Collaborative Study for the Validation of Serological Methods for Potency Testing of Diphtheria Toxoid Vaccines - Part 2

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ABSTRACT

The study is a contribution to the EDQM's efforts to meet some of the expectations of the 3 Rs: Replacement, Reduction and Refinement of animal assays as proposed by Russell and Burch in 1959 and adopted by the European Union in 1986, and specifically to validate alternative assays to replace, for batch-release purposes, the European Pharmacopoeia (Ph. Eur.) in vivo direct challenge procedures for the potency determination of diphtheria toxoid vaccines. The study results may be used in support of the replacement of the multi-dilution direct challenge procedures in different animal models by a single dilution serology test, where appropriate, and to use sera from the same animals for potency testing of several components in combined vaccines. With regard to the latter, the present study explores the possibility of testing both diphtheria and tetanus toxoid potencies using serum from the same animals.

KEYWORDS

diphtheria vaccine; tetanus vaccine; combined vaccines; potency test; Vero cell assay; ELISA; ToBI; toxin neutralisation; serology; biological standardisation; collaborative study; European Pharmacopoeia; Council of Europe.

1. INTRODUCTION

A collaborative study to assess the relevance and reliability of the functional toxin neutralisation assay (Vero cell assay) and the non-functional Enzyme-Linked Immunosorbent Assay (D-ELISA) for potency testing of diphtheria toxoid-containing combined vaccines was initiated in January 2001. The study was performed under the aegis of the Biological Standardisation Programme and supported by the Council of Europe and the European Commission as an extension of the collaborative study that led to the refinement of the Ph. Eur. *in vivo* direct challenge assays for potency testing of tetanus toxoid vaccines for human use. Both ELISA and toxin-binding inhibition assay (ToBI) were deemed valid methods for routine batch release testing of combined tetanus vaccines [1], although the results of the 2 methods differed somewhat, e.g. for anti-tetanus responses in vaccines containing *Haemophilus influenzae* type b (Hib) component where tetanus toxoid (TT) is used as a carrier of polyribosylribitol phosphate (PRP).

The principal aim of these 2 studies was to explore the possibility of considerably reducing the number of animals used for potency determination of vaccines containing diphtheria and tetanus toxoid components, and to refine the Ph. Eur. potency assays for routine use. In order to reduce the number of animals required for valid statistical calculations of a multi-dilution assay, the present project was designed to investigate whether sera from the same animals could be used for potency determination of both diphtheria and tetanus toxoid components of combined vaccines.

In the present study, as in the previous study on tetanus toxoid [1], the same animals were used for the *in vivo* challenge test and the serological assays in order to avoid bias by individual differences in immune responses when comparing different methods.

Guinea pigs were chosen as the species for immunisation as they are used in Ph. Eur. procedures for potency testing of diphtheria and tetanus vaccines [2,3,4,5] and have previously been used for validation of tetanus potency assays [1]. Mice, although used for potency testing of tetanus vaccines, are insensitive to diphtheria toxin and show great strain differences in the serological responses to tetanus toxoid [3], in particular when the whole-cell pertussis [6] or Hib components [7] are present in combinations.

Furthermore, previous studies [8] indicated that guinea pigs, in contrast to Balb/c and NIH strains of mice, have a similar response to fragment B of diphtheria toxin, harbouring the receptor-binding domain, as man does, and could provide comparable information regarding immunogenicity of vaccines as in clinical trials [9]. The relationship between the potency of tetanus toxoid measured in the guinea pig model and the antitoxin titre induced in infants was reported by Japanese researchers in the 1970s [10]. More-recent studies confirmed that age- and sex-determined differences in the establishment of tetanus antitoxin production observed in guinea pigs were comparable to observations made on the induction of response in humans [11].

To allow the interim evaluation of test results and to monitor study progress, the study was divided into 3 consecutive phases. The pre-validation (Phase I) study was performed in 2 laboratories to verify protocols and select the optimal vaccine dilutions for immunisation of guinea pigs that would allow potency testing by challenge and serological methods. The results from the Phase I study [12] indicated that comparable diphtheria toxoid potency estimates were obtained in the Ph. Eur. direct intra-dermal challenge assay in guinea pigs, in the Vero cell assay and in D-ELISA for 5 vaccines of different potencies. The correlation between the challenge and the Vero cell assays corresponded to that between the challenge and D-ELISA, confirming that the antibodies play an important role in protection and that predominantly protective/neutralising antibodies are present in guinea pigs 6 weeks after immunisation. The study also provided preliminary information that sera from the same guinea pigs may be used for potency determination of both diphtheria and tetanus toxoid components of vaccines.

An extension to this study documented the high correlation between antitoxin potencies obtained by the Vero cell, D-ELISA and T-ELISA and neutralising potency as determined by the *in vivo* toxin neutralisation test (TNT) [13].

Sufficient information was obtained to recommend the continuation to Phase II, in which suitability of the Vero cell and D-ELISA methods for potency testing of diphtheria toxoid-containing vaccines were investigated in an additional 5 laboratories [12]. Four laboratories performed the assay by lethal challenge and 1 laboratory carried out the

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intra-dermal challenge assay. All laboratories performed the diphtheria Vero cell assay and ELISAs for both diphtheria and tetanus antitoxins measurement. One laboratory also performed the ToBI assay for tetanus. The correlation coefficient (*r*) between Vero cell assay and D-ELISA ranged from 0.76 to 0.91 in different laboratories. The correlation between diphtheria serological assays and lethal challenge assays were confirmed satisfactory as ca. 90 per cent of serum estimates led to a correct prediction of mortality. All laboratories found identical ranking of the vaccines in all serological assays and in the valid challenge assays. The ranking order was identical to assumed/provided potency for the highest and the lowest vaccine.

Combined vaccines used in this study, containing an inactivated poliovirus vaccine (IPV) component in addition to DTaP, did not always meet the present Ph. Eur. requirements for diphtheria in the serological assays, and it was proposed to investigate further in Phase III whether this is a general feature of combinations with IPV.

As the vaccine doses were optimised for the diphtheria component, serum anti-tetanus toxoid/toxin activities varied widely between the vaccines, making it difficult to apply a parallel-line model to calculate exact potencies. However, the dose levels used showed a clear regression and good linearity in general. The Phase I/II studies confirmed a considerably smaller effect of Hib component on anti-tetanus response than previously reported for mouse challenge assays [7], and indicated possible differences between T-ELISA and ToBI assays in detecting antibodies to TT carrier protein in Hib-containing combined vaccines [12], which requires further investigation.

The reliability of the serological assays was investigated in the Phase III study by obtaining information on repeatability and reproducibility. The suitability of using serum from the same animals for serological assays of both diphtheria and tetanus toxoid components was also examined for several multi-component vaccines currently marketed in Europe.

2. PARTICIPANTS

Initially, 28 laboratories agreed to participate in the Phase III study, but 2 laboratories had to withdraw at a later stage. Of the 26 participants, 8 represented manufacturers and 18 the public health sector (see section 7 for details). In this report, each participant is identified by a code number (1-26) allocated at random and not corresponding to the order of listing in section 7. The majority of participants were from Europe with representations from Austria, Croatia, Belgium, Denmark, Germany, Italy, Finland, France, the Netherlands, Norway, Poland, Portugal and the United Kingdom. The study also included participants from Australia, Canada, Brazil, India and the United States of America.

