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A comparison of ELISA methods for the determination of human serum antibodies to *Haemophilus influenzae* type b.

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Contents

Abbreviations

BSA	Bovine Serum Albumin
CBER	Center for Biological Evaluation and Review
CPS	Capsular Polysaccharide
DPT	Diphtheria, Pertussis and Tetanus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
FDA	Food and Drug Administration
GMT	Geometric Mean Titer
HbO-HA	Hib Oligosaccharide-Human Serum Albumin conjugate
HibPs	*Haemophilus influenzae* type b Polysaccharide
Hib	*Haemophilus influenzae* type b
HSA-Hib	Human Serum Albumin conjugated HibPs
Ig	Immunoglobulin
IPV	Inactivated Polio Vaccine
LVM	Laboratory for Vaccine Research and Research on Immune Mechanisms
LVO	Laboratory for Clinical Vaccine Research
n	population
nd	not done
p-NPP	*para*-Nitrophenylphosphaat
PBS	Phosphate Buffered Saline
PRP-T	Polyribosylribitolphosphate-Tetanus Toxoid conjugate
R	correlation coefficient
RABA	Radio Antigen Bindings Assay
RIVM	National Institute of Public Health and the Environment

Abstract

The vaccination efficacy of individuals against *Haemophilus influenzae* type b is usually determined with the established radio-antigen binding assay (RABA). In 1990 an ELISA was proposed by Phipps *et al.*, which correlated well with the RABA and which was clearly an improvement in terms of assay feasibility. This Phipps' ELISA was used at the RIVM as a replacement of the RABA for the determination of anti-Hib polysaccharide (HibPs) antibody concentrations in human sera. In a collaboration with CHIRON, it was shown that their newly developed competitive ELISA resulted in a better correlation with the RABA than the Phipps' ELISA performed by the LVM (RIVM). Furthermore, the correlation between both ELISA's was poor. This prompted to investigate the Chiron competitive ELISA at the RIVM and to compare it with the Phipps' ELISA and the RABA.

Anti-Hib antibody concentrations of 4 different serum panels, all from the same clinical trial, were determined with the Chiron competitive ELISA and the Phipps' non-competitive ELISA in our laboratory and with the Chiron competitive ELISA and RABA by CHIRON. The results were compared with the original trial data, determined with the Phipps' ELISA by the LVM.
Reliability, reproducibility plus a good correlation with the RABA demonstrate that both the Chiron competitive ELISA and the Phipps' non-competitive ELISA can replace the classical RABA for anti-Hib total Ig determination. However, the clear advantage of the somewhat more laborious competitive ELISA, as proposed by Mariani *et al.*, is that overestimation of the percentage of subjects with antibody titers > 0.15 μg/ml is absent, as compared to the non-competitive ELISA. Competition with free HibPs, appeared necessary to avoid false positive binding for anti Hib antibody concentrations ≤ 1.0 μg/ml. Although the non-competitive ELISA showed differences with the RABA in the evaluation of low titer sera and thus the problem might be restricted for a large part to pre-immune sera it can still influence the amount of subjects falsely considered sufficiently protected against Hib. The Phipps' non-competitive ELISA could be improved by changing the incubation time, incubation temperature, incubation buffers, conjugate and ELISA plate towards the conditions of the Chiron competitive ELISA.

Based on the results presented in this report we propose an improved ELISA for determining Hib antibodies which uses the HbO-HA antigen from PRAXIS (or the HSA-Hib antigen from CHIRON) coated overnight to a Maxisorp microtiter plate. Samples are tested in a twofold serial dilution, preferably with 8 dilutions. Control wells with free soluble *Haemophilus influenzae* type b polysaccharide are used for samples with Hib antibody concentrations ≤ 1.0 μg/ml. The sample- and conjugate buffer consists of PBS with 1% (w/v) BSA and 0.05% (v/v) Tween-20 (pH 7.4) and incubation is performed at 37°C for at least 2 hours. Goat anti-human immunoglobulin (IgG, IgM, IgA) alkaline phosphatase from Sigma is used as conjugate. The anti-HibPs antibody concentrations are calculated by interpolations of the sample absorbance, and can be corrected for a-specific binding, in the linear part of a 4-parameter fit from the reference curve (^{10}log concentration vs. absorbance). The quantitation limit in undiluted sera corresponds to 0.10 μg/ml.

Samenvatting

De vaccinatie efficacy van *Haemophilus influenzae* type b in individuen wordt gewoonlijk bepaald met de klassieke radio-antigeen bindings assay (RABA). In 1990 werd door Phipps *et al.* een ELISA geïntroduceerd, die goed correleerde met de RABA en welke een duidelijke vooruitgang betekende in de uitvoerbaarheid van de assay. Deze Phipps' ELISA werd op het RIVM gebruikt als vervanger van de RABA voor de bepaling van anti-Hib polysaccharide (HibPs) antistof concentraties in humane sera. Uit een samenwerkingsverband met CHIRON bleek dat de door hen nieuw ontwikkelde competitieve ELISA beter correleerde met de RABA dan de Phipps' ELISA uitgevoerd door het LVM (RIVM). Verder was de correlatie tussen beide ELISA's matig. Dit was de aanleiding voor verder onderzoek aan de Chiron competitieve ELISA op het RIVM en voor een vergelijking met de Phipps' ELISA en de RABA.

Anti-HibPs antistof concentraties van 4 serum panels, alle afkomstig uit hetzelfde klinische veldonderzoek, werden binnen ons laboratorium bepaald met de Chiron competitieve ELISA en de Phipps' non-competitieve ELISA en door CHIRON met de competitieve ELISA en de RABA. De resultaten werden vergeleken met de originele veldonderzoek gegevens, bepaald m.b.v. de Phipps' ELISA door het LVM.
Betrouwbaarheid, reproduceerbaarheid plus een goede correlatie met de RABA tonen aan dat zowel de Chiron competitieve ELISA als de Phipps' non-competitieve ELISA de gevestigde RABA kunnen vervangen voor de bepaling van anti-HibPs totaal Ig. Het duidelijke voordeel van de enigszins meer bewerkelijke, competitieve ELISA, zoals voorgesteld door Mariani *et al.*, is echter dat overschatting van het percentage individuen met antistof titers > 0,15 µg/ml achterwege blijft in vergelijking met de non-competitieve ELISA. Competitie met vrij HibPs bleek noodzakelijk om vals positieve binding te voorkomen bij antistof concentraties ≤ 1,0 µg/ml. Hoewel de non-competitieve ELISA slechts verschillen oplevert t.o.v. de RABA bij de evaluatie van laag titerige sera en het probleem daardoor mogelijk grotendeels beperkt blijft tot pre-immuun sera, kan het toch wel degelijk van invloed zijn op het aantal mensen dat ten onrechte als voldoende beschermd wordt beschouwd (tegen Hib). De Phipps' non-competitieve ELISA is te verbeteren door veranderingen in de incubatie tijd, incubatie temperatuur, incubatie buffers, conjugaat en type ELISA plaat in de richting van de Chiron competitieve ELISA toe te passen.

Op basis van de in dit rapport gepresenteerde resultaten introduceren we een verbeterde ELISA voor het bepalen van Hib antistoffen, waarbij gebruik wordt gemaakt van het HbO-HA antigeen van PRAXIS (of het HSA-Hib antigeen van CHIRON) dat overnacht wordt gebonden aan een Maxisorp microtiter plaat. Monsters worden getest in een tweevoudige verdunningsreeks, bij voorkeur bestaande uit 8 verdunningen. Controle wells met vrij oplosbaar *Haemophilus influenzae* type b polysaccharide worden ingezet voor monsters met een Hib antistof concentraties ≤ 1,0 µg/ml. De monster en conjugaat buffer bestaat uit PBS met 1% (w/v) BSA en 0,05% (v/v) Tween-20 (pH 7.4). De incubatieduur bedraagt tenminste 2 uur bij 37°C. Als conjugaat wordt het geit anti-humaan immuun globuline (IgG, IgM, IgA) van Sigma gebruikt, geconjugeerd met alkalische fosfatase. De anti-HibPs antistof concentraties worden berekend door interpolatie van de monster extincties, al dan niet gecorrigeerd voor a-specifieke binding, in het lineaire deel van een 4-parameter fit van de referentie curve (^{10}log concentratie vs. extinctie). Als detectie limiet voor onverdunde sera wordt 0,10 µg/ml aangehouden.

1. Introduction

1.1 General

The gram negative bacteria *Haemophilus influenzae* were first identified during the influenza pandemic of 1890. At that time, they were ascribed to be the cause of epidemic influenza and originally named as the "Pfeiffer influenza bacillus". Since the great influenza pandemic of 1918-1919 it became apparent that this bacillus was part of the normal flora of the upper respiratory tract and did not always cause disease. The organism was therefore renamed *Haemophilus influenzae* (figure 1); *Haemophilus* (blood loving) because it requires blood factors for growth in vitro, and *influenzae* for its historical association with influenza. The genus *Haemophilus influenzae* is divided in capsulated and non-capsulated (non-typable) strains (1,2). The capsulated strains are subdivided into sero types a through f, based on the antigenic specificity of their capsular polysaccharide. Ninety-five percent of systemic infections in childhood are caused by serotype b. World wide about fifty percent of all *Haemophilus influenzae* type b (Hib) invasive infections result in bacterial meningitis especially among infants and young children up to five years of age. Hib also causes other invasive infections including epiglottitis, cellulitis, arthritis and pneumonia. Infections by non-capsulated *Haemophilus influenzae* occur much more frequent than capsulated Hib infections. They are however restricted to the upper (otitis media, sinustitis, nasopharyngitis) and lower (bronchitis, alveolitis) respiratory tract (3,4) and less dangerous.

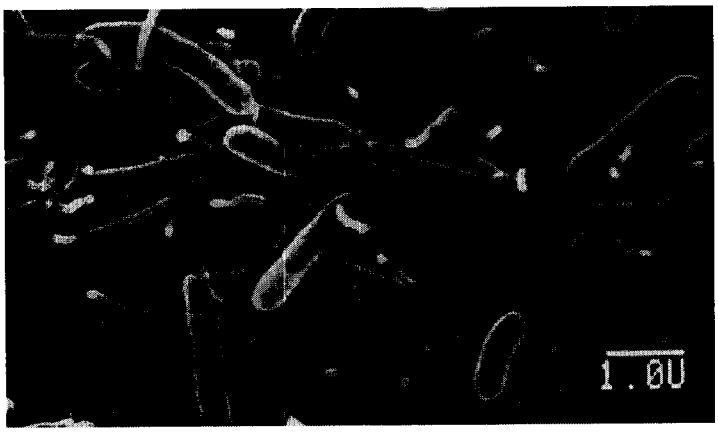

Figure 1.
EM scan of *Haemophilus influenzae* (National Center for Genome Resourses, NCGR, Santa Fe, NM, US)

1.2 Immunity and vaccine

Natural immunity to *Haemophilus influenzae* is multifactorial and involves a complex integration of many components of the immune system. Most individuals acquire protective immunity in the first few years of life without ever having developed invasive Hib disease. Although natural immunity to Hib undoubtedly involves immunity to several surface antigens of the organism, antibodies to the type b capsular polysaccharide polyribosylribitolphosphate (PRP) appear to be of primary importance; they protect against invasive disease caused by this organism (4-6). A vaccine that contained the PRP of *Haemophilus influenzae* was developed more than 20 years ago. This plain polysaccharide vaccine was shown to be immunogenic in children 2 years of age or older. However, there is no booster response upon repeated administration of the vaccine since the polysaccharide acts as a T-cell independent immunogen.

1.3 Conjugate vaccine and vaccination

H. influenzae type b conjugate vaccines were developed to enhance the immunogenicity of the polysaccharide PRP by employing the immunological principles of carrier-hapten linkage. The PRP is covalently linked to an immunogenic T-cell dependent protein carrier. Currently available *Haemophilus influenzae* type b conjugate vaccines differ significantly in several respects, like: type of protein carrier, polysaccharide size, chemical linkage of the polysaccharide to the carrier, polysaccharide/protein ratio and the three dimensional structure of the conjugate. All conjugate vaccines demonstrate the improved immunogenicity characteristics of most T-cell dependent immunogens and they prime for a booster response (2). In the Netherlands, vaccination with *Haemophilus influenzae* type b conjugate vaccine started in April 1993. The vaccine used is the PRP-Tetanus toxoid conjugate vaccine manufactured by Pasteur Mérieux Sérums et Vaccines. In the nation-wide vaccination program the vaccine is administrated to new-borns at 3, 4, 5 and 11 months of age simultaneously but separate with DPT-IPV (7). The effect of vaccination against Hib was monitored by the Netherlands Reference Laboratory for Bacterial Meningitis and the Dutch Paediatric Surveillance Unit, over a period of three years. The yearly number of invasive Hib infections decreased from an estimated 700 before vaccination down to 40 in 1996. This decrease took place in every clinical manifestation. Furthermore, a shift in the age distribution took place and the peak incidence of meningitis no longer occurred in children under one year. This can be explained by the introduction of immunisation from April 1993 onward (4,8,9). In this period 7 cases of true vaccine-failure, 4 of apparent- and 2 of possible vaccine failure occurred (9,10).

