccination of individuals against Hib is conventionally determined with a RABA. This assay is time-consuming and involves the problems connected with handling radioactivity and waste. In 1990, an ELISA was proposed by Phipps et al. (16) that was clearly an improvement in term of assay feasibility and also paved the way to the replacement of RABA with ELISA for the estimation of anti-HibCPS antibody titers in human sera.

In our hands, human sera were found to give different background values, with day-to-day variability. The noncompetitive ELISA might overestimate the percentage of subjects with antibody titers of >1.0 μg/ml. This fact caused discrepancies in the evaluation of low-titer sera, and although the problem is essentially restricted to preimmune sera, it may not exclude a low response after vaccine administration. In these cases, a marked overestimation was introduced when the conventional indirect (noncompetitive) ELISA was used, because it was clearly shown in the low range of titers with the noncompetitive-competitive ELISA data correlation. To solve this problem, we decided to test the serum samples in duplicate: one well with dilution buffer and, in parallel, one well with dilution buffer containing an excess of purified HibCPS. To determine serum anti-HibCPS antibody concentrations, absorbance values in wells containing serum dilutions incubated with soluble HibCPS were subtracted as specific background from the corresponding values of the wells in which sera were diluted with buffer only. Antibody concentrations of sera were calculated from the standard curve and were expressed in micrograms per

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**TABLE 5. Quality control serum reproducibility of the ELISA used in this study with sera A143, A144, A146, and A148**

| Run  | Operator | No. of replicates/run | No. of points/run | Within run | Between run | Overall |
|------|----------|-----------------------|-------------------|------------|-------------|---------|
|      |          |                       |                   | %CVa       | %CVb        | %CVa   | %CVb   |
| A143 |          |                       |                   |            |             |         |         |
| Series 1 | 1 | 6 | 1 | 13.1 | 19.4 | 17.1 | 24.3 | 25.6 | 26.1 |
|         | 2 | 23.6 | 19.9 | 3 | 5.1 | 28.2 |
| Series 2 | 2 | 6 | 1 | 13.1 | 16.3 | 28.1 | 26.4 |
|         | 2 | 38.5 | 17.1 | 3 | 8.4 | 10.9 |
| A144 |          |                       |                   |            |             |         |         |
| Series 1 | 1 | 6 | 1 | 7.7 | 7.3 | 11.7 | 12.1 | 17.3 | 17.5 |
|         | 2 | 5.7 | 11 | 3 | 2.6 | 8.4 |
| Series 2 | 2 | 6 | 1 | 12.4 | 18.2 | 14.7 | 20.8 |
|         | 2 | 11.8 | 17.3 | 3 | 5.7 | 19.8 |
| A146 |          |                       |                   |            |             |         |         |
| Series 1 | 1 | 6 | 1 | 8.6 | 19.2 | 10.2 | 19.8 | 13.1 | 17.2 |
|         | 2 | 10.6 | 7.8 | 3 | 5.0 | 6.1 |
| Series 2 | 2 | 6 | 1 | 5.4 | 9.4 | 13.6 | 12.7 |
|         | 2 | 9.4 | 12.8 | 3 | 5.7 | 15.2 |
| A148 |          |                       |                   |            |             |         |         |
| Series 1 | 1 | 6 | 1 | 3.1 | 16.3 | 8.4 | 18.4 |
|         | 2 | 11.1 | 11 | 3 | 4.6 | 11.9 |
| Series 2 | 2 | 6 | 1 | 10.0 | 14.4 | 12.3 | 13.2 |
|         | 2 | 14.4 | 9 | 3 | 3.7 | 8.8 |

*a Average of the individual point %CVs calculated by the absorbancy of each point.

*b Average of the individual point %CVs calculated by the absorbancy of each inhibited point.
milliliter. This approach has been shown to be useful in obtaining a very good correlation between ELISA and RABA values on a panel of sera from different clinical trials representative of a wide range of antibody levels with various isotype and subclass compositions. The correlation of the competitive ELISA with RABA was better than that found for the ELISA from Phipps. Moreover, the overestimation of sera in the lower concentration range was still present when the Phipps' ELISA was performed under the incubation conditions of the competitive ELISA, indicating that competition with free HibCPS is necessary to avoid this false-positive binding.

The reliability, ruggedness, and reproducibility of the competitive ELISA and the absence of background, plus the good correlation and regression line with RABA, demonstrate that the proposed competitive ELISA can replace RABA for measuring levels of anti-HibCPS total Ig in vaccinated populations.

ACKNOWLEDGMENTS

We are indebted to Dan Granoff (Chiron Vaccines, Emeryville, Calif., and Children's Hospital Oakland Research Institute, Oakland, Calif.) and Giuseppe Del Giudice (Chiron S.p.A., Siena, Italy) for helpful discussion and suggestions.

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