For ease of presentation of the data, the 26 laboratories were coded into 2 groups depending on the panel of serum that was provided for testing and on the choice of assays that they agreed to perform. Laboratories 1 to 8 agreed to carry out the ToBI assay and were provided with serum panel 2, whereas all other laboratories were provided with serum panel 1 (see section 3.1 for details).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Sera and vaccines

Table 1 provides a summary of serum codes and vaccines from which they were produced for this study.

The 13 currently marketed vaccines from which the sera originated were assigned vaccine codes A to M. Manufacturers donating the vaccines and sera were identified with Roman numeral codes I to IV. When sera were produced at one of the organisers' sites, the manufacturer code was followed by the letter 'a'. Four vaccine doses, or 3 in one case, with 2.5 or 3.0 dilution steps were used for immunisation. The 1st vaccine dose varied and an undiluted dose was used in diphtheria vaccines with reduced antigen content (such as vaccines for adults and adolescents) whereas a 14-fold dilution was used in vaccines with whole-cell pertussis (wP). Most other combinations were immunised starting from ca. 5- or 10-fold dilutions for the 1st dose. Sera were from animals bled after 6 weeks, except those produced by 1 organising laboratory, coded a, where animals were bled after 5 weeks.

A panel of 55 samples was used in the Phase III study (panel 1). Because the ToBI assay requires a larger volume of serum, laboratories that agreed to perform the ToBI assay were provided with only 47 samples (panel 2, excluding S17-S20 and S29-S32). Code N was used to refer to sera S56-S59, prepared from a single pool obtained by immunisation of guinea pigs with 10 IU of Diphtheria Toxoid, Adsorbed, 3rd IS/Ph. Eur. BRP batch No. 3 [4] and 15 IU of Tetanus Toxoid, Adsorbed, 3rd IS/Ph. Eur. BRP batch No. 2 [5] by serial 2-fold dilution with normal guinea pig serum. The purpose of including these 4 samples was to confirm how well each method could discriminate between exactly 2-fold diluted sera.

3.1.2. Reference standards and reagents

Positive control guinea pig (GP) reference serum

Guinea pig serum, Diphtheria and Tetanus Antitoxin, NIBSC Code No: 98/572, (GP) with a mean estimate of 3 IU/ampoule for diphtheria antitoxin and 3.5 IU/ampoule for tetanus antitoxin, both measured by *in vivo* toxin neutralisation methods.

(GP: A homologous, stable guinea pig reference serum has been prepared for the purpose of the collaborative study by NIBSC. It was produced using liquid formulations of IS/Ph. Eur. standards [4,5] for immunisation. This reference was calibrated in 5 laboratories by *in vivo* toxin neutralisation method in guinea pigs and mice against the Diphtheria antitoxin (DI 98, 97/762) and TE-3 (I.S. for tetanus antitoxin, human), respectively.)

Diphtheria toxins for Vero cell assay

- 1st International Reference Reagent for Diphtheria (SCHICK) test toxin, NIBSC Code: STT, containing 900 IU/ampoule (0.9 Lf/ampoule and 20,000 Lr/ampoule), or
- Diphtheria Toxin Aventis FA16723 containing 400 Lf/ml, or
- In-house Diphtheria Toxin used in in-house validated Vero cell assay procedure.

D-ELISA critical provided/recommended reagents

- Diphtheria toxoid (NIBSC 02/176) at 900 Lf/vial to be used at 0.5 Lf/ml for coating of ELISA plates
- Positive control guinea pig reference serum (NIBSC 98/572)
- Negative control guinea pig serum (EDQM 02/11-71 or NIBSC 98/686)
- Goat anti-guinea pig IgG (Sigma A 7289 or equivalent) or
- Rabbit anti-guinea pig IgG (Sigma A 5545 or equivalent)

T-ELISA critical provided/recommended reagents

- Tetanus toxoid from Aventis lot no. FA 065598 liquid form at 10,000 Lf/ml (EDQM 03/10-68) to be used at 0.5 Lf/ml for coating of ELISA plates (NIBSC 02/126)

Table 1 - *Overview of sera and the vaccines from which they originate*

Vaccine code	Components	Manufacturer code	Potency† Diphtheria	Potency† Tetanus	First vaccine dilution	Dilution step	Respective serum codes
A	D T	I	20	41	1/2	2.5	S01 S03 S02 S04
B	D T aP HepB IPV	II	73	163	1/10	2.5	S05 S06 S07 S08
C	d T	III	8 (4-16)	78 (57-105)	1/1	2.5	S09 S10 S11 S12
D	D T aP HepB IPV Hib*	II	102 (79-173)	206 (154-278)	1/10	2.5	S13 S14 S15 S16
E	D T	IV	63	126	1/10	2.5	S17 S18 S19 S20
F	D T aP HepB Hib* IPV	III	44 (33-60)	833 (469-1249)	1/5.5	2.5	S21 S22 S23 S24
G	D T aP IPV Hib*	III	56 (36-82)	353 (234-542)	1/10	2.5	S25 S26 S27 S28
H	dT	IV	10	63	1/1.81	2.5	S29 S30 S31 S32
I	D T wP	IIa	50 (36-71)	337 (241-473)	1/14	2.5	S33 S34 S35
J	D T aP	IIa	84 (62-135)	120 (83-177)	1/5	3	S36 S37 S38 S39
K	D T aP IPV	IIa	68 (62-135)	98 (66-147)	1/5	3	S40 S41 S42 S43
L	D T aP IPV	IIIa	46 (35-89)	54 (66-147)	1/5	3	S48 S49 S50 S51
M	D T aP IPV Hib*	IIIa	41 (31-55)	179 (119-268)	1/5	3	S52 S53 S54 S55

† Potencies are in mean IU/0.5 ml with 95% confidence intervals given in brackets. The dilution of the highest dose is listed together with the subsequent dilution steps

* Hib component corresponding to tetanus toxoid-polyribosylribose (PRP) conjugate (Hib-TT)

- Positive control guinea pig reference serum (NIBSC 98/572)
- Negative control guinea pig serum (EDQM 02/11-71 or NIBSC 98/686)
- Goat anti-guinea pig IgG (Sigma A 7289 or equivalent) or
- Rabbit anti-guinea pig IgG (Sigma A 5545 or equivalent)

ToBI critical provided/recommended reagents

- Tetanus toxin (lot T445, RIVM) at 200Lf/ml to be used at 0.1 Lf/ml
- Positive control guinea pig reference serum (NIBSC 98/572)
- Negative control guinea pig serum (EDQM 02/11-71 or NIBSC 98/686)
- Horse anti-tetanus (lot GTL 34 RIVM), at 200 IU/ml to be used at 1.0 IU/ml
- Horse anti-tetanus peroxidase (lot 32-33, RIVM) to be used at 1/4000

3.2. Methods

Each participating laboratory was provided with either 55 (panel 1) or 47 (panel 2) test sera. Critical reagents and controls required for serology assays were provided (or a commercial source was recommended) together with a protocol and Standard Operating Procedures for methods that had to be performed.