1.4 Hib antibody protection level and -assays

Serum antibodies to Hib polysaccharide have been quantitatively determined with the radio antigen binding assay (RABA) technique first described by Farr in 1958 (11) and later modified for specificity and labelling (12-14). The exact concentration of serum anti-HibPs antibody sufficient to evoke protection is not known (15). Estimates varied from concentrations of 0.1 μg/ml to 1.0 μg/ml (12,16-18). Because of qualitative differences in antibody functions attributable to a combination of differences in isotype and avidity (15), precise estimates are probably not possible. However, vaccinated subjects are considered protected when a level of 1.0 μg/ml anti-HibPs antibodies is measured (17,18). The use of conjugate Hib vaccines able to elicit a T-dependent immune response, may lower this level in the future because of their ability to prime for memory serum antibody responses, as recently suggested by Käyhty (15). In 1990, Phipps *et al.* published an ELISA measurement of total Ig to HibPs that showed a good correlation with RABA results (19). This ELISA procedure was an improvement in term of feasibility of assaying large numbers of serum samples while it avoids the use of radioisotopes, this assay also depends on antibody avidity (20). This ELISA was introduced at the RIVM as the standard for determining anti-HibPs antibodies. It was used in a multi center clinical study on the simultaneous administration of DPT-IPV and Hib PRP-T vaccine in 1994 (7). During this vaccine study a collaboration was started with CHIRON in Italy for establishing a correlation between RABA, Phipps' ELISA and a newly developed competitive ELISA by CHIRON. The aim of this competitive ELISA was to reduce variability in background by addition of a saturating amount of soluble HibPs to the serum and subtracting the result from the unbound fraction (21). From the first collaboration, it was found that results of the competitive ELISA correlated significantly better with the

RABA than the Phipps' ELISA, respectively 0.976 and 0.839. Furthermore, the correlation between both ELISA's was also poor with a factor 0.852. These findings inspired for further investigations; the collaboration was continued by LVO. In this report we describe a comparative study of the RABA with the Phipps' ELISA and the competitive ELISA methods for quantitative measurement of serum antibodies to HibPs. Aspects of this study were described in the paper "A competitive ELISA method for the determination of serum antibody to *Haemophilus influenzae* type b" (21).

2. Materials and methods

2.1 Materials

- EDTA, Ethylenediaminetetraacetic acid (Sigma, St. Louis, Missoury, US)
- Sterile non-pyrogenic water (NPBI BV, Hospital Products Division, Amstelveen, the Netherlands)
- *p*-NPP tablets (Sigma, St. Louis, Missoury, US)
- HbO-HA antigen, *Haemophilus influenzae* type b oligosaccharide-Human Serum Albumin conjugate (Lederle-PRAXIS Biologicals, Rochester, New York, US) (19)
- HSA-Hib antigen, Human Serum Albumin conjugated *Haemophilus influenzae* type b Capsular Polysaccharide conjugate (CHIRON, Siena, Italy) (21)
- HibPs, capsular polysaccharide purified from *Haemophilus influenzae* type b, lyophilized (CHIRON, Siena, Italy) (21)
- Goat anti-human immunoglobulin (polyspecific) alkaline phosphatase conjugated, working dilution 1/2000, (Caltag, San Francisco, California, US, H17008)
- Goat anti-human immunoglobulin (IgG, IgM, IgA) alkaline phosphatase conjugated, working dilution 1/10,000, (Sigma, St. Louis, Missoury, US, A-3313)
- Reference serum US standard human anti-*Haemophilus influenzae* type b capsular polysaccharide antibodies (serum) Lot 1983 with an assigned value of 70 μg/ml (Center for Biological Evaluation and Review, CBER), Food and Drug Administration, Bethesda, Maryland, US)
- Control sera, determined with the radio-antigen bindings-assay (RABA), kindly donated by Dr C. Frasch (Food and Drug Administration, Bethesda, Maryland, US) en Dr H. Käyhty (National Institute of Public Health, Helsinki, Finland)
- All other chemicals were purchased from Merck
- Microtiterplates for ELISA 96-well, flat bottom (NUNC Polysorp, Life technologies, Breda, the Netherlands)
- Microtiterplates for ELISA 96-well, flat bottom (NUNC Maxisorp, Roskilde, DK)
- HSA-Hib antigen pre-coated microtiterplates for ELISA 96-well, flat bottom (NUNC Maxisorb F8 modules framed) were obtained from CHIRON, Siena, Italy
- Spectrophotometer Bio-Tek Multiplate reader EL312e with KC3, Kineticalc for Windows (Bio-Tek Instruments, Winooski, Vermont, US)

2.1.1 Sera

The serum samples (panel 1-4) used for this study originated from the multi-center study on the simultaneous administration of DPT-IPV and Hib PRP-T vaccine (7). The serum panels consisted of pre-vaccination sera, post sera after the 3rd vaccination (post dose 3), pre sera before the 4th vaccination (pre dose 4) and post sera after the 4th vaccination (post dose 4). Panel 1 consisted of 20 sera from a total amount of 146 sera which were shipped to and sent back from Chiron in Italy for the first collaboration. In Italy all 146 sera were tested for HibPs antibody levels with the RABA and the competitive ELISA developed by Chiron. Panel 2 and 3 consisted of serum samples that were chosen from the same vaccine study using the original HibPs antibody level data from LVM to ensure a broad range of antibody concentrations. For panel 4, the serum samples were especially selected to cover an antibody range as broad as

possible, with a special interest for antibody concentrations < 1.0 µg/ml. In appendix 1 the antibody concentration distribution from panel 4 is shown. From most serum donors the pre-vaccination sera, post dose 3, pre dose 4 and post dose 4 were used. All sera were stored at –20°C in small aliquots. To avoid repeated freeze-thawing sera were temporarily stored at 4°C during the experiments.

2.2 Phipps' non-competitive ELISA

Total Ig anti-HibPs antibodies were measured as described by Phipps *et al.* (19). The HbO-HA antigen was diluted in PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.0-7.4 prepared with sterile, non-pyrogenic water) to a concentration of 1 µg /ml (based on HbO). 100 µl aliquots of this solution were dispensed into the wells of polystyrene microtiter plates (Polysorp). After an incubation of 90 min. at 37°C, the plates were washed with PBS / 0.05% (v/v) Tween-20 (pH 7.4), 4 times with 200 µl aliquots/well. Two-fold serial dilutions of each serum sample were prepared in duplicate on the plate in PBS / 0.3% (v/v) Tween 20 / 0.01 M EDTA, starting with a dilution of 1/20 up to 1/2560 and for the reference serum starting with a dilution of 1/400 resulting in a concentration range from 0.175 to 0.0014 µg/ml. The plate configuration is shown in table 1. Control sera were used in each experiment. Plates were incubated at room temperature for 60 min. After the previously described washing procedure, Goat anti-human alkaline phosphatase conjugated Ig (polyspecific), diluted in PBS / 0.05 % Tween-20 was added to the plates (100 µl/well and incubated at room temperature for 60 min. To prepare the chromogen-substrate solution, *p*-nitrophenylphosphate tablets were dissolved in a solution of 1 M diethanolamine and 0.5 mM $MgCl_2$ (pH 9.8) according to the manufacturer's instructions. After the final wash procedure 100 µl of this *p*-NPP solution was added to each well. After a 30-60 min. colour development at room temperature, the maximum absorbance of the reference should be between 1.500 and 2.500, the absorbance was measured at 405 nm.

	1 2	3 4	5 6	7 8	9 10	11 12
	sample 1	sample 2	sample 3	sample 4	sample 5	reference
A	1/20	1/20	1/20	1/20	1/20	0.175 µg/ml
B	1/40	1/40	1/40	1/40	1/40	0.0875 µg/ml
C	1/80	1/80	1/80	1/80	1/80	0.0438 µg/ml
D	1/160	1/160	1/160	1/160	1/160	0.0219 µg/ml
E	1/320	1/320	1/320	1/320	1/320	0.0109 µg/ml
F	1/640	1/640	1/640	1/640	1/640	0.0055 µg/ml
G	1/1280	1/1280	1/1280	1/1280	1/1280	0.0027 µg/ml
H	1/2560	1/2560	1/2560	1/2560	1/2560	0.0014 µg/ml

Table 1. Plate configuration for the Phipps' non-competitive ELISA

Anti-HibPs antibody levels were calculated with the CBER reference serum as standard (assigned value of 70 µg/ml total anti-HibPs). A standard curve was calculated from the serial dilution of the reference (^{10}log concentration vs. absorbance). The 4-parameter fit of this curve was used to interpolate the serum sample absorbance in the linear part of the curve only. An example of a 4-parameter fitted reference curve is illustrated in figure 2. Antibody concentrations of sera calculated from this standard curve were expressed in µg/ml. The quantitation limit of undiluted sera corresponded to 0.10 µg/ml.

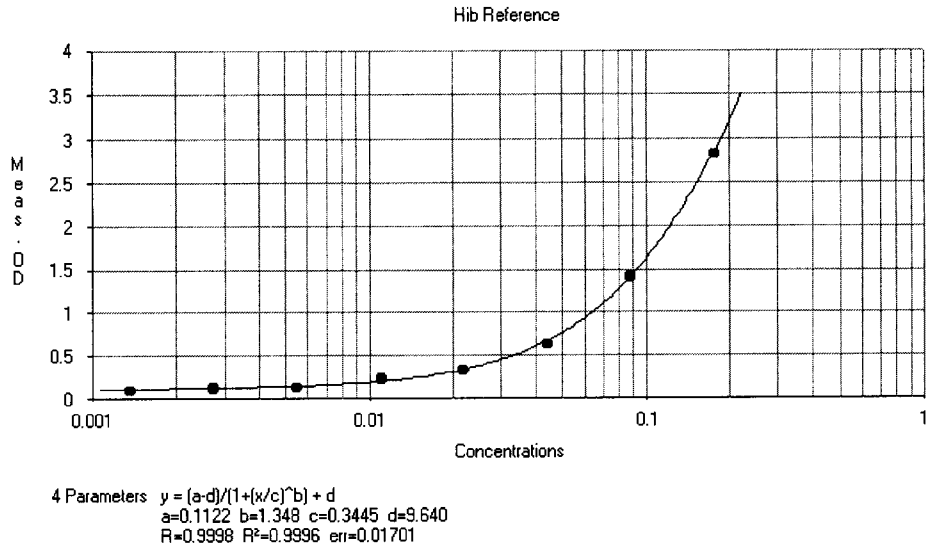

Figure 2. Example of a 4-parameter fit from a reference curve of the Phipps' non-competitive ELISA. Concentrations of the reference are actual amounts in the assay in µg/ml

The Phipps' ELISA anti-HibPs antibody levels measured by LVM were calculated with a linear fit (concentration vs. absorbance) of the reference (CBER) curve instead of a 4-parameter fit. Antibody concentrations of sera calculated from the standard curve were expressed in µg/ml. By using extrapolation antibody concentrations up to 0.01 µg/ml could be calculated which was used as quantitation limit.

2.3 Chiron competitive ELISA

Anti-HibPs antibodies were measured as described by Mariani *et al* (21). The HSA-Hib antigen was diluted in PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4 prepared with sterile, non-pyrogenic water) to a concentration of 1 µg/ml (based on the saccharide concentration). 100 µl aliquots of this solution were dispensed into the wells of polystyrene microtiter plates (Maxisorp). After overnight incubation at 4°C, plates were washed 4 times with 200 µl of PBS / 0.05% (v/v) Tween-20 (pH 7.4).
Wells were overcoated with 250 µl of a 1% w/v gelatin solution in PBS (pH 7.4), for 3 hours at 37°C. After washing, 250 µl of a fixative solution (saline containing 4% w/v polyvinyl pyrrolidone, 10% w/v saccharose) was added to each well. After 2 hours incubation at room temperature the fixative solution was removed from the wells, and the plates were dried overnight at room temperature. Dried plates could be stored up to 8 months at 4°C, in sealed plastic bags.
For each plate reference serum and sample sera were diluted in PBS / 1% (w/v) BSA / 0.05% (v/v) Tween-20, pH 7.4 (PBS / BSA). Each plate contained reference serum in duplicate, a two-fold serial dilution with a starting dilution of 1/280 and a concentration range from 0.250 to 0.0019 µg/ml. Each plate contained 8 serum samples in duplicate in four 4-fold serial dilutions, final dilutions 1/50, 1/200, 1/800 and 1/3200. One of the duplicates was used to determine non-specific binding by addition of an excess soluble HibPs (final concentration 50 µg/ml) in PBS / BSA. Soluble HibPs was added to the wells before the serum sample. Control sera were incorporated in each experiment. The plate configuration is shown in table 2.