Participants were expected to perform:

- Vero cell toxin neutralisation assay (Vero cell assay)
- ELISA for diphtheria serology (D-ELISA)
- ELISA and/or ToBI for tetanus serology (T-ELISA and/or ToBI)

Two independent assays with each method were required. Results were to be reported to the EDQM on provided electronic data sheets. Detailed standard operating procedures were provided, but available in-house methods could be used for the Vero cell assay provided that comparable sensitivity was confirmed.

3.2.1. Vero cell assay

2 procedures of the Vero cell assay were described in the protocol, which can be obtained from the EDQM. Briefly, the methods were based either on the publication by Miyamura *et al.* [14], relying on metabolic inhibition as the end point and on visual (colour) inspection of the cultures, confirmed by cell morphology; or relying on cytotoxicity as the end point [15] and the addition of thiazolyl blue dye [MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] for visual detection [16,17]. The limit of detection for guinea pig positive control serum (NIBSC 98/572) was confirmed as 0.05 IU/ml and 0.015 IU/ml for these methods, respectively [12].

Participants were requested to use 1 of the 2 provided procedures or, provided it had demonstrated suitable sensitivity, their in-house validated method.

In all the methods, the end point was taken as the highest serum dilution protecting the cells from the fixed concentration of diphtheria toxin. The antitoxin activity was calculated with respect to GP reference serum and expressed in IU/ml. Whenever the next well showed

partial neutralisation, the relative activity was corrected by half a dilution-step. In serum titration rows where inversion was observed (i.e. lower concentrations showing full neutralisation whereas at least 1 higher concentration showed no neutralisation), no activity was calculated for that serum sample. For laboratory 3, reporting OD-readings rather than negative and positive readings, the EDQM classified the readings as 'protected', 'partially protected' or 'not protected' by subdividing the range between the maximum and minimum readings in 3 equal intervals.

3.2.2. ELISA

The methods used for ELISA titration of diphtheria and tetanus antitoxin in guinea pig sera were essentially as described elsewhere [1,7,12]. Individual antitoxin titres were calculated with respect to GP reference and expressed in IU/ml.

Activities were calculated by fitting logistic curves to the data using non-linear least-squares techniques (PROC NLIN, The SAS System). Four parameters were estimated to characterise the standard curve, and 1 parameter per sample to characterise the horizontal distance between the curves appearing on the same plate [18]. In cases where the algorithm failed to converge, it was first attempted to force convergence by selecting an optimal convergence path by eye. If this still did not work due to 1 sample being on the edge of the space of convergence (e.g. close to 0), this parameter was eliminated, and the procedure repeated with the remaining data.

For laboratory 11 there were not sufficient readings in the upper part of the curve for T-ELISA to estimate the upper asymptote from the data. After close examination of the data it was decided to fix the asymptotes at 0.000 and 2.000 for all assays from this laboratory.

3.2.3. ToBI

The method used for titration of tetanus antitoxin in guinea pig sera was as published elsewhere [1,7] and activities were calculated in an identical way as for D-ELISA and T-ELISA.

4. RESULTS AND DISCUSSION

Results from a total of 1,177 micro-titre plates were submitted, representing more than 100,000 individual readings. Most laboratories carried out at least 2 assays of a given method, as requested. Additional assays were usually performed with a subset of sera if the dilution range was not optimal. Laboratory 9 submitted only 1 Vero cell assay, due to lack of time. Laboratory 14 did not carry out the Vero cell assay, laboratory 1 did not carry out T-ELISA and laboratories 2, 7 and 8 (which received the 47 samples panel) did not carry out the ToBI assay. Laboratory 6 carried out only 1 ToBI assay.

4.1. Vero cell assay

Twenty-five laboratories carried out the Vero cell assay on at least 2 occasions, and 1 (laboratory 9) performed only 1 assay. Results from 479 micro-titre plates were submitted.

The cell control wells were as expected on all plates in all laboratories except in laboratory 8 and in 1 well on 1 plate in laboratory 2. Laboratory 8 commented that "under the microscope a degenerating, detaching monolayer was recognised and after MTT addition and sodium dodecyl sulphate (SDS)-lysis no reduction of MTT was revealed". A partial toxic effect on the cells in all serum wells was observed in the 1st assay. The 2nd assay was considered suitable for calculations although the control cells were not as expected for a valid test. The unexpected inferior quality of the plates may be due to the use of an alternative extraction buffer containing less-pure SDS.

The negative serum control showed no protection in any of the wells except in 1 well on 2 different plates in laboratory 13, where partial protection was observed. As the quality of the rest of the plates did not seem to be affected, the results from these 2 plates were used for calculations.

Laboratory 12 reported that, based on visual inspection (colour), the control wells for the respective sera, containing cells and no toxin, showed partial toxicity of the cells for all test serum samples and indicated that neat serum may have had a toxic effect on the cells in the in-house Vero cell assay. Since the wells with neat test serum, containing toxin, do not reveal partial toxicity after microscopic examination, it is suspected that neat sera could interfere with the in-house viable-cells indicator used by Laboratory 12. Laboratory 4 observed partial toxicity in the wells containing cells, serum sample 8, 16 or 43 and no toxin. As the quality of the rest of the plates did not seem to be affected, this partial toxicity did not lead to exclusion of any of the sera from further calculations.

Even the highest concentration of toxin, in the toxin titration column, did not kill the cells on 2 plates in laboratory 24, whereas the lowest concentration of the toxin killed the cells on some plates in laboratories 2, 4, 8 and 12 (1 plate) and partially killed the cells on most plates in laboratories 15 and 26.

Despite the above reported problems with the various controls, no results, except assay 1 of laboratory 8, were excluded from further calculations, unless the test sera themselves showed inversion or other irregularities.

Five to 8 laboratories reported problems in assays with serum samples S30-S32 and 2 laboratories experienced problems with serum sample S29. Additional tests by the organising laboratories revealed that samples S30-S32 could have been contaminated *a priori* with up to 4 different bacteria. Bacterial contamination most likely contributed to irregular results in some of the laboratories, regardless of the Vero cell method used. For routine control, analysis of contaminated serum samples are generally not accepted when using a method that promotes bacterial growth over 5-6 days. However, for the specific purpose of this study, all the results have been included in subsequent analysis because the influence on the pooled results was negligible.

Calculated activities for laboratory 17 were much lower than for other laboratories, despite acceptable sensitivity of assay with GP reference and proper behaviour of controls. The laboratory was asked if this could be due to a mistake in the pre-dilution reported for the reference serum, but no conclusive answer has been received.

For each serum and each laboratory the geometric means of the calculated activities from the 2 assays together with the overall medians are listed in Table 2. To enable visual evaluation of intra-laboratory variation, the following convention was used. If the difference between the 2 assays was 2.83-fold (1.5 dilution step), the results were printed on a grey background. If the difference was 4-fold or more (2 dilution steps or more), the results were printed on a black background. (Cases in which only 1 assay was available are clearly marked with an asterisk.) The difference between the assays varied on average from 1.03-fold in laboratory 11 to 1.91-fold in laboratory 10, and was on average 1.39-fold for all laboratories. These results confirm the findings of the Phase II study, where it was concluded that a reasonable target for repeatability between the 2 assays should not be more than 2.83-fold. The Phase III study showed that many of the participating laboratories may set this target at 2-fold.

Although the results vary considerably from laboratory to laboratory, the data gives reasonable median dose response relationships, as illustrated in Figure 1, where a graphical impression of the distribution of results for all vaccines is given. The results are shown as box-and-whisker plots per serum (see Annex for explanations). They are grouped by

vaccine and in order of descending activity per serum. A clear regression can be observed for each vaccine although the shape of the curves can be very different from one another. It can also be seen that, although there are quite a lot of 'outlying' results, in general 50 per cent of the laboratories differ from each other by not more than a factor of 3-4.

4.2. D-ELISA

Twenty-five laboratories carried out the D-ELISA. Results from 303 micro-titre plates were submitted. Whenever more than 2 assays were carried out, in order to optimise pre-dilutions, the 1st assays were regarded as preliminary assays. Only 2 assays with the optimal serum dilution range were used for further calculations.

The quality of fit was in general good with correlation coefficients *(r)* above 0.99 in 95.7 per cent of the assays, above 0.995 in 79.2 percent of the assays and above 0.999 in 13.5 per cent of the assays.

For each serum and each laboratory the geometric means of the calculated activities from the 2 assays are listed together with the overall medians in Table 3. The following convention was used. If the difference between the 2 assays was between 2.38-fold and 3.36-fold (1.25 to 1.75 dilution step) the results were printed on a grey background, and if the difference was more, the results were printed on a black background. These criteria have been used to enable visual evaluation and a fair comparison with the results of the Vero cell assay in terms of intra-laboratory variation. If the methods had equal repeatability, the same amount of grey and black cells should appear in the tables. It is very clear, however, that ELISA is of superior repeatability. The difference between the assays varied on average from 1.09-fold in laboratory 14 to 1.70-fold in laboratory 24 and was on average 1.23-fold. This confirms the conclusion of the Phase II study that a reasonable target for intra-assay repeatability would be not more than 2-fold.

A graphical impression of the results is given in Figure 2. As observed by the Vero cell assay a clear regression can be observed for each of the vaccines. Although the shape of the curves can be very different from one another, it is noteworthy that, by comparing Figures 1 and 2, the curves appear to have a rather similar shape and extension by both methods. For example, vaccine C extends by both methods over a smaller range than any of the other vaccines. It is also evident that reproducibility is much better with D-ELISA than with the Vero cell assay. The results of most laboratories do not differ from each other by more than a factor of 2-3, whereas a factor of 3-4 was observed by the Vero cell assay.

4.3. T-ELISA

Twenty-four laboratories carried out T-ELISA. Results from 321 micro-titre plates were submitted. Many laboratories performed additional assays, either at the request of the project leaders or at their own initiative, because the recommended pre-dilution for some sera was not optimal. Only the 2 assays with the most optimal serum dilution range were used for further calculations.

The quality of fit between the standard curve and the respective samples was in general good, with correlation coefficients (*r*) above 0.99 in 93.0 per cent of the assays, above 0.995 in 79.6 per cent of the assays and above 0.999 in 25.7 per cent of the assays.

For each serum and each laboratory the geometric means of the calculated activities from the 2 assays are listed in Table 4a together with the overall medians. The same criteria as for D-ELISA were used to print results on a grey or black background. The differences between the assays varied on average from 1.05-fold in laboratory 14 to 1.59-fold in laboratory 10, and were on average 1.20-fold. Poor repeatability was mainly observed in laboratories 10 and 19. It was noticed that the large differences in laboratory 19 only occurred for sera with a 400-fold pre-dilution, which may indicate a technical error.

A graphical impression of the results is given in Figure 3. A clear regression can be observed for each vaccine. This is an important observation because the vaccine doses were optimal for the diphtheria toxoid component, and may not necessarily be optimal for the tetanus component. Results are in line with what was observed in the Phase II study, and it strongly supports the idea that the same sera can be used to determine the potency of both components, possibly even in a single-dose assay. It can also be observed that the results of most laboratories do not differ from each other by more than a factor of 2-3.

4.4. ToBI

Five laboratories carried out the ToBI assay, 1 of which (laboratory 6) carried out only 1 assay. Results from 74 micro-titre plates were submitted for calculation.

Plates including sera S49-S59 in laboratory 4 were, in both assays, of such poor quality that it was not possible to calculate activities. The quality of fit was less good than with T-ELISA with correlation coefficients (*r*) above 0.99 in 68.6 per cent of the assays, above 0.995 in 28.6 per cent of the assays and above 0.999 in none of the assays.

For each serum and each laboratory the geometric means of the calculated activities from the 2 assays are listed in Table 4b together with the overall medians. The same criteria as for D-ELISA and T-ELISA were used to print results on a grey or black background. The difference between the assays varied on average from 1.13-fold in laboratory 5 to 1.55-fold in laboratory 4, and was on average 1.34-fold.

A graphical impression of the results is given in Figure 4. Because of the low number of laboratories having performed this method, no box-and-whisker plots are shown, but rather all individual results and their median. Again, a clear regression can be observed for each of the vaccines. The shape of the respective curves in Figure 4 resembles that of the curves in Figure 3. The results of most laboratories do not differ from each other by more than a factor of 2-3.

4.5. Agreement between serological methods

In order to investigate the agreement between the various methods, 2-way plots were generated. For each serum the median outcome of all laboratories were plotted.

4.5.1. Vero cell assay and D-ELISA

Agreement between the Vero cell assay and D-ELISA is illustrated by a two-way plot, using the median outcome of all laboratories (Figure 5). The axes are shown on a $\log_2$ scale so that every unit represents a 2-fold difference. Each serum is indicated by the vaccine code from which it was produced. The most striking feature is that almost all points are located above the diagonal line of agreement, indicating that D-ELISA gives higher outcome than the Vero cell assay. The degree of disagreement varies for the respective vaccines. As a measure of agreement the antilogarithm of the following quantity was calculated:

$$\sqrt{\frac{1}{3}\sum_{i=1}^{3}\left(\ln\left(m_i\right)-\ln\left(n_i\right)\right)^2}$$

where m_i is the median result of the Vero cell assay with respect to dose i, n_i is the median result of the D-ELISA assay

with respect to dose *i*, and *i* indicates each of the 3 highest vaccine doses. This quantity expresses the average absolute factor difference between the methods for a specific vaccine.

Table 5 gives the vaccines ranked in order from closest to furthest agreement. No obvious relationship between the degree of agreement and the number of vaccine components can be identified, although vaccines containing only the diphtheria and tetanus toxoid components may show a better agreement than vaccines containing more components.

It is striking, however, that all sera with the highest degree of disagreement (albeit not statistically significant) were produced at the site of 1 of the project leaders (marked a). This observation may indicate that several factors such as strain, health condition, diet, immunisation technology and others may play a role in determining the ratio of functional to non-functional antibodies. However, the most plausible explanation is the fact that animals in the laboratory of 1 of the project leaders were bled 1 week earlier, i.e. at 5 weeks rather than 6 weeks. Previous studies have confirmed that a longer time post-immunisation narrows differences between apparent functional and non-functional antibody response to diphtheria toxoid in similar vaccine combinations and with equivalent adjuvant [19]. Although antibody titres can be very different for the same vaccine type and dose, immunised in different laboratories by an identical method, vaccine potency will not be different as long as responses to the reference and test vaccine are equally affected.