	1	2	3 4	5 6	7 8	9 10	11 12
			reference	Ps	Ps	Ps	Ps
A	B	NSB	0.250 µg/ml	spl 1 1/50	spl 3 1/50	spl 5 1/50	spl 7 1/50
B	B	NSB	0.125 µg/ml	spl 1 1/200	spl 3 1/200	spl 5 1/200	spl 7 1/200
C	B	NSB	0.0625 µg/ml	spl 1 1/800	spl 3 1/800	spl 5 1/800	spl 7 1/800
D	B	NSB	0.0.312 µg/ml	spl 1 1/3200	spl 3 1/3200	spl 5 1/3200	spl 7 1/3200
E	B	NSB	0.0156 µg/ml	spl 2 1/50	spl 4 1/50	spl 6 1/50	spl 8 1/50
F	B	NSB	00078 µg/ml	spl 2 1/200	spl 4 1/200	spl 6 1/200	spl 8 1/200
G	B	NSB	0.0039 µg/ml	spl 2 1/800	spl 4 1/800	spl 6 1/800	spl 8 1/800
H	B	NSB	0.0019 µg/ml	spl 2 1/3200	spl 4 1/3200	spl 6 1/3200	spl 8 1/3200

Table 2. Plate configuration for the Chiron competitive ELISA. The B labelled column is the blank. The NSB labelled column is the control for non specific binding. The Ps labelled columns contain soluble HibPs in a final concentration of 50 µg/ml. Spl. represents sample

Plates were then incubated for 3 hours at 37°C. After the wash procedure 100 µl aliquots of alkaline phosphatase conjugated goat IgG anti-human Ig (IgG, IgM, IgA), diluted in PBS / BSA were added to each well. Plates were incubated for 3 hours at 37°C. The preparation of the chromogen-substrate solution, the color development and plate reading were identical to the Phipps' ELISA procedure described in paragraph 2.2. Anti-HibPs antibody levels were calculated with the CBER reference serum as standard (assigned value of 70 µg/ml total anti-HibPs) by interpolation of the serum sample absorbance in the standard curve (linear fit, concentration vs. absorbance). Concentrations of the samples were determined using absorbance values in the linear part of the standard curve. An example of a linear fitted reference curve is illustrated in figure 3. The absorbance value of the wells with soluble HibPs were subtracted as background from the corresponding values of the wells in which sera were diluted with buffer only. Antibody concentrations of sera calculated from the standard curve were expressed in µg/ml. The quantitation limit in undiluted sera was first set on 0.34 µg/ml, which was used in serum panel 1 – 3. During the collaboration the limit was lowered to 0.10 µg/ml which was used for serum panel 4.

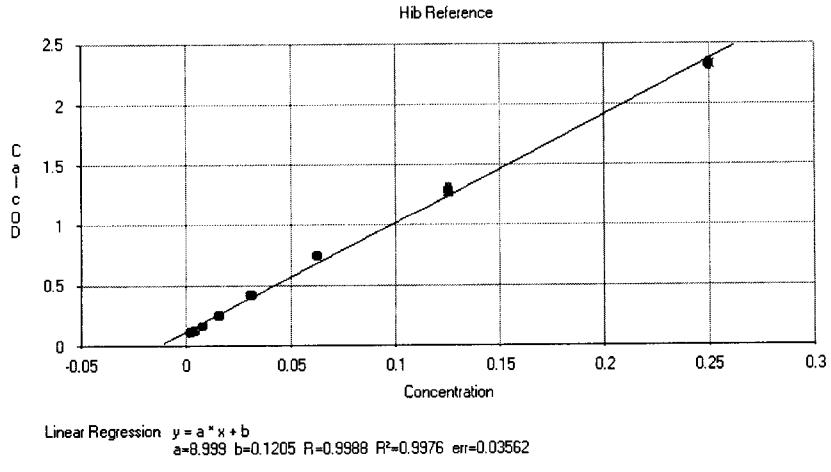

Figure 3. Example of a linear fit from a reference curve of the competitive ELISA. Concentrations of the reference are actual amounts in the assay in µg/ml

2.4 Radio Antigen binding assay

Several sera were analysed by RABA for the determination of total anti-HibPs antibody concentration. These experiments were performed at CHIRON (21). The general procedure of Robbins *et al.* was followed (12). In brief, ^{125}I-labelled HibPs was obtained following the chloramin-T method (22) by labelling a HibPs-tyramine conjugate conform the specifications established in 1987 by Dr. Carl Frash (CBER, FDA). Purified capsular polysaccharide from *Haemophilus influenzae* type b strain Eagan was fractionated on a Sepharose CL-2B (Pharmacia, Uppsala, Sweden) column and fractions in the 0.3 - 0.7 K_d range were pooled to exclude the very high molecular weight outer membrane protein containing material. Purity of the radio labelled antigen was assayed for binding to rabbit serum prepared against a capsular deficient variant (S-2) of strain Eagan (average 3.8% binding). Antigen concentration was estimated in RABA by competition with cold HibPs, at concentrations ranging from 7.8 to 2000 ng/ml in presence of an antibody concentration of 0.875 µg/ml. The antigen concentration used in the RABA was about 50-60 ng/ml. Antibody concentrations of test sera were determined by comparison of binding with the standard CBER reference serum (70 µg/ml). For calculation, the average was used of values determined by serum dilutions within the 15-80 percent binding range.

2.5 Statistical analysis

The paired samples T-Test (2-tailed) with logarithmically transformed values of antibody concentrations was used to access the similarity of the different assay methods, resulting in correlation coefficients and p-values. Statistical analysis was performed with the statistical program SPSS 7.0. Since the limit of the sensitivity of the ELISA methods was 0.10 µg/ml all values < 0.10 µg/ml were assigned 50% of the minimum (0.05 µg/ml). For the results of the Chiron competitive ELISA a quantitation limit of 0.34 µg/ml was used in serum panel 1 - 3; values < 0.34 µg/ml were assigned the value of 0.17 µg/ml.

3. Results

3.1 Serum panel 1

The correlation between the Phipps's ELISA, used at the RIVM, the RABA and the Chiron competitive ELISA as found in a first collaboration study between the RIVM and CHIRON, was a reason to further investigate these Hib antibody assays. The first panel we investigated was a small amount of sera (n = 20) which were shipped back from Italy and were used in the first collaborative study (total sera = 146). In spite of the poor condition of the sera we analysed the samples with the Chiron competitive ELISA. For this assay HSA-Hib antigen and HibPs was used which was supplied by CHIRON. The Chiron competitive ELISA procedure was followed with the exception that the Polysorp ELISA plate and the conjugate (Caltag) from the Phipps' ELISA were used. To investigate the influence of the HibPs control the Chiron competitive ELISA was performed with and without this control. Results were compared with the original Hib antibody data from LVM, who used the Phipps' ELISA and data from CHIRON, who performed the competitive ELISA and RABA on these 146 sera. The GMT and the correlation are shown in table 3. All data from panel 1 are listed in appendix 2.

Table 3. Comparison of anti-Hib antibody assays presented as GMT and correlation coefficient (R), with 20 serum samples. An assay, represented as an ">" in the column, is compared with the other assays in the corresponding horizontal grey bar. All antibody assays were performed in duplicate except for the Chiron competitive ELISA LVO which was performed as an independent triplicate. *In the Chiron competitive ELISA procedure the standard Maxisorp microtiter plate and the Sigma conjugate were replaced by a Polysorp microtiter plate and Caltag conjugate

assay	Phipps' ELISA	Chiron ELISA		Chiron ELISA	RABA
laboratory	*LVM/RIVM*	*LVO/RIVM*		*CHIRON*	*CHIRON*
remarks		Praxis/Chiron materials*			
		with HibPs control	without HibPs control		
GMT [µg/ml]	2.1	4.4	7.9	4.8	5.7
Correlation (R)	>	0.814	0.836	0.829	0.826
		>	0.990	0.920	0.918
			>	0.895	0.896
				>	0.992

The results show that the correlation for this sub panel of 20 sera, with regard to the comparison of both ELISA's with the RABA, was not markedly different from that found for the total panel (n = 146) as described in the introduction. The correlation between the Chiron competitive ELISA and the RABA was good. However, the correlation between the Chiron competitive ELISA results performed by CHIRON and LVO was moderate (0.920) and about the same when the Chiron competitive ELISA LVO was compared to the RABA (0.918). The poor condition of the sera could be responsible for the observed differences. The use of the HibPs control slightly improved the correlation with the competitive ELISA and the RABA. Furthermore, the GMT of the sera with HibPs control was markedly lower than without HibPs control which indicates reduced a-specific binding. On the other hand the GMT of the RABA was also higher than for the Chiron competitive ELISA's, whereas the GMT of the original Hib antibody data from LVM was about 50 % lower. There was a good correlation between the Chiron competitive ELISA results (LVO) with or without HibPs control (0.990). In practice, the Chiron competitive ELISA proved more laborious than the Phipps' ELISA

mainly because of the dilution method. The two-fold serial dilution as used in the Phipps' ELISA was not only less laborious than a fourfold dilution, it also showed better duplicate values after calculation. Thus the reliability of the calculated average antibody concentration of samples is improved. Furthermore, we observed a much longer colour development time (substrate/conjugate interaction) compared to the Phipps' ELISA but also compared to the assay information from CHIRON. This effect could be due to the ELISA plate used and/or to the conjugate specificity or -dilution we used in the Chiron competitive ELISA.

3.2 Serum panel 2

To investigate the Chiron competitive ELISA more profound we composed a new serum panel of 30 samples. These sera were tested with the Phipps' ELISA and the slightly adapted Chiron competitive ELISA (see 3.1). We could compare the results with the original Hib antibody data measured with the Phipps' ELISA (LVM). Both Hib ELISA assays were performed in duplicate on different days. The results are shown in table 4. All data from panel 2 are listed in appendix 3.

Table 4. Comparison of anti-Hib antibody assays presented as GMT and correlation coefficient (R), with 30 serum samples. All antibody assays were performed in duplicate. *Remark for Chiron competitive ELISA see table 3

assay	Phipps' ELISA	Phipps' ELISA	Chiron ELISA
laboratory	*LVM/RIVM*	*LVO/RIVM*	*LVO/RIVM*
remarks			Praxis/Chiron materials*
GMT [µg/ml]	31.0	38.2	38.5
Correlation (R)	>	0.950	0.814
		>	0.858

The inter assay variation (correlation between duplicates on different days) for the competitive and non-competitive ELISA was good with R = 0.992 (Phipps' ELISA) and R = 0.971 (Chiron competitive ELISA, data not shown). The reproducibility of the Phipps' ELISA (inter laboratory variation) between LVO and LVM was also high (R = 0.950). Even though the GMT between the Phipps' ELISA and Chiron competitive ELISA was almost identical the correlation was low (0.858). The correlation with the original LVM data was even lower (0.814). These results again show that there is a difference between both ELISA's. To exclude the influence of the antigen in the ELISA assay, we tested a subset of 14 sera from serum panel 2 with the Phipps' ELISA and the adapted Chiron competitive ELISA with both antigens (HbO-HA from Praxis and HSA-Hib from CHIRON). The results are shown in table 5. All data from subset 2 are listed in appendix 4.
The use of the two different antigens in the ELISA assays did not introduce a great difference in correlation within the same test, 0.972 for the Phipps' ELISA and 0.954 for the Chiron competitive ELISA. Furthermore, the correlation between the Phipps' ELISA and the Chiron competitive ELISA, for all "antigen" combinations, was comparable with the result found in table 4 and varied between 0.844 and 0.919. However, the GMT's for both Chiron competitive ELISA's showed a difference (43.6 and 66.2 µg/ml respectively), especially when compared to the GMT's from both Phipps' ELISA's, 27.4 and 28.7 µg/ml respectively. These findings were in contrast with the results found in tabel 2, where the GMT of both ELISA methods for serum panel 2 was almost identical, 38.2 and 38.5 µg/ml respectively. This could possibly be due to the choice of the sera in the subset.

Table 5. Influence of the coated antigen on the correlation of anti-Hib antibody assays presented as GMT and correlation coefficient (R), with a subset of 14 serum samples. *Remark for Chiron competitive ELISA see table 3

assay	Phipps' ELISA		Chiron competitive ELISA	
laboratory	*LVO/RIVM*		*LVO/RIVM**	
remarks	HbO-HA antigen	HSA-Hib antigen	HSA-Hib antigen	HbO-HA antigen
GMT [µg/ml]	27.4	28.7	43.6	66.2
Correlation (R)	>	0.971	0.867	0.919
		>	0.844	0.895
			>	0.954

The inter assay variation for both ELISA's (results from panel 2 compared to this subset) resulted in R = 0.983 (Phipps' ELISA) and R = 0.960 (Chiron competitive ELISA). These correlation's where slightly lower when compared to the inter assay variation for these assays described earlier. The time span (2 weeks) between the assays from serum panel 2 and it's subset could have introduced this small decrease in correlation as the sera were stored at 4°C during the experiments to avoid repeated freeze-thawing.