Previous studies comparing potencies in guinea pig challenge and serology were performed in conjunction with calibration of replacement WHO IS and BRP for diphtheria toxoid [4] and for standardisation of Japanese reference diphtheria vaccine [20]. The studies confirm that comparable potency estimates can be obtained in guinea pig challenge assays as in guinea pig serology, although lower potencies were obtained in ELISA (competition assay) than in toxin challenge and Vero cell methods. Both studies reported higher diphtheria potency estimates in the mouse Vero cell assay model compared to direct challenge in guinea pigs, which were statistically significant [4].

4.5.2. T-ELISA and ToBI

Agreement between T-ELISA and ToBI is illustrated by a 2-way plot, using the median outcome of all laboratories (Figure 6). Axes and vaccine indications are as described for Figure 5. Also in this case almost all points of the T-ELISA assay are located above the diagonal line of agreement, indicating that a broader range of specific antibodies are detected by ELISA than by ToBI. However, the extended part of this collaborative study confirmed excellent correlation ($r = 0.9$) between ELISA and the *in vivo* TNT assay for an independently generated set of samples (n = 20) in 2 different laboratories [13].

In previous studies [1] it was observed that the ELISA/ToBI ratio deviates from 1, and that a statistically significant difference in antitoxin titre may be obtained by ELISA and ToBI. Divergence in titres particularly occurred in the low antitoxin range where ELISA titres tended to be higher than ToBI titres. In the high antitoxin range ToBI titres tended to be higher than ELISA, although some opposite examples were also noted.

In the present study it appears that vaccines with more components tend to give more disagreement than vaccines containing only tetanus and diphtheria toxoid. Since the multi-component vaccines giving the most pronounced disagreement were mostly produced by manufacturer III, it is difficult to presume if this observation is truly component-related or manufacturer-related, i.e. related to the vaccine manufacturing method or the immunisation procedure and strain of animals used and their health and immunological status. It is noticeable, however, that vaccines containing Hib show a significant ($p<0.02$) difference between the 2 methods compared to vaccines not containing this component, even when produced in different laboratories. This observation is in line with that of the previous study [1], where serum P (DTP-Hib) showed a similar discrepancy, whereas serum O (DTP), from the same manufacturer, indicated no difference between the 2 serological methods. Results from the Phase II study [12] also indicate a different ranking order for potency of vaccines C and F in ToBI and ELISA assays. Although antibody titres can be very different for the same vaccine type and dose, immunised in different laboratories by an identical method, vaccine potency will not be different as long as responses to the reference and test vaccine are equally affected. However, comparable potencies in 2 methods for combined vaccines containing Hib-TT component will only be achievable if a product-specific reference is used.

Previous studies comparing potencies in guinea pig or mouse challenge and serology were limited and some information is available from replacement WHO IS and BRP for the tetanus toxoid study [5] and for standardisation of Japanese reference diphtheria vaccine [20]. The latter study confirms that highly comparable potency estimates can be obtained in guinea pig challenge assays as in guinea pig serology.

Unlike results with diphtheria serology, no additional differences were noted for serum provided by 1 organising laboratory and when the animals were bled at 5 weeks instead of 6 weeks.

In the previous study [1] inversion of ELISA and ToBI titres was seen in some cases when using different dilutions of the same vaccine as immunising preparations (e.g. sera A and B). That observation could be explained by the degree of dilution of adsorbed vaccines, the composition of the diluent used, and, in particular, the time interval between the dilution of the vaccine and the immunisation. All these factors may have an impact on the amount and nature of the antibodies induced. Preparation of the vaccines for immunisation for the present study has been performed *lege artis*, and no inversion was seen.

5. CONCLUSIONS

The repeatability and reproducibility of D-ELISA and T-ELISA was generally superior to that of the Vero cell and ToBI assays.

For the tetanus serological potency assay the observations made in this study were essentially in agreement with conclusions made in previous studies [1].

An interesting observation is that earlier bleeding time (5 weeks instead of 6 weeks) may explain larger albeit not statistically significant differences observed between the ELISA and Vero cell methods for diphtheria antitoxins, but this should not affect potency calculation, provided that the response to reference and test vaccines are equally affected.

In general, no obvious relationship between the degree of agreement between Vero cell assay and D-ELISA and the number of vaccine components could be identified for diphtheria, but responses to vaccines containing Hib-TT component measured by T-ELISA and ToBI show significant differences, suggesting a differential ability of the 2 methods for detection of anti-tetanus antibodies to the carrier protein. These observations suggest that a product-specific reference should be used in the serological potency assays.

T-ELISA tended to give systematically higher anti-tetanus titres than the ToBI assay, indicating that a broader range of specific antibodies may be detected by T-ELISA. This was not in line with the previous study, where T-ELISA tended to give higher values than ToBI in the low antitoxin range samples, and the reverse was obtained in the high antitoxin range

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samples [1]. However, additional studies in 2 laboratories confirmed a high correlation between T-ELISA and TNT in the mouse model of functional antibody response for the same type of vaccines, including for a vaccine containing Hib-TT [13].

In summary, the present study successfully explored the possibility of testing both diphtheria and tetanus toxoid potencies using serum from the same animals, and supports the replacement of the multi-dilution direct challenge procedures performed in different animal models by a single-animal-model serological potency test that could, where appropriate, be adapted to a single-dilution serology test.

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8. ABBREVIATIONS

aP: Acellular pertussis; BSP: Biological Standardisation Programme; c.i.: Confidence interval; c.l.: Confidence limit; D-ELISA: ELISA for diphtheria antitoxin; DI: Diphtheria antitoxin, equine, 1st IS; EDQM: European Directorate for the Quality of Medicines; ELISA: Enzyme-linked immunosorbent assay; gcv: Geometric coefficient of variation; GM: Geometric mean; GP: Guinea pig serum, diphtheria and tetanus antitoxin (NIBSC Code 98/572); Hib: *Haemophilus influenzae* type b; IPV: Inactivated poliovirus vaccine; IS: International Standard; MTT: Thiazolyl blue; NIBSC: National Institute for Biological Standards and Control; NoMA: Norwegian Medicines Agency; OMCL: Official Medicines Control Laboratory; PA_{50}: 50 per cent predictive dose; Ph. Eur.: European Pharmacopoeia; PRP: Polyribosylribitol phosphate; RSD: Relative standard deviation; SD: Standard deviation; SDS: Sodium docecyl sulphate; T-ELISA: ELISA for tetanus antitoxin; TNT: Toxin neutralisation test; TT: Tetanus toxoid; US-FDA: United States Food and Drug Administration; Vs.: Versus; WHO: World Health Organisation; wP: Whole-cell pertussis.

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Table 2 - Estimated activities per serum and per laboratory by Verocell assay (in IU/ml)

		Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	Lab 18	Lab 19	Lab 20	Lab 21	Lab 22	Lab 23	Lab 24	Lab 25	Lab 26	Med
Vac A (DT)	S01	0.68	3.84	4.57	0.81	2.72	3.81	0.81	1.92	3.20	3.20	1.92	2.72	1.80	n.p.	1.92	2.69	0.06	3.20	0.68	5.43*	2.72	0.68	1.61	2.28	2.26	2.26	**2.26**
	S03	0.06	1.14	1.92	0.48	1.36	1.60	0.34	.	1.60	0.95	0.96	1.36	1.20	n.p.	0.96	1.35	0.03	1.35	0.34	1.14	0.81	0.29	0.96	1.14	1.90	1.13	**1.14**
	S02	0.12	0.48	0.81	0.17	0.48	0.40	0.12	0.68	0.40	0.67	0.29	0.40	0.30	n.p.	0.48	0.40	0.01	0.57	0.17	0.57	0.40	0.10	0.40	0.40	0.57	0.40	**0.40**
	S04	0.01	0.04	0.08	0.03	0.06	0.06	0.01	0.12	0.05	0.08	0.03	0.04	0.06	n.p.	0.06	0.03	0.00	0.07	0.01	0.04	0.05	0.01	0.03	0.05	0.06	0.04	**0.04**
Vac B (DTaP HepB IPV)	S05	0.68	1.92	3.23	0.48	1.92	2.69	0.96	1.92	3.20	3.20	1.92	2.72	2.40	n.p.	1.92	2.26	0.05	3.20	0.48	2.28	2.28	0.96	1.92	1.61	2.26	2.26	**1.92**
	S06	0.24	0.81	1.36	0.24	0.96	0.80	0.24	0.96	0.80	0.80	0.48	0.68	0.60	n.p.	0.48	0.95	0.03	1.13	0.24	0.68*	0.57	0.17	0.57	0.96	0.80	0.57	**0.68**
	S07	0.12	0.24	0.34	0.10	0.34	0.28	0.17	0.68	0.20	0.24	0.24	0.34	0.30	n.p.	0.24	0.28	0.04	0.40	0.08	0.40	0.29	0.06	0.20	0.57	0.34	0.20	**0.24**
	S08	0.00	0.02	0.02	0.03	0.01	0.02	0.00	0.02	0.01	0.03	0.00	0.01	0.02	n.p.	0.01	0.01	0.02	0.02	0.01	0.01	0.00	0.00	0.02	0.02	0.01	0.02	**0.01**
Vac C (dT)	S09	0.48	0.96	1.61	0.20	1.36	1.60	0.40	1.92	0.80	1.60	0.96	1.36	1.20	n.p.	0.96	1.35	0.03	1.35	0.34	1.61	1.14	0.40	0.57	3.23	0.95	1.13	**1.13**
	S10	0.17	0.57	0.81	0.20	0.68	0.67	0.20	1.36	0.80	0.80	0.48	0.68	0.85	n.p.	0.48	0.67	0.03	0.67	0.17	0.81	0.57	0.17	0.57	0.81	0.80	0.67	**0.67**
	S11	0.12	0.48	0.48	0.10	0.48	0.48	0.12	0.96	0.20	0.48	0.24	0.40	0.42	n.p.	0.40	0.57	0.03	0.57	0.12	0.40	0.17	0.12	0.34	0.81	0.40*	0.40	**0.40**
	S12	0.06	0.24	0.24	0.08	0.24	0.17	0.06	0.68	0.20	0.28	0.12	0.20	0.24	n.p.	0.24	0.28	0.01	0.28	0.06	0.24	0.20	0.06	0.14	0.57	0.28	0.20	**0.20**
Vac D (DTaP HepB IPV Hib)	S13	0.24	0.96	0.96	0.29	0.96	1.13	0.68	0.96	0.80	1.60	0.96	0.96	1.01	n.p.	0.57	0.67	0.03	0.80	0.24	1.14	0.81	0.24	0.81	0.96	0.80	0.80	**0.81**
	S14	0.12	0.48	0.48	0.12	0.48	0.48	0.12	0.48	0.40	0.57	0.48	0.40	0.42	n.p.	0.29	0.34	0.02	0.57	0.17	0.48	0.14	0.10	0.29	0.48	0.40	0.40	**0.40**
	S15	0.02	0.06	0.07	0.03	0.06	0.07	0.02	0.12	0.10	0.14	0.06	0.06	0.06	n.p.	0.08	0.04	0.01	0.12	0.02	0.07	0.07	0.01	0.04	0.07	0.10	0.05	**0.06**
	S16	0.00	0.02	0.02	0.03	0.01	0.02	0.00	0.02	0.01	0.03	0.00	0.01	0.00	n.p.	0.01	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.02	0.00	0.01	0.02	**0.01**
Vac E (DT)	S17									0.80	0.80	0.96	1.36	1.70	n.p.	1.14	1.35	0.06	1.90	0.24	1.14	0.96	0.48	0.81	1.36	0.95	1.13	**0.96**
	S18									0.40	0.48	0.40	0.48	0.85	n.p.	0.48	0.40	0.03	0.95	0.17	0.68	0.34	0.24	0.48	0.57	0.48	0.67	**0.48**
	S19									0.20	0.14	0.24	0.17	0.42	n.p.	0.20	0.24	0.03	0.20	0.12	0.24	0.10	0.07	0.17	0.20	0.20	0.10	**0.20**
	S20									0.01	0.04	0.01	0.07	0.06	n.p.	0.01	0.03	0.01	0.02	0.02	0.01	0.00	0.00	0.04	0.01	0.01*	0.02	**0.01**
Vac F (DTaP HepB Hib IPV)	S21	0.68	2.72	3.23	0.81	1.92	3.81	1.61	1.92	1.60	3.20	1.92	2.72	2.40	n.p.	2.28	2.26	0.06	2.69	1.92	2.28	1.92	0.81	1.92	2.28	1.60	2.69	**1.92**
	S22	0.24	1.14	1.36	0.48	0.96	1.60	0.29	0.96	0.80	1.13	0.81	0.96	0.85	n.p.	0.57	0.80	0.03	1.13	0.48	1.14	0.48	0.29	0.68	0.81	0.80	0.80	**0.80**
	S23	0.06	0.24	0.29	0.12	0.24	0.20	0.08	0.68	0.20	0.40	0.24	0.34	0.17	n.p.	0.24	0.34	0.02	0.48	0.17	0.29	0.12	0.08	0.17	0.34	0.40	0.24	**0.24**
	S24	0.03	0.12	0.12	0.05	0.12	0.12	0.04	0.34	0.10	0.14	0.24	0.12	0.17	n.p.	0.12	0.10	0.01	0.24	0.06	0.14	0.06	0.04	0.08	0.34	0.20	0.12	**0.12**
Vac G (DTaP IPV Hib)	S25	0.17	0.40	0.68	0.29	0.48	0.80	0.20	1.36	0.80	0.80	0.48	0.68	0.30	n.p.	0.48	0.57	0.04	0.95	0.34	0.81	0.40	0.20	0.48	0.68	0.57	0.67	**0.48**
	S26	0.06	0.24	0.24	0.14	0.24	0.28	0.05	0.68	0.20	0.28	0.24	0.34	0.15	n.p.	0.24	0.34	0.02	0.34	0.12	0.29	0.20	0.06	0.17	0.40	0.40	0.24	**0.24**