3.3 Serum panel 3

As previous results showed that the correlation of the Chiron competitive ELISA with the RABA was much better than with the Phipps' ELISA the aim was try and improve the Phipps' ELISA. Therefore a new serum panel was composed which consisted of 28 sera. We focussed on the main differences between both ELISA's besides the antigens. The major differences between both procedures are summarised in table 6 and are incubation time, temperature and buffer. In this tabel are not included differences in materials and suppliers.

Table 6. Major differences between the Chiron competitive ELISA and the non-competitive Phipps' ELISA

Materials and timing	Chiron competitive ELISA	Phipps' ELISA
ELISA plate type	MaxiSorp	PolySorp
Coating	HSA-Hib	HbO-HA
Coat time	Overnight at 4°C	90 minutes at 37°C
Overcoating and fixing	Yes	No
Serum binding buffer	PBS, 1% (w/v) BSA, 0.05% (v/v) Tween-20	PBS, 0.3% (v/v) Tween-20, 0.01 M EDTA
Serum dilution	4-fold serial 1/50 to 1/3200	2-fold serial 1/20 to 1/5120
Serum incubation time	3 hours 37°C	1 hour 22°C
Conjugate	Alkaline phosphatase conjugated goat IgG anti-human Ig (IgG, IgM, IgA) (Sigma), dilution 1/10,000	Alkaline phosphatase conjugated goat IgG anti-human polyspecific (Caltag), dilution1/2000
Conjugate incubation time	3 hours 37°C	1 hour 22°C

A Phipps' ELISA was performed with 3 variations namely 1. the serum- and conjugate incubation time and temperature increased to 3 hours and 37°C (as in the Chiron competitive ELISA); 2. the serum- and incubation buffer changed to PBS / 1% (w/v) BSA / 0.05% (v/v) Tween-20 (like the Chiron competitive ELISA) and 3. variation 1 and 2 combined. These variables were compared to the standard Phipps' ELISA and the adapted Chiron competitive ELISA procedure. The results are shown in table 7. All data from panel 3 are listed in appendix 5. The changes made to the incubation time/temperature and buffers decreased the correlation with the standard Phipps' ELISA down to 0.923 (inter assay variations with 0.992 and 0.983 were previously found, see 3.2). On the contrary, the correlation with the adapted

Chiron competitive ELISA increased from 0.902 up to 0.962 due to the changes in the assay. Furthermore, the effect of the changes in incubation time, incubation temperature and buffer on the correlation seemed cumulative. As a result of the changes on the Phipps' ELISA the GMT increased from 17.8 to 31.0 µg/ml. The GMT of the Phipps' ELISA and the Chiron competitive ELISA was comparable.

Table 7. Influence of incubation time, -temperature and -buffer on the Phipps' ELISA, presented as GMT and correlation coefficient (R), with 28 serum samples. All antibody assays were performed at least in duplicate. [1]The serum- and conjugate incubation time and -temperature were changed to 3 hours and 37°C. [2]The serum- and incubation buffers were changed to PBS / 1% (w/v) BSA / 0.05% (v/v) Tween-20. [3]Variation [1] and [2] combined. *Remark see table 3

assay	Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Chiron ELISA
laboratory	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*
remarks	Chiron incubation temperature and time[1]	Chiron incubation buffers[2]	Chiron incubation[3]		Praxis/Chiron materials*
GMT [µg/ml]	20.3	24.2	31.0	17.8	20.3
Correlation (R)	>			0.952	0.924
		>		0.965	0.925
			>	0.923	0.962
				>	0.902

Considering the effect of the changes in incubation time/temperature and buffer on the Phipps' ELISA we compared these results with the RABA and the Chiron competitive ELISA, independently performed by CHIRON on the same serum panel. The results are shown in table 8. All data are listed in appendix 5. The improved correlation for the Phipps' ELISA, after adapting the incubation time/temperatures and buffers was not found when compared to the Chiron competitive ELISA results from CHIRON. The correlation slightly decreased from 0.955 down to 0.938. However, compared to the RABA an improvement in correlation from 0.928 up to 0.965 was seen. More remarkable was the correlation between the Phipps' ELISA variations and the Chiron competitive ELISA and the RABA (both CHIRON) which was a considerably higher than previously seen with the original Hib antibody data (LVM), 0.829 and 0.826 for table 3 and 0.852 and 0.839 when all 146 sera where considered (see 3.1 and 1.4). Correlation values found now, were about 0.1 higher with 0.927 and 0.921 (vs. LVM data) and 0.955 and 0.928 (vs. LVO Phipps' ELISA), respectively. These results for the comparison with the RABA were even better than the R = 0.917 reported by Phipps *et al.* (19). The adapted Chiron competitive ELISA compared with the CHIRON data for the ELISA/RABA also showed an improved correlation, 0.923/0.954 vs. 0.920/0.918 in table 3 respectively. There was again a good correlation between the RABA and the Chiron competitive ELISA (CHIRON), 0.970 compared to 0.992 in table 3. The inter laboratory variation between LVO and LVM on the Phipps' ELISA was comparable with the results from table 4 (0.954).

The GMT for the Chiron competitive ELISA (CHIRON) was roughly 6 µg/ml higher than the GMT values for both Phipps' ELISA's. The GMT for the RABA results was even higher, at least a factor 2. Appendix 5 shows that almost all concentrations obtained with the RABA are twice as high compared to the concentrations in other assays, which was also noted for most sera of panel 1. However, compared to panel 1, serum panel 3 contained more sera with high Hib antibody concentrations, which could increase the GMT. The results in tabel 6 might be influenced by the time span (> 5 weeks) between the assays performed by LVO and

CHIRON. Sera were stored at 4°C during the experiments to avoid repeated freeze-thawing. A similar effect was already observed for serum panel 2.

Table 8. Comparison of anti-Hib antibody assays presented as GMT and correlation coefficient (R), with 28 serum samples. All antibody assays were performed at least in duplicate. [3]For remark Phipps' ELISA see table 7. *Remark for Chiron competitive ELISA see table 3

assay	Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Chiron ELISA	Chiron ELISA	RABA
laboratory	*LVM/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*CHIRON*	*CHIRON*
remarks			Chiron incubation[3]	Praxis/Chiron materials*		
GMT [µg/ml]	19.2	17.8	31.0	20.3	25.7	45.5
Correlation (R)	>	0.954		0.862	0.927	0.921
		>	0.923	0.902	0.955	0.928
			>	0.962	0.938	0.965
				>	0.923	0.954
					>	0.970

Besides the difference in incubation time, -temperature and -buffer between the Phipps' ELISA and the Chiron competitive ELISA, they also differ in conjugate and microtiter plate. The original materials for the Chiron competitive ELISA (used by CHIRON), Sigma conjugate and HSA-Hib antigen pre-coated and non-precoated microtiterplates were obtained from CHIRON. The conjugate from Sigma (Goat anti-human immunoglobulin, IgG, IgM, IgA) seemed more specific as it could be used in a higher working dilution than the Caltag conjugate, while the Maxisorp microtiter plate was claimed to have a higher affinity for polar groups and excellent binding of various proteins. With these materials preliminary experiments were performed on the Chiron competitive ELISA with a subset of panel 3. Unfortunately, the experiments (results not shown) with this subset were not conclusive due to the serum quality. The reproducibility of the adapted Chiron competitive ELISA (LVO) compared with the data in table 7 was decreased to R = 0.911 (the time span between the assays was 4 months), while a correlation of 0.971 was found earlier (see 3.2). Practical conclusions that could be drawn from these experiments were that the Sigma conjugate in spite of the higher dilution showed a faster colour development (substrate/conjugate interaction) and an improved linearity of the reference curve. So the specificity of this conjugate might indeed be higher compared to the Caltag conjugate. Furthermore, we found no difference between pre-coating and overnight coating of the HSA-Hib antigen. To investigate the effect of conjugate and Maxisorp microtiter plate on the Chiron competitive ELISA more profound we composed a new serum panel of 96 sera, serum panel 4.

3.4 Serum panel 4

Serum panel 4, which consisted of 96 sera, was composed in such a way that a broad antibody concentration range was covered, with a special interest for antibody concentrations < 1.0 µg/ml. In serum panel 1, 2 and 3 no sera with anti Hib concentrations < 1.0 µg/ml were included. The panel was tested with the standard Phipps' ELISA, the Phipps' ELISA with adapted incubation time/temperatures and buffers with Caltag conjugate and Polysorp microtiter plate but also with Sigma conjugate and Maxisorp microtiter plate, the LVO adapted Chiron competitive ELISA (Caltag conjugate and Polysorp microtiter plate) and the Chiron competitive ELISA without adaptations (Sigma conjugate and pre-coated Maxisorp microtiter plate). These assays were compared to the original Hib antibody data with the Phipps' ELISA (LVM) and the Chiron competitive ELISA and the RABA performed by CHIRON in Italy, on this serum panel. The sera for LVO and CHIRON were divided in small

aliquots and stored at –20°C before analysis in the Netherlands and Italy. In this way, repeated freeze-thawing and long time storage at 4°C were avoided, which might influence the assay results. Results are shown in table 9, some of which are illustrated as correlation charts in figure 4. All data from panel 4 are listed in appendix 6. Like in table 8, the results in table 9 showed a high correlation for all the different Phipps' ELISA's with the Chiron competitive ELISA and the RABA. Correlation values were even higher ranging between 0.952 and 0.972, except for the original Phipps' ELISA data (LVM), which were only 0.921 (vs. Chiron competitive ELISA) and 0.934 (vs. RABA). The inter laboratory variation for the Phipps' ELISA data between LVO and LVM was lower than usual with a factor 0.920 (0.950 and 0.954 for panel 2 and 3 respectively). This might be explained by the high amount of sera with concentrations < 1.0 µg/ml in panel 4. Calculation of the original Phipps' ELISA anti-HibPS antibody concentrations by LVM was done with a linear fit from the reference curve which enabled extrapolation of low antibody concentrations. Antibody concentrations lower than the quantitation limit of the other assays could be reached when calculated in this way. Compared to the Chiron competitive ELISA and the RABA (both CHIRON) the Chiron competitive ELISA assayed by LVO (without any adaptations) showed a very high correlation, considerably better than with the adapted Chiron competitive ELISA used for panel 1, 2 and 3. The inter laboratory variation of this ELISA between LVO and CHIRON was R = 0.973, which was even higher than was found for the Phipps' ELISA (0.950, 0.954 and 0.920, respectively). The correlation between the Chiron competitive ELISA and the RABA (CHIRON) was 0.977, which was comparable to the 0.992 found in panel 1 and 0.970 in panel 3. The results with this ELISA from LVO were similar (R = 0.982). In contrast, the correlation's for the same comparisons with the adapted Chiron competitive ELISA (LVO) were very poor with R values of 0.847 and 0.853, respectively. This indicated that the more specific Sigma conjugate and the Maxisorp mirotiter plate markedly influenced the ELISA results. Even the Phipps' ELISA seemed to benefit from these changes as the already "improved" Phipps' ELISA (incubation from the Chiron competitive ELISA) showed an even higher correlation with the Chiron competitive ELISA's when Sigma conjugate and the Maxisorp plate were used (from 0.953 to 0.972). Compared to the RABA this effect was less profound (from 0.967 to 0.968). Furthermore, the influence of the adaptations in incubation time/temperatures and buffers was comparable with the results found with panel 3 when compared to the RABA.

The differences between the assays in GMT were relatively small. The GMT for the RABA and the adapted Phipps' ELISA's were almost identical with 2.2 µg/ml, while the GMT for the standard Phipps' ELISA was a fractionally lower with 2.0 µg/ml. The GMT for the Phipps' ELISA data from LVM was lower with 1.2 µg/ml which can be explained by the lower quantitation limit they used. The GMT's for the Chiron competitive ELISA's were also lower compared to the RABA, 1.2, 1.5 and 1.4 µg/ml, respectively. This might be explained by the new quantitation limit of 0.10 µg/ml which was used in panel 4 instead of the limit of 0.34 µg/ml used in panel 1, 2 and 3.

Table 9. Comparison of anti-Hib antibody assays presented as GMT and correlation coefficient (R), with 96 serum samples. All antibody assays were performed in duplicate. [1]For remark Phipps' ELISA see table 7. [2]As [1], but the standard Polysorp microtiter plate and the Caltag conjugate were replaced by a Maxisorp microtiter plate and Sigma conjugate. *Remark for Chiron competitive ELISA see table 3

assay	**Phipps' ELISA**	**Phipps' ELISA**	**Phipps' ELISA**	**Phipps' ELISA**	**Chiron ELISA**	**Chiron ELISA**	**Chiron ELISA**	**RABA**
laboratory	*LVM/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*CHIRON*	*CHIRON*
remarks			Chiron incubation[1]	Chiron method[2]	Praxis/Chiron materials*	Chiron materials		
GMT [µg/ml]	1.2	2.0	2.2	2.2	1.2	1.5	1.4	2.2
Correlation (R)	>	0.920	0.917	0.933	0.843	0.942	0.921	0.934
		>	0.944	0.966	0.849	0.938	0.952	0.955
			>	0.957	0.843	0.959	0.953	0.967
				>	0.874	0.967	0.972	0.968
					>	0.869	0.847	0.853
						>	0.973	0.982
							>	0.977

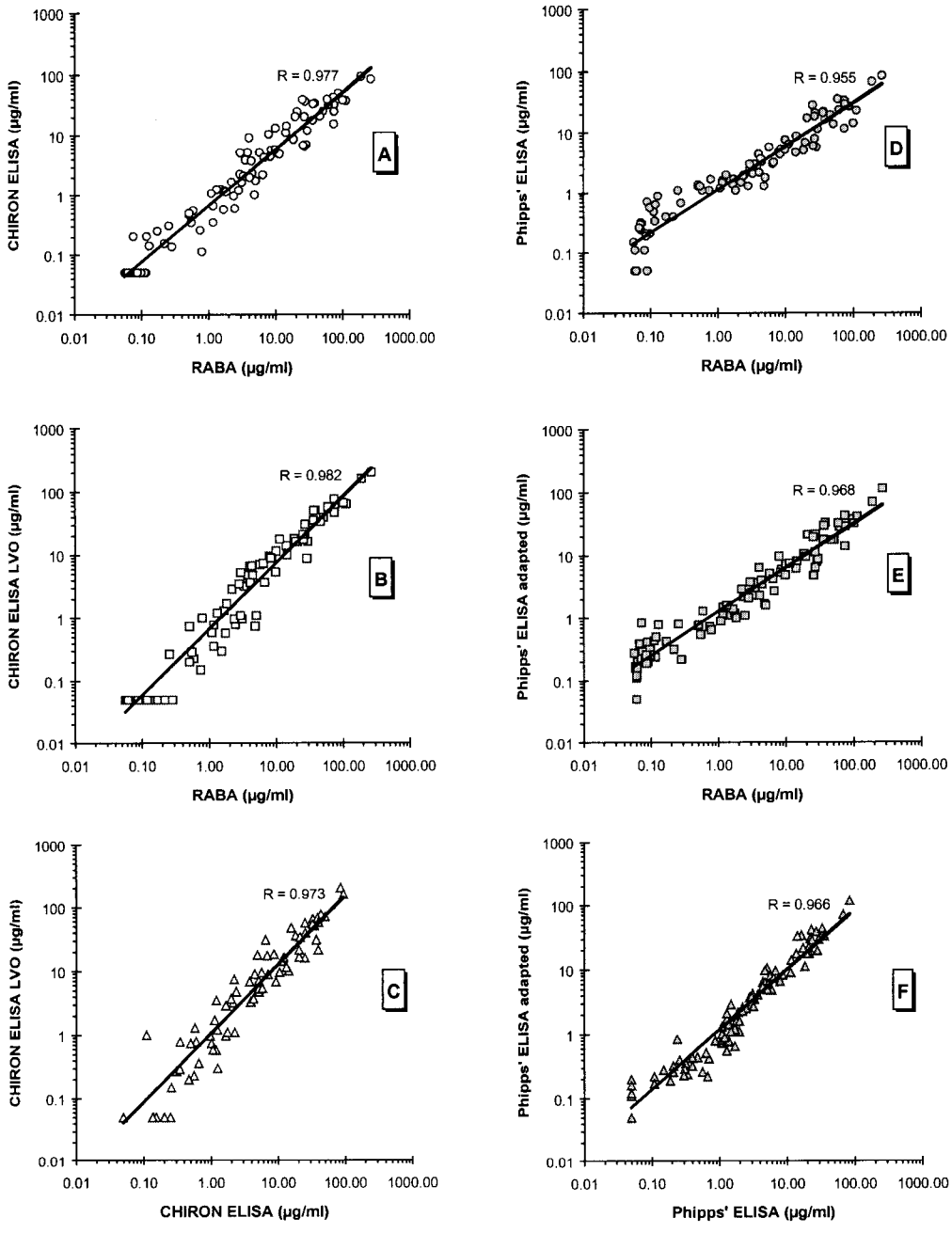

Figure 4. Correlation (R) of anti-Hib antibodies in human sera (n = 96) as determined by Chiron competitive ELISA (CHIRON) and RABA (A), Chiron competitive ELISA (LVO) and RABA (B), Chiron competitive ELISA performed by LVO and CHIRON (C), Phipps' ELISA and RABA (D), Phipps' ELISA adapted (Chiron method, see remark 2 table 9) and RABA (E) and Phipps' ELISA adapted and Phipps' ELISA (F).

In table 10 the p-values for the different Hib antibody assays are presented. In spite of the good correlation's, only a minority of the assays appeared not significantly different (p-value >0.05). These assays all showed similar GMT's as seen for the comparison between the RABA and all Phipps' ELISA's (LVO), p-values of 0.279, 0.963 and 0.905, respectively. Both Chiron ELISA's were not significantly different (p-value 0.221). However, when compared to the RABA p-values < 0.05 were found. In spite of the high correlation between these assays a number of individual serum Hib antibody concentrations differed significantly also represented by the difference in GMT. This effect was induced by the changed quantitation limit for the competitive ELISA (results not shown). The same effect was found when the Phipps' ELISA's were compared to the original data from LVM. In spite of a correlation factor of 0.920 most individual LVM data were lower in antibody concentration, probably due to the calculation method. In contrast, when these LVM data were compared to the Chiron competitive ELISA (CHIRON, R = 0.921) no significant difference was found.

Table 10. Comparison of anti-Hib antibody assays presented as p-values (2-tailed), with 96 serum samples. For other remarks see table 9. Two assays are significantly different with a p-value < 0.05. P-values that indicate significance are underlined

assay	Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Chiron ELISA	Chiron ELISA	RABA
laboratory	*LVM/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*CHIRON*	*CHIRON*
remarks			Chiron incubation[1]	Chiron method[2]	Chiron materials		
p-value	>	0.000	0.000	0.000	0.020	0.107	0.000
		>	0.118	0.027	0.025	0.000	0.279
			>	0.907	0.000	0.000	0.963
				>	0.001	0.000	0.905
					>	0.221	0.000
						>	0.000

As the classical RABA is still the standard assay for the determination of anti-Hib antibodies in human sera, the correlation results of table 9 were presented in an ELISA/RABA ratio as a function of RABA concentrations in figure 5. The differences found in correlation between the ELISA methods compared to the RABA were elucidated in figure 5. The charts clearly showed an overestimation of sera in the lower concentration range (< 1.0 µg/ml) for the Phipps' ELISA, even when performed with incubation circumstances and materials according to the Chiron competitive ELISA (chart 7A, 7B and 7C). This effect was absent for the Chiron competitive ELISA with CHIRON materials (chart 7E and 7F). These results confirmed the findings of Mariani *et al.* (21) that specific binding inhibition, with purified HibPs, induces a decrease in overestimation of low concentration samples.

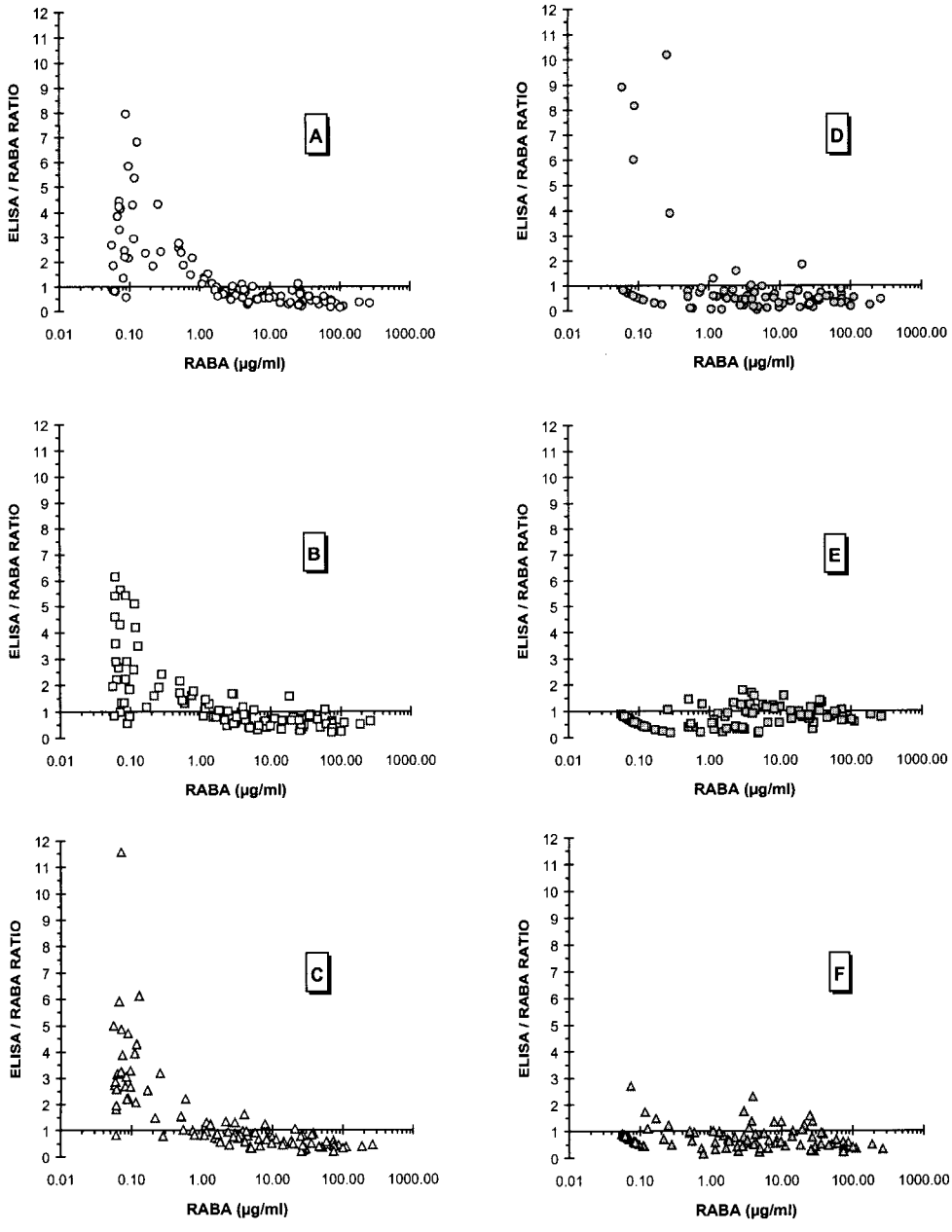

Figure 5. ELISA/RABA ratio as a function of RABA concentrations, Phipps' ELISA (A), Phipps' ELISA with Chiron competitive ELISA incubation procedure (B), Phipps' ELISA with Chiron competitive ELISA incubation procedure and materials (Maxisorp microtiter plate and Sigma conjugate) (C), Chiron competitive ELISA with adaptations LVO (Polysorp microtiter plate and Caltag conjugate) (D), Chiron competitive ELISA performed by LVO (E) and Chiron competitive ELISA performed by CHIRON (F)

Although the exact concentration of serum anti-HibPs antibody sufficient to evoke protection is not known (15), unvaccinated individuals are considered protected with a level of 0.15 µg/ml anti-Hib antibodies (17-18). Vaccinated individuals are considered protected with a level of 1.0 µg/ml anti-Hib antibodies. These protection levels were used in the multi center clinical study on the simultaneous administration of DPT-IPV and Hib PRP-T vaccine in

1994 (7). As illustrated in figure 5 the Phipps' non-competitive ELISA might overestimate the percentage of subjects with antibody concentration > 1.0 µg/ml. In table 11 the percentage of sera with an antibody concentration lower or equal to 0.15 and to 1.0 µg/ml were determined of the total of 96 sera.