	S27	0.04	0.06	0.08	0.03	0.12	0.08	0.02	0.24	0.10	0.08	0.06	0.08	0.08	n.p.	0.08	0.06	0.01	0.08	0.06	0.07	0.07	0.02	0.08	0.14	0.17	0.10	**0.08**
	S28	0.01	0.02	0.03	0.03	0.03	0.02	0.01	0.08	0.01	0.04	0.02	0.01	0.02	n.p.	0.04	0.02	0.00	0.04	0.02	0.04	0.03	0.00	0.02	0.04	0.05	0.03	**0.02**
Vac H (DT)	S29									.	0.80	0.96	0.68	0.85	n.p.	0.34	0.48	0.07	0.80	0.34	0.68	0.68	0.12	0.57	1.36	0.95	0.67	**0.68**
	S30									.	0.14	0.48	7.68	0.21	n.p.	0.24	0.48	0.03	.	0.02	0.57	0.00	0.24	1.36	0.68*	0.57	.	**0.36**
	S31									0.05	.	0.08	0.34	0.21	n.p.	0.10	0.10	0.02	.	0.02	0.12	0.00	0.06	0.24	0.20	0.12	.	**0.10**
	S32									0.01	.	0.01*	0.68	0.11	n.p.	0.05	0.08	0.03	.	0.02	0.04	0.00	0.10	0.34	0.14	0.05	.	**0.05**
Vac I (DTwP)	S33	0.24	0.96	1.36	0.40	1.36	1.13	0.24	1.36	0.80	0.40	0.68	1.36	0.85	n.p.	0.48	1.35	0.06	1.35	0.34	0.96	1.14	0.40	0.96	1.14	1.60	0.80	**0.96**
	S34	0.12	0.40	0.57	0.20	0.48	0.57	0.12	0.96	0.40	0.57	0.34	0.48	0.42	n.p.	0.24	0.40	0.03	1.13	0.24*	0.40	0.57	0.17	0.34	0.29	0.57	0.34	**0.40**
	S35	0.00	0.02	0.03	0.03	0.03	0.03	0.01	0.04	0.01	0.04	0.04	0.01	0.03	n.p.	0.02	0.01	0.00	0.04	0.02	0.03	0.06	0.00	0.02	0.04	0.01*	0.02	**0.02**
Vac J (DTaP)	S36	0.96	1.92	3.23	2.28	2.72	4.53	0.68	2.72	1.60	2.69	1.92	3.84	3.39	n.p.	1.43	3.20	0.12	4.53	1.36	3.23	2.28	0.96	1.61	1.61	3.20	2.26	**2.28**
	S37	0.08	0.24	0.40	0.24	0.24	0.34	0.06	0.48	0.10	0.28	0.24	0.24	0.30	n.p.	0.24	0.40	0.03	0.24	0.17	0.29	0.17	0.05	0.24	0.40	0.34	0.24	**0.24**
	S38	0.02	0.04	0.07	0.06	0.06	0.05	0.01	0.12	0.05	0.10	0.06	0.04	0.06	n.p.	0.05	0.06	0.01	0.05	0.03	0.05	0.05	0.01	0.06	0.06	0.08	0.05	**0.05**
	S39	0.00	0.02	0.02	0.03	0.01	0.02	0.00	0.02	0.01	0.03	0.00	0.01	0.00	n.p.	0.01	0.01	0.00	0.01	0.02	0.01	0.00	0.00	0.02	0.01	0.01	0.02	**0.01**
Vac K (DTaP IPV)	S40	0.48	1.36	1.36	0.96	2.72	1.60	0.57	1.92	0.80	3.20	1.61	1.61	1.70	n.p.	0.85	2.26	0.04	1.35	1.36	1.36	1.14	0.57	1.92	1.61	2.26	1.60	**1.36**
	S41	0.24	0.81	0.96	0.81	0.96	1.60	0.29	1.36	0.80	1.13	0.96	0.81	1.20	n.p.	0.68	1.35	0.03	1.35	0.48	1.14	0.96	0.34	0.96	1.36	2.26	0.80	**0.96**
	S42	0.02	0.06	0.08	0.03	0.08	0.07	0.01	0.24	0.05	0.12	0.06	0.04	0.08	n.p.	0.03	0.03	0.01	0.06	0.03	0.07	0.08	0.01	0.06	0.10	0.07	0.04	**0.06**
	S43	0.00	0.02	0.02	0.03	0.01	0.02	0.00	0.02	0.01	0.02	0.00	0.01	0.00	n.p.	0.01	0.00	0.00	0.01	0.02	0.01	0.00	0.00	0.02	0.00	0.01	0.02	**0.01**
Vac L (DTaP IPV)	S48	0.34	1.36	1.36	0.96	1.36	1.35	0.40	1.92	0.80	2.26	1.92	1.14	1.20	n.p.	0.96	1.90	0.04	1.13	0.48	1.14	1.36	0.34	0.68	1.36	2.69	1.60	**1.20**
	S49	0.12	0.48	0.48	0.48	0.48	0.57	0.14	0.48	0.40	0.57	0.48	0.48	0.42	n.p.	0.40	0.40	0.03	0.48	0.12	0.34	0.40	0.10	0.34	0.40	0.95	0.40	**0.40**
	S50	0.02	0.06	0.07	0.06	0.08	0.06	0.02	0.12	0.05	0.07	0.06	0.05	0.08	n.p.	0.08	0.06	0.01	0.04	0.02	0.04	0.06	0.01	0.06	0.04	0.14	0.05	**0.06**
	S51	0.00	0.02	0.02	0.03	0.01	0.03	0.00	0.02	0.01	0.02	0.00	0.05	0.01	n.p.	0.01	0.00	0.00	0.01	0.01	0.01	0.00	0.01	0.02	0.00	0.01	0.02	**0.01**
Vac M (DTaP IPV Hib)	S52	0.24	0.96	1.14	0.48	1.36	1.90	0.48	0.96	0.80	0.80	0.96	1.14	1.20	n.p.	0.60	1.35	0.03	0.80	0.48	0.96	1.14	0.34	0.81	0.81	1.60	0.80	**0.81**
	S53	0.12	0.48	0.68	0.34	0.96	0.80	0.17	0.96	0.40	0.57	0.48	0.48	0.60	n.p.	0.48	0.67	0.02	0.48	0.34	0.48	0.48	0.17	0.48	0.40	0.67	0.48	**0.48**
	S54	0.03	0.12	0.12	0.08	0.12	0.14	0.03	0.17	0.10	0.10	0.12	0.07	0.12	n.p.	0.12	0.04	0.01	0.12	0.04	0.12	0.07	0.02	0.08	0.12	0.17	0.10	**0.10**
	S55	0.00	0.02	0.02	0.03	0.01	0.02	0.00	0.02	0.01	0.02	0.00	0.40	0.00	n.p.	0.01	0.00	0.00	0.01	0.02	0.01	0.00	0.00	0.02	0.01	0.01	0.03	**0.01**
Ref N (R-D and R-T)	S56	0.17	0.68	0.48	0.24	0.68	0.95	0.20	0.96	0.40	0.40	0.48	0.48*	0.21	n.p.	0.36	0.67	0.02	0.95	0.17	0.40	0.57	0.12	0.40	0.81	0.48	0.57	**0.48**
	S57	0.12	0.06	0.07	0.12	0.34	0.28	0.07	0.48	0.20	0.20	0.24	0.29	0.17	n.p.	0.24	0.34	0.02	0.40	0.12	0.24	0.29	0.06	0.17	0.48	0.28	0.24	**0.24**
	S58	0.06	0.12	0.05	0.12	0.17	0.17	0.02	0.24	0.10	0.14	0.12	0.10	0.12	n.p.	0.14	0.12	0.02	0.14	0.08	0.12	0.14	0.03	0.08	0.40	0.17	0.10	**0.12**
	S59	0.02	0.05	0.05	0.06	0.08	0.06	0.01	0.17	0.05	0.05	0.06	0.05	0.06	n.p.	0.08	0.03	0.02	0.08	0.06	0.06	0.06	0.01	0.06	0.10	0.04	0.05	**0.06**