Table 11. Percentage of sera on a total of 96 lower or equal to the Hib antibody protection level 0.15 µg/ml (protection unvaccinated individuals) and 1.0 µg/ml (protection vaccinated individuals)

anti-Hib antibody assay	laboratory	GMT	Percentage of total [%]	
			≤ 0.15 µg/ml	≤ 1.0 µg/ml
Phipps' ELISA	LVM/RIVM	1.19	21.9	46.9
Phipps' ELISA	LVO/RIVM	1.96	10.4	29.2
Phipps' ELISA *(Chiron incubation)*	LVO/RIVM	2.18	9.4	34.4
Phipps' ELISA *(Chiron incubation and materials)*	LVO/RIVM	2.20	3.1	37.5
Chiron competitive ELISA *(Praxis/Chiron materials)*	LVO/RIVM	1.24	26.0	46.9
Chiron competitive ELISA *(Chiron materials)*	LVO/RIVM	1.51	30.2	45.8
Chiron competitive ELISA	CHIRON	1.39	26.0	41.7
RABA	CHIRON	2.17	26.0	36.5

The percentage of sera ≤ 0.15 µg/ml for the Chiron competitive ELISA fluctuated between 26.0 and 30.2 % and was comparable to the percentage obtained with the RABA (26.0 %). For the Phipps' ELISA this percentage was at least a factor 2 lower, 10.4, 9.4 and 3.1 %, respectively. The Phipps' ELISA data from LVM showed a much higher percentage with 21.9 % probably due to the calculation method and quantitation limit. The percentage of sera ≤ 1.0 µg/ml for the Chiron competitive ELISA's lay between 41.7 and 46.9 %, somewhat higher than the RABA result (36.5 %). For the Phipps' ELISA this percentage was slightly lower, 29.2, 34.4 and 37.5 %, respectively. In this case the percentage for the Phipps' ELISA with Chiron incubation and materials was comparable with the RABA (37.5 %). Overestimation for unvaccinated subjects (compared to the RABA), with antibody concentrations > 0.15 µg/ml, was considerably higher when measured with the Phipps' ELISA, and increased when the Phipps' ELISA was adapted to Chiron ELISA specifications. Furthermore, these results showed a slight overestimation of the percentage of subjects with antibody concentration > 1.0 µg/ml determined with the Phipps' non-competitive ELISA compared to the RABA. This effect seemed to decrease when the Phipps' ELISA was adapted to Chiron ELISA specifications. The competitive ELISA slightly underestimated the amount of subjects with antibody concentration > 1.0 µg/ml.

In table 12 the basic anti-Hib antibody assays (without adaptations) were compared considering the percentages of sera which were protected in one assay and not protected in the other assay, for both protection levels. Compared to the RABA as standard assay the differences found for the Chiron competitive ELISA's were smaller than for the Phipps' ELISA's, 4.2 up to 7.3 % compared to 7.3 up to 14.6 %, respectively. This effect was independent of the level of protection (0.15 or 1.0 µg/ml). Furthermore, when the Phipps' ELISA's were compared to the Chiron competitive ELISA the percentages were even higher, 11.5 up to 14.6 %. Even when both Phipps' ELISA's (LVO and LVM) were compared, the percentages were high, 9.4 up to 15.6 %. These results show that in spite of a good correlation between two assays, they still can give different answers to the question of sufficient protection. Furthermore, table 12 confirmed that overestimation of protected individuals (unvaccinated and vaccinated) was almost absent with the competitive ELISA which also showed a higher similarity with the RABA than the non-competitive ELISA.

Table 12. Differences between anti-Hib antibody assays on the percentage (total = 96) of sera for the protection levels 0.15 and 1.0 µg/ml. [a]Protection level for the Phipps' ELISA (LVM) in all cases lower. [b]Protection level for the Chiron competitive ELISA in all cases lower. [c]Protection level for the RABA in all cases lower. [d]Protection level for the Chiron competitive ELISA (LVO) in all cases lower

		differences in protection level as percentage of total [%]		
anti-Hib antibody assay	**Protection level (µg/ml)**	**Phipps' ELISA (LVO)**	**Chiron competitive ELISA**	**RABA**
Phipps' ELISA (LVM)	0.15	9.4[a]	11.5	7.3
	1.0	15.6[a]	11.5	11.5
Phipps' ELISA (LVO)	0.15		14.6[b]	14.6[c]
	1.0		11.5[b]	7.3[c]
Chiron competitive ELISA (LVO)	0.15		5.2	4.2[d]
	1.0		5.2	7.3[d]
Chiron competitive ELISA	0.15			4.2
	1.0			4.2

4. Discussion

The vaccination efficacy of individuals against *Haemophilus influenzae* type b is normally determined with the established radio-antigen binding assay (RABA). This assay is time and sample consuming and involves the problems connected with radioactivity handling and waste. In 1990 an ELISA was proposed by Phipps *et al.* (19), which correlated well with the RABA and which was clearly an improvement in terms of assay feasibility. This ELISA could be used as a replacement of the RABA for the determination of anti-HibPs antibody concentrations in human sera. At the RIVM the Phipps' ELISA was used by LVM in a multi center clinical study. During this clinical trial a collaboration was started with CHIRON in Italy to establish a correlation between Phipps' ELISA, RABA and CHIRON's newly developed competitive ELISA. The aim of the competitive ELISA was to reduce variability in background and day to day variation by addition of a saturating amount of purified soluble Hib capsular polysaccharides to the serum. To determine serum anti-HibPs antibody concentrations, absorbance values in wells containing serum dilutions incubated with soluble HibPs were subtracted as specific background from the corresponding values of the wells in which sera were diluted with buffer only. They showed that their approach resulted in a very good correlation between ELISA and RABA values within a panel of 146 sera from the clinical trial, representative for a wide range of antibody levels. The correlation of the competitive ELISA with the RABA in this study appeared much better than for the non-competitive ELISA from Phipps (about 0.14). Furthermore, the correlation between both ELISA's was poor. This was the reason to investigate the Chiron competitive ELISA ourselves and to compare it with the Phipps' ELISA and the RABA using sera from the same clinical trial.

Serum panel 1

Serum panel 1, a small selection of the 146 previously tested sera, was tested at the RIVM with the competitive ELISA mainly according to the Chiron procedure. The antigen and purified Hib polysaccharide were obtained from CHIRON, the conjugate and microtiter plate (Polysorp) were applied from the Phipps' ELISA procedure. The correlation factor of the competitive ELISA with the RABA (Chiron) was 0.07 lower compared to the results from CHIRON with the competitive ELISA. The correlation with the original Hib data determined with the Phipps' ELISA by LVM was comparably low, which supported the results found for all 146 sera. Furthermore, competition with purified Hib polysaccharide appeared useful considering that the correlation factor decreased by 0.025 when this competition (non-competitive) was omitted. This effect was also found by an increased GMT for the ELISA without HibPs. The condition of the tested sera had become poor, due to the shipments between Italy and the Netherlands, which might explain for some of the observed differences between the ELISA results. In practice, the new competitive ELISA appeared more laborious than the Phipps' ELISA, mainly because of the impractical four fold serum dilution method instead of a regular twofold dilution. The use of a twofold serial dilution is also supported by an increase of the reliability of the average antibody concentration, due to more available extinction's for interpolation and calculation. The development time in the competitive ELISA was longer compared to the standard ELISA, which could partially be masked by decreasing the conjugate dilution. Furthermore, the linearity of the reference line was not always good.

Serum panel 2

The newly composed serum panel 2, a selection of new sera from the same clinical trial, was tested with the Chiron competitive ELISA with adaptation from LVO, the Phipps' ELISA and again compared to the original Hib antibody data (LVM). The correlation between the competitive ELISA and the original ELISA data for this panel was identical to the one found in panel 1. The correlation factor with the new Phipps' ELISA results was about 0.04 better. A correlation factor between both Phipps' ELISA's (inter assay variation) of 0.950 was found, in spite of the nearly two year time interval between the performance of the assays.

To exclude the influence of the antigen on the ELISA both assays were performed again with the different antigens (HbO-HA from Praxis and HSA-Hib from CHIRON) on a subset of panel 2. No great differences in correlation were found within both assays. The GMT's for the competitive ELISA's were higher compared to the Phipps' ELISA's. The low correlation between both ELISA's could not be improved with the switch of the antigen.

Serum panel 3

In order to improve the correlation between the Phipps' ELISA, the Chiron competitive ELISA and the RABA changes in our ELISA procedure were applied and a new serum panel was used. The results showed an increased correlation for all variations applied on the procedure of the Phipps' ELISA's towards the method of the competitive ELISA. This increase seemed cumulative considering the best correlation (0.962) was obtained with the combination of changes in incubation time, -temperature and -buffer. With an improved correlation towards the competitive ELISA, it was obvious that the correlation factor between the Phipps' ELISA's decreased, down to 0.923, while 0.990 was found earlier. The improved correlation we found between the competitive ELISA (LVO) and the adapted Phipps' ELISA was not observed with the competitive ELISA results from CHIRON. On the contrary, the correlation slightly decreased (0.017). This again showed that a considerable difference existed between the performance of the competitive ELISA's. When compared to the RABA the correlation factor of the adapted Phipps' ELISA increased with 0.037. Remarkably, the correlation between the standard Phipps' ELISA (LVO and LVM) and both RABA and competitive ELISA (results CHIRON) was much better (at least 0.1 higher) than found with panel 1 and for all 146 sera. The correlation values exceeded the one found by Phipps *et al* (19) for their ELISA compared to the RABA on 214 sera. The large difference in correlation factors found in the first collaboration (all 146 sera) towards the RABA and between both ELISA's was now strongly diminished in panel 3. The inter laboratory variation between both Phipps' ELISA performances (LVM and LVO) was again high with R = 0.954. The competitive ELISA performed by LVO in this panel showed an increased correlation with the RABA compared to panel 1, up with 0.036, whereas the comparison with the Chiron competitive ELISA data from Italy remained the same. The correlation between the two assays performed by CHIRON was as good as the result found in panel 1. The GMT of the assays performed by CHIRON was however rather high, especially for the RABA. This could be introduced by the relatively increased number of sera with high Hib antibody concentrations in panel 3. The 2 month time interval between the performance of the assays by LVO and CHIRON might also be the cause. Serum quality might have decreased during prolonged storage at 4°C, which was done to prevent repeated freeze thawing. The negative effect of prolonged serum storage was demonstrated in experiments where the reproducibility of the Chiron competitive ELISA, with a time interval between the assays of 4 months, was decreased with a factor 0.06.

A competitive ELISA performed with Sigma conjugate and pre-coated and non-coated microtiter plates (Maxisorp) showed that the development time of the ELISA could be decreased while less conjugate was needed. This elucidated the problems mentioned for serum panel 1. The conjugate from Sigma seemed to be more specific. Furthermore, the linearity of the reference curve improved which has a direct influence on the calculated antibody concentrations. No difference was found between pre-coating and overnight coating of the HSA-Hib antigen.

Serum panel 4

This serum panel of 96 sera was composed to elucidate the remaining questions from panel 3 and conclusively confirm previous results. The panel covered a broad range of antibody concentrations (concentrations < 1.0 μg/ml included). To prevent the influence of long time storage at 4°C the sera for both laboratories were filled separately in small aliquots and stored at –20°C until analysis. In addition to comparing the same assays as used in serum panel 3, a standardised competitive ELISA (no differences with the Chiron procedure) was performed and a Phipps' ELISA with the incubation time, temperature, buffer, conjugate and microtiter plate changed to the Chiron procedure. This experiment confirmed the results from panel 3. Again high correlation's between the Phipps' ELISA and the Chiron competitive ELISA/RABA were observed even better than for panel 3, with an exception for the original Hib data from the clinical trial (LVM, about 0.025 lower). The inter assay variation between both Phipps' ELISA's (LVO and LVM) was also about 0.025 lower than in serum panel 2 and 3. A high number of sera with concentrations < 1.0 μg/ml in this panel might explain this, as LVM used extrapolation for calculation and a lower quantitation limit. It should be mentioned that CHIRON after evaluating their competitive ELISA changed to a new quantitation limit, 0.10 μg/ml instead of 0.34 μg/ml, which was used in panel 4. The detection limit for both ELISA's was now the same.

The correlation factor between the two standardised Chiron competitive ELISA's (LVO and CHIRON) was very good, 0.973, which highlighted the reproducibility of this assay. The correlation of both assays with the adapted competitive ELISA (LVO), used in panels 1 to 3, was still poor with at least a factor 1.0 lower. This showed that the microtiter plate and conjugate used in the assay were very important and that the adaptations on the competitive ELISA we used from the start had a profound negative influence on the results. Both standardised competitive ELISA's showed excellent correlation factors with the RABA, 0.977 for CHIRON and 0.982 for LVO, in striking contrast to the 0.853 for the adapted ELISA. The effect of the changes in incubation time, temperature and buffer on the Phipps' ELISA were partly confirmed. The correlation with the adapted competitive ELISA (LVO) did not improve, but compared with the RABA it did. Compared with the standardised competitive ELISA (LVO) the correlation increased while compared to the Chiron ELISA (CHIRON) no increase was found, which was also observed in panel 3. The variation of the Phipps' ELISA with the extra adaptations (conjugate and the microtiter plate) showed an improved correlation with all competitive ELISA's and the RABA. The correlation with the RABA was 0.968 and thus almost identical to the result for the standardised competitive ELISA's. The GMT values of the Phipps' ELISA's (LVO) and the RABA were similar while the GMT of the competitive ELISA's and the original Hib data from LVM were lower. This effect might be due to the quantitation limit changes in the competitive ELISA.

In spite of good correlation's, a majority of the Hib antibody assays was found significantly different (p-value < 0.05) like both competitive ELISA's compared with the RABA. Assays

which were not significantly different all showed similar GMT's, like the RABA compared to all Phipps' ELISA's (LVO) as well as between both Chiron competitive ELISA's. It appeared that in spite of a similar trend between the assays, represented by a good correlation, the height of a number of individual serum Hib antibody concentrations differed significantly. This effect was induced by the changed quantitation limit for the competitive ELISA. The same effect was found for the comparison between the Phipps' ELISA's and the original data from LVM. Most LVM antibody concentration were lower, probably due to the calculation method.

The figures that present the ELISA/RABA ratio as a function of the RABA concentrations. clearly showed the difference between the competitive and non-competitive ELISA. A clear overestimation was found with the Phipps' ELISA for sera in the lower concentration range (< 1.0 μg/ml). However, this effect did not disappear with the changes made to the assay procedure, changes which showed to improve the correlation. This effect was not found for the competitive ELISA which confirmed the findings of Mariani *et al* (21) that competition with free HibPs is necessary to avoid this false positive binding.

When the protection level 0.15 μg/ml for unvaccinated subjects (17-18) was employed, the percentage of sera ≤ 0.15 μg/ml for the Phipps' ELISA was at least a factor 2 lower than for the competitive ELISA and the RABA. This again showed that with the non-competitive ELISA the percentage of subjects with antibody titers > 0.15 μg/ml might be overestimated. When the protection level 1.0 μg/ml for vaccinated subjects (17-18) was used, the percentage of sera ≤ 1.0 μg/ml for the Phipps' ELISA and the RABA was comparable, while the percentage for the competitive ELISA was slightly higher. This indicated a slight, however non serious, underestimation of subjects with antibody concentrations > 1.0 μg/ml. The results clearly showed that the similarity for the different protection levels was higher between the Chiron competitive ELISA and RABA than for the Phipps' non-competitive ELISA and RABA.

Conclusions

Reliability, reproducibility plus a good correlation with the RABA demonstrate that both the non-competitive Phipps' ELISA and the newly introduced competitive ELISA by CHIRON can replace the RABA for anti-Hib total Ig determination in populations. However, a clear advantage of the more laborious competitive ELISA, as proposed by Mariani *et al.*, is that the overestimation of the percentage of subjects with antibody titers > 0.15 μg/ml is absent, as compared to the non-competitive ELISA. Competition with free HibPs, appeared necessary to avoid false positive binding for anti Hib antibody concentrations ≤ 1.0 μg/ml. Although the non-competitive ELISA showed differences with the RABA in the evaluation of low titer sera and thus the problem might be restricted to pre-immune sera it could influence the number of subjects considered sufficiently protected against Hib.
The Phipps' non-competitive ELISA used at the RIVM for the detection of Hib antibodies could be improved by increasing the incubation time from 60 to 180 minutes, raising the incubation temperature from 22 to 37°C, changing the incubation buffers to PBS / 1% (w/v) BSA / 0.05% (v/v) Tween-20 (pH 7.4) as used in the Chiron competitive ELISA, together with the use of the more specific conjugate from Sigma and the Maxisorp microtiter plate. The serial twofold dilution of the sera as used in the Phipps' ELISA is preferred over a four fold dilution in handling and by an increase of the reliability of the average antibody concentration, due to more available extinction's for interpolation and calculation. Prolonged

storage of sera at 4°C and repeated freeze thawing effect the ELISA result negatively and should therefore be avoided.

Based on the results of the four serum panels we propose an improved ELISA for determining Hib antibodies (at the RIVM) with the following characteristics:

- The HbO-HA antigen from the Phipps' ELISA (PRAXIS) or HSA-Hib from the competitive ELISA (CHIRON) is coated overnight to a Maxisorp microtiter plate (Nunc).
- The samples are tested in a twofold serial dilution, preferably with 8 dilutions.
- The control wells with HibPs, free soluble polysaccharide purified from *Haemophilus influenzae* type b (CHIRON) are used for samples with Hib antibody concentrations ≤1.0 µg/ml.
- The sample and conjugate are incubated at least 2 hours at 37°C with PBS / 1% (w/v) BSA / 0.05% (v/v) Tween-20 (pH 7.4) as buffer.
- The goat anti-human immunoglobulin (IgG, IgM, IgA) alkaline phosphatase from Sigma (working dilution 1:10,000) is used as conjugate.
- The anti-HibPs antibody concentrations are calculated by interpolations of the sample absorbance, possibly corrected for a-specific binding, in the linear part of a 4-parameter fit from the reference curve (^{10}log concentration vs. absorbance).
- The quantitation limit in undiluted sera corresponds to 0.10 µg/ml.

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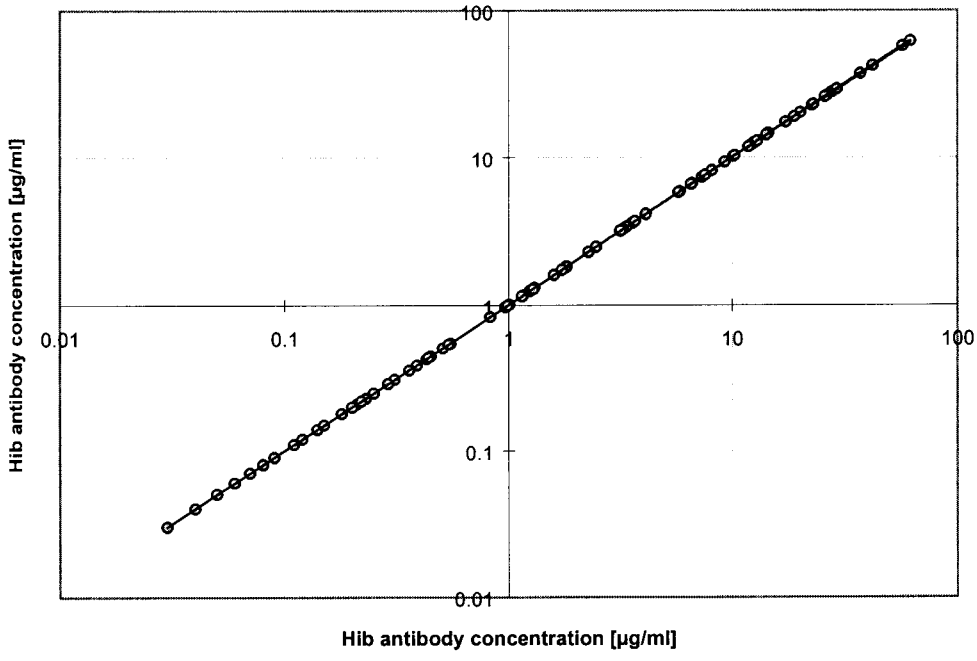
Appendices

Appendix 1.

Logarithmical distribution of Hib antibody concentration from sera in panel 4 in µg/ml, based on the clinical trial data from LVM measured with the Phipps' non-competitive ELISA.



Appendix 2.

Serum panel 1. Comparison of anti-Hib antibody assays presented as antibody concentrations, average and GMT [µg/ml] (n = 20). All antibody assays were performed in duplicate except the Chiron competitive ELISA *LVO/RIVM* which was performed as an independent triplicate. *In the Chiron competitive ELISA procedure the standard Maxisorp microtiter plate and the Sigma conjugate were replaced by a Polysorp microtiter plate and Caltag conjugate.

assay	Phipps' ELISA	Chiron ELISA		Chiron ELISA	RABA
laboratory	*LVM/RIVM*	*LVO/RIVM*		*CHIRON*	*CHIRON*
	94/95	*23-26/04/96 and 02/05/96*		*18/12/95*	
remarks		Praxis/Chiron materials*			
		with HibPs control	without HibPs control		
UTN no.					
22 VP 1	0.31	0.71	1.8	0.17	0.08
22 VP 2	6.1	0.26	1.7	0.17	0.11
22 VP 3	1.6	3.1	4.9	15.0	15.1
22 VP 4	44.9	55.5	60.8	108.4	158.5
33 VP 1	0.2	0.38	1.4	0.17	0.08
33 VP 2	0.11	0.54	1.4	0.42	0.14
33 VP 3	2.9	1.3	2.6	2.4	3.9
33 VP 4	32.9	19.0	23.2	44.1	91.6
59 VP 1	0.14	0.46	1.2	0.17	0.09
59 VP 2	17	7.1	8.2	74.6	91.9
59 VP 3	1.0	1.7	5.9	4.4	7.4
59 VP 4	56.7	97.1	110.4	84.8	369.4
123 VP 1	0.56	0.40	0.99	1.2	1.0
123 VP 2	1.2	6.1	7.6	8.4	15.9
123 VP 3	1.1	6.6	9.5	1.0	1.1
123 VP 4	26.6	123.6	130.6	52.1	80.7
170 VP 1	0.01	0.17	0.51	0.17	0.16
170 VP 2	13.9	79.3	83.9	97.5	343.1
170 VP 3	1.3	17.3	19.5	22.5	34.0
170 VP 4	16.3	157.1	172.7	136.6	246.9
average [µg/ml]	11.2	28.9	32.4	32.7	73.1
GMT [µg/ml]	2.1	4.4	7.9	4.8	5.7

Appendix 3.

Serum panel 2. Comparison of anti-Hib antibody assays presented as antibody concentrations, average and GMT [µg/ml] (n = 30). All antibody assays were performed in duplicate. *Remark for Chiron competitive see appendix 1.

assay	Phipps' ELISA	Phipps' ELISA	Chiron ELISA
laboratory	*LVM/RIVM*	*LVO/RIVM*	*LVO/RIVM*
	94/95	*6-14/06/96*	*7-20/06/96*
remarks			Praxis/Chiron materials*
serum no.			
1	25.8	20.6	23.6
2	24.4	38.9	27.4
3	2.7	3.3	4.8
4	40.2	40.4	44.2
5	41.8	37.6	41.8
6	8.6	14.7	26.8
7	64.5	40.5	17.9
8	11.2	16.1	67.0
9	11.5	12.2	10.2
10	19.7	11.5	12.5
11	36.5	42.0	46.7
13	17.6	15.1	68.1
14	x	87.7	108.5
15	99.1	120.6	75.7
16	7.9	6.4	3.6
20	37.7	36.7	38.5
21	35.9	86.7	66.8
22	325.6	459.5	405.3
23	25.5	49.9	71.8
24	18.2	16.0	10.1
25	191.0	155.6	111.8
27	9.6	25.9	11.6
29	125.3	145.6	353.6
30	100.1	140.3	161.6
31	8.1	8.8	26.0
32	101.4	152.1	88.0
33	46.7	68.9	38.7
34	147.2	198.9	108.6
35	8.4	13.5	10.0
36	40.9	55.7	33.0
average [µg/ml]	56.3	70.7	70.4
GMT [µg/ml]	31.0	38.2	38.5

Appendix 4.

Serum sub panel 2. Influence of the coated antigen on the correlation of anti-Hib antibody assays represented as antibody concentrations [µg/ml], average and GMT (n = 14). *Remark for CHIRON competitive ELISA see appendix 1.

assay	Phipps' ELISA	Phipps' ELISA	Chiron ELISA	Chiron ELISA
laboratory	*LVO/RIVM*		*LVO/RIVM**	
	27/6/96		*16/7/96*	
remarks	HbO-HA antigen	HSA-Hib antigen	HSA-Hib antigen	HbO-HA antigen
serum no.				
1	22.7	25.3	32.6	43.9
2	50.2	34.5	30.7	81.1
3	3.6	4.5	6.0	6.0
4	46.9	36.3	43.5	87.6
5	44.2	44.4	53.0	84.4
6	12.7	19.1	29.4	57.0
7	41.0	52.3	38.8	105.4
8	16.5	17.3	59.4	100.6
9	11.7	14.9	11.8	18.5
10	11.0	12.6	13.3	19.3
13	14.7	11.1	56.3	60.7
25	93.9	109.9	174.3	210.8
29	100.6	79.3	308.4	298.9
32	101.5	127.6	199.9	238.9
average [µg/ml]	40.8	42.1	75.5	100.9
GMT [µg/ml]	27.4	28.7	43.6	66.2

Appendix 5.

Serum panel 3. Influence of incubation time, temperature and buffer on the Phipps' ELISA in comparison with anti-Hib antibody assays presented as antibody concentrations, average and GMT [µg/ml] (n = 28). All antibody assays were performed at least in duplicate. [1]The serum- and conjugate incubation time and temperature were changed to 3 hours and 37 °C. [2]The serum- and incubation buffer were changed to PBS / 1% (w/v) BSA / 0.05% (v/v) Tween-20. [3]Combination of [1] and [2]. *Remark for Chiron competitive ELISA see appendix 1.

assay	Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Chiron ELISA	Chiron ELISA	RABA
laboratory	*LVM/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*CHIRON*	*CHIRON*
	94/95	*11/9/96*	*17/9/96*	*20/9/96*	*2-4/10/96*	*13/9/96*	*8-14/11/96*	*13-14/11/96*
remarks			Chiron incubation	Chiron incubation	Chiron incubation[3]	Praxis/Chiron materials*		
			temperature and times[1]	buffers[2]				
serum no.								
2	5.0	2.5	1.5	1.8	2.6	1.5	1.7	2.1
3	101.0	86.8	195.2	108.4	227.5	119.0	125.0	188.8
6	14.0	19.5	21.7	22.1	22.8	18.8	20.6	32.3
9	5.0	4.6	5.9	11.6	21.0	33.0	13.5	29.5
10	187.0	90.1	184.2	235.9	435.9	307.5	263.3	673.6
13	23.0	19.5	15.4	17.8	20.3	17.6	23.4	31.1
15	10.0	10.7	8.5	14.2	14.7	5.8	17.6	31.0
17	10.0	5.7	5.7	14.1	20.1	17.9	13.5	28.6
26	10.0	24.2	6.9	52.8	17.1	8.7	32.2	30.4
28	6.0	5.7	14	5.4	9.85	6.0	3.8	11.2
32	11.0	9.3	10.9	12.5	18.4	10.2	15.0	26.9
41	28.0	16.1	24.5	21.3	24.6	8.7	23.0	25.6
42	3.0	4.5	3.3	7.1	8.3	5.9	6.3	11.5
43	143.0	141.7	232.5	153.9	232.9	165.0	133.9	283.9
50	48.0	29.9	45.2	72.9	94.2	39.2	48.8	168.5
53	18.0	19.5	22.9	28	63.8	27.8	30.7	73.6
54	15.0	15.0	23.1	25.8	50.6	21.5	28.2	55.0
57	2.0	2.0	2.1	2.0	3.4	2.4	1.1	2.2
58	32.0	20.8	41.1	23.4	53.6	23.5	24.0	49.8
62	15.0	18.2	15.6	24.5	22.6	8.5	30.5	31.7
65	10.0	7.3	13.2	10.7	15.0	9.7	17.0	33.8
66	191.0	257.8	362.7	250.8	415.6	312.5	428.9	842.1
69	11.0	13.1	7.4	16.8	14.5	10.9	24.5	35.6
72	8.0	8.9	4.9	12.9	8.7	8.2	16.8	28.2
73	26.0	15.3	19.8	22.8	24.5	17.7	n.d.	n.d.
75	85.0	152.9	162.3	134.7	108.8	157.5	160.8	242.1
77	189.0	150.2	280.8	155.1	191.3	160.4	167.6	283.2
83	8.0	7.8	7.5	9.3	10.8	11.0	13.7	24.4
average [µg/ml]	43.4	41.4	62.1	52.5	76.9	54.9	62.4	121.3
GMT [µg/ml]	19.2	17.8	20.3	24.2	31.0	20.3	25.7	45.5

Appendix 6.

Serum panel 4. Comparison of anti-Hib antibody assays represented as antibody concentrations, average and GMT [µg/ml] (n = 96). All antibody assays were performed in duplicate. [1]For remark Phipps' ELISA see appendix 5. [2]As [1], but the standard Polysorp microtiter plate and the Caltag conjugate were replaced by a Maxisorp microtiter plate and Sigma conjugate. *Remark for Chiron competitive ELISA see appendix 1.

assay		Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Chiron ELISA	Chiron ELISA	Chiron ELISA	RABA
laboratory		*LVM/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*CHIRON*	*CHIRON*
		94/95	*20-21/3/97*	*24-26/3/97*	*28-29/4/97*	*1-3/4/97*	*27-28/3/97*	*4/97*	*4/97*
remarks				Chiron incubation[1]	Chiron method[2]	Praxis/Chiron materials*	Chiron materials		
serum no.									
1	1	0.21	0.24	0.41	0.84	0.05	0.05	0.05	0.07
2	2	3.3	2.0	2.5	1.1	3.9	0.8	0.60	2.5
3	4	7.3	5.1	29.2	10.6	14.5	18.3	8.6	18.5
4	1	0.08	0.05	0.38	0.16	0.05	0.05	0.05	0.06
5	2	0.25	0.34	0.59	0.24	0.05	0.05	0.05	0.12
6	1	0.15	0.05	0.14	0.20	0.05	0.05	0.05	0.06
7	2	0.97	2.2	1.8	2.6	0.61	0.97	1.7	3.1
8	3	0.45	1.1	1.2	0.73	0.53	0.15	0.26	0.75
9	4	27.8	22.8	64.0	41.6	58.0	64.3	38.0	112.2
10	2	5.8	8.7	9.5	8.2	5.4	10.1	14.3	14.5
11	3	0.83	1.1	0.78	1.3	0.05	0.23	0.56	0.59
12	4	42.0	27.0	33.5	38.4	28.5	70.9	49.8	87.0
13	1	0.14	0.32	0.31	0.35	0.05	0.05	0.05	0.07
14	2	10.1	2.8	3.7	3.7	3.5	4.8	5.2	3.8
15	4	37.0	83.8	168.2	116.9	119.0	203.8	85.1	267.3
16	1	0.11	0.31	0.10	0.29	1.0	0.05	0.20	0.07
17	2	3.3	20.7	19.6	22.0	8.9	31.0	37.1	27.4
18	2	1.8	4.4	4.7	6.4	4.0	6.8	9.2	4.0
19	4	3.6	16.3	29.7	33.7	27.0	51.6	34.4	38.4
20	1	0.09	0.30	0.07	0.23	0.05	0.05	0.05	0.07
21	2	0.25	0.71	0.26	0.42	0.73	0.05	0.05	0.09
22	3	0.18	1.6	1.3	1.5	1.5	0.36	0.66	1.2
23	4	2.5	3.3	3.3	3.5	3.1	4.8	2.4	4.5
24	1	0.03	0.15	0.11	0.28	1.6	0.05	0.05	0.06
25	2	17.3	22.0	32.0	30.1	14.4	51.3	33.5	36.3
26	3	3.6	5.7	6.1	5.2	5.5	7.1	5.2	5.7
27	4	26.5	33.5	47.9	35.2	48.2	65.7	32.5	74.9
28	1	0.03	0.05	0.05	0.20	0.05	0.05	0.05	0.09
29	2	0.31	0.48	0.29	0.44	0.05	0.05	0.05	0.11
30	3	4.1	4.7	4.7	9.7	4.4	9.6	10.5	7.9
31	1	0.07	0.88	0.45	0.79	2.5	0.05	0.14	0.13
32	4	25.8	33.1	40.6	43.4	62.9	77.5	43.0	73.8
33	1	0.08	0.11	0.11	0.22	0.05	0.05	0.05	0.08
34	4	6.6	4.7	4.7	6.2	8.3	14.2	11.1	14.1
35	2	0.22	1.7	1.2	1.1	0.05	0.30	1.3	1.5
36	4	3.4	3.1	2.0	4.2	3.3	7.4	2.2	6.4
37	1	0.2	0.26	0.18	0.4	1.0	0.05	0.05	0.07
38	2	0.23	0.21	0.18	0.32	0.05	0.05	0.05	0.10
39	3	12.5	5.3	3.5	6.0	5.6	9.5	5.7	8.6
40	1	0.05	0.05	0.05	0.16	1.3	0.05	0.05	0.06
41	2	8.1	7.6	6.9	7.3	1.0	11.5	13.4	9.9
42	3	0.55	1.1	0.49	0.81	2.6	0.27	0.31	0.26
43	4	3.3	3.0	2.6	2.9	2.0	3.3	3.9	3.5
44	3	1.0	1.9	1.1	2.2	1.1	1.0	0.98	2.4
45	4	61.7	14.2	24.8	32.8	16.3	67.6	38.7	100.8
46	1	0.14	0.57	0.08	0.26	0.05	0.05	0.05	0.10
47	2	0.51	2.5	1.6	2.5	0.78	1.1	2.3	2.9
48	3	1.0	1.5	1.5	2.9	1.8	2.9	1.7	2.2
49	4	20.1	13.7	20.9	17.7	29.4	39.7	25.4	50.9
50	2	1.3	1.7	1.6	1.2	0.91	0.58	1.2	1.7
51	4	29.1	23.3	32.3	28.7	19.8	53.8	32.4	61.7
52	1	0.25	0.40	0.20	0.43	0.05	0.05	0.25	0.17

Appendix 6 continues on next page

assay		Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Phipps' ELISA	Chiron ELISA	Chiron ELISA	Chiron ELISA	RABA
laboratory		*LVM/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*LVO/RIVM*	*CHIRON*	*CHIRON*
		94/95	*20-21/3/97*	*24-26/3/97*	*28-29/4/97*	*1-3/4/97*	*27-28/3/97*	*4/97*	*4/97*
remarks				Chiron incubation[1]	Chiron method[2]	Praxis/Chiron materials*	Chiron materials		
serum no.									
53	4	14.5	17.3	16.3	21.6	38.4	16.1	25.0	20.9
54	1	0.43	1.3	0.86	0.76	0.39	0.20	0.47	0.51
55	2	0.4	1.3	2.1	1.7	0.05	0.74	1.0	4.8
56	4	3.2	5.7	11.9	8.0	11.8	8.9	7.0	28.7
57	2	0.22	1.8	1.9	1.6	0.67	1.1	1.8	5.0
58	1	0.06	0.40	0.35	0.32	0.05	0.05	0.2	0.22
59	2	3.7	3.0	4.9	3.8	1.3	5.3	5.1	3.0
60	3	12.0	11.3	14.5	8.9	3.6	16.4	12.1	29.8
61	1	1.3	1.4	1.1	0.78	0.28	0.74	0.5	0.51
62	3	3.2	2.1	2.6	2.3	1.4	3.2	2.1	3.3
63	4	22.6	7.9	9.1	6.5	11.1	30.9	6.5	26.9
64	1	0.04	0.11	0.05	0.17	0.53	0.05	0.05	0.06
65	3	7.6	4.4	5.3	5.4	4.1	9.0	4.5	8.3
66	4	14.2	29.3	27.7	29.4	33.3	56.4	25.0	75.3
67	1	0.07	0.64	0.50	0.51	0.05	0.05	0.2	0.12
68	2	1.3	3.7	2.9	4.1	0.70	6.8	3.8	4.3
69	4	6.6	6.4	8.3	7.5	2.8	17.9	4.9	11.3
70	1	0.15	0.21	0.19	0.26	0.05	0.05	0.05	0.09
71	2	2.3	3.2	3.1	2.7	0.59	3.7	4.3	6.7
72	3	1.6	1.4	1.3	1.4	1.3	1.3	0.6	1.7
73	4	22.9	11.6	15.7	14.0	21.0	47.5	15.6	74.1
74	1	0.03	0.05	0.33	0.11	0.05	0.05	0.05	0.06
75	2	11.8	21.2	30.4	17.9	17.5	35.8	18.0	35.8
76	1	0.08	1.3	0.78	0.55	0.05	0.29	0.3	0.55
77	2	1.8	6.8	12.9	9.6	2.2	16.5	20.5	19.6
78	3	0.44	1.3	4.7	2.1	0.56	3.5	1.2	2.8
79	4	18.8	35.4	63.8	33.1	18.0	58.1	39.9	59.1
80	1	0.12	0.19	0.47	0.19	0.52	0.05	0.05	0.09
81	2	0.99	1.1	1.9	1.0	0.86	1.7	1.1	1.8
82	3	0.54	1.7	1.4	0.65	0.70	1.0	0.1	0.79
83	4	10.3	6.0	7.1	4.8	5.1	17.6	6.9	25.8
84	2	5.8	18.4	13.5	11.2	6.7	21.3	20.6	27.0
85	3	1.2	2.2	3.6	2.3	1.8	3.7	2.0	4.1
86	4	9.3	19.5	32.2	17.7	25.5	34.0	21.1	46.3
87	1	0.03	0.05	0.28	0.05	0.05	0.05	0.05	0.06
88	3	0.20	0.68	0.68	0.22	1.1	0.05	0.1	0.28
89	4	12.9	28.1	16.8	19.6	14.2	21.4	39.6	25.3
90	3	0.29	2.0	1.7	1.6	0.72	1.2	1.3	1.3
91	4	57.0	67.4	98.1	71.7	40.9	161.3	93.4	189.2
92	1	0.05	0.05	0.18	0.16	0.05	0.05	0.05	0.06
93	2	0.06	0.05	0.22	0.12	0.05	0.05	0.05	0.06
94	3	0.36	1.5	1.7	1.1	0.66	0.78	0.3	1.2
95	1	1.2	1.2	0.91	0.89	0.05	0.59	1.1	1.1
96	2	1.7	5.2	4.4	4.9	2.9	5.4	5.8	9.8
average [µg/ml]		6.6	7.6	10.9	9.0	8.2	15.7	9.9	18.9
GMT [µg/ml]		1.2	2.0	2.2	2.2	1.2	1.5	1.4	2.2

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