Explanations: Listed are the estimated activities per serum and per laboratory. Each value represesents the geometric mean of 2 assays provided that at least 2 assays were available.
Values that represent only one assay: Laboratory 9 performed only one assay. The quality of assay 1 by laboratory 8 was insufficient for inclusion. Other cases where only one result was available are marked with a *.
Grey boxes indicate that the difference between the 2 assays was 2.83-fold (1.5 dilution step) and black boxes indicate that the difference was 4-fold (2 dilution steps) or more.
n.p. = not performed.
Med = Median value of all laboratories.

Table 3 - Estimated activities per serum and per laboratory by D-ELISA (in IU/ml)

		Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	Lab 18	Lab 19	Lab 20	Lab 21	Lab 22	Lab 23	Lab 24	Lab 25	Lab 26	Med
Vac A (DT)	S01	2.05	2.19	1.96	2.27	1.63	2.06	1.86	1.44	n.p.	1.80	1.69	1.85	2.12	2.04	2.23	1.93	1.97	1.71	2.36	1.84	1.63	1.77	3.60	1.96	2.25	2.20	**1.96**
	S03	0.88	1.36	1.05	1.33	1.35	1.19	1.16	0.63	n.p.	0.95	1.08	1.09	0.93	0.98	1.03	0.92	1.05	1.28	1.39	0.96	0.81	0.81	1.52	0.92	1.31	1.31	**1.05**
	S02	0.39	0.50	0.42	0.57	0.33	0.45	0.53	0.21	n.p.	0.37	0.51	0.44	0.41	0.46	0.46	0.33	0.35	0.42	0.58	0.32	0.34	0.37	0.70	0.38	0.48	0.45	**0.42**
	S04	0.07	0.08	0.07	0.10	0.06	0.08	0.07	0.05	n.p.	0.05	0.03	0.08	0.06	0.08	0.07	0.06	0.06	0.10	0.10	0.05	0.05	0.07	0.09	0.02	0.12	0.09	**0.07**
Vac B (DTaP HepB IPV)	S05	1.39	1.75	1.60	2.58	1.66	1.73	1.59	0.90	n.p.	1.44	1.07	1.76	1.49	1.66	1.44	1.40	1.87	1.91	2.03	1.57	1.21	1.46	2.80	1.77	1.86	2.09	**1.66**
	S06	0.93	1.16	0.98	1.54	0.82	1.21	1.02	0.56	n.p.	0.94	0.72	1.11	0.98	1.00	0.91	0.84	1.12	1.12	1.14	0.95	0.80	0.83	1.72	0.95	1.20	1.16	**0.98**
	S07	0.26	0.34	0.32	0.50	0.27	0.29	0.31	0.15	n.p.	0.30	0.25	0.35	0.24	0.33	0.31	0.23	0.39	0.26	0.38	0.26	0.26	0.25	0.41	0.21	0.36	0.32	**0.30**
	S08	0.03	0.03	0.03	0.05	0.03	0.03	0.04	0.02	n.p.	0.03	0.02	0.03	0.03	0.04	0.03	0.02	0.04	0.03	0.04	0.02	0.02	0.02	0.04	0.00	0.06	0.04	**0.03**
Vac C (dT)	S09	0.90	1.35	1.14	2.22	0.90	1.18	1.18	0.50	n.p.	0.91	1.17	1.34	1.04	1.14	1.23	0.84	0.96	1.13	1.46	1.11	1.23	1.03	1.70	1.60	1.64	1.52	**1.17**
	S10	0.78	1.04	0.83	1.22	0.85	0.87	1.18	0.35	n.p.	0.86	1.05	1.19	0.82	0.96	0.95	0.68	0.67	0.77	1.14	0.62	1.23	0.88	1.09	1.49	1.21	1.10	**0.95**
	S11	0.61	0.74	0.71	0.77	0.77	0.38	0.70	0.50	n.p.	0.70	0.70	0.58	0.65	0.66	0.64	0.51	0.46	0.66	0.59	0.56	0.67	0.61	0.62	0.74	0.78	0.76	**0.66**
	S12	0.29	0.37	0.30	0.49	0.27	0.26	0.38	0.22	n.p.	0.34	0.34	0.21	0.32	0.36	0.35	0.26	0.24	0.29	0.31	0.29	0.29	0.27	0.26	0.28	0.37	0.35	**0.29**
Vac D (DTaP HepB IPV Hib)	S13	0.90	0.92	0.83	1.06	0.60	0.74	0.94	0.44	n.p.	1.03	0.81	0.68	0.82	0.93	0.80	0.68	0.76	0.66	0.77	0.70	0.86	0.71	1.38	0.87	0.88	0.92	**0.82**
	S14	0.53	0.48	0.45	0.57	0.30	0.32	0.56	0.18	n.p.	0.50	0.44	0.33	0.44	0.49	0.43	0.33	0.26	0.33	0.41	0.35	0.42	0.34	0.87	0.55	0.44	0.43	**0.43**
	S15	0.14	0.14	0.14	0.36	0.09	0.10	0.16	0.06	n.p.	0.15	0.11	0.08	0.11	0.17	0.12	0.10	0.07	0.08	0.13	0.09	0.11	0.09	0.13	0.15	0.03	0.13	**0.11**
	S16	0.01	0.01	0.01	0.04	0.01	0.01	0.01	0.01	n.p.	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.04	0.01	0.01	0.01	0.01	0.01	0.00	0.00	0.02	0.01	**0.01**
Vac E (DT)	S17									n.p.	1.72	1.01	1.10	1.14	1.41	1.57	1.01	1.36	0.69	1.46	1.16	1.70	1.29	2.55	1.71	1.74	1.83	**1.41**
	S18									n.p.	0.91	0.80	0.66	0.60	0.82	0.97	0.63	0.71	0.47	0.74	0.69	1.04	0.87	0.98	1.35	1.08	1.09	**0.82**
	S19									n.p.	0.39	0.39	0.25	0.28	0.41	0.41	0.30	0.36	0.29	0.32	0.26	0.38	0.34	0.34	0.45	0.42	0.39	**0.36**
	S20									n.p.	0.06	0.07	0.05	0.04	0.08	0.07	0.05	0.14	0.03	0.05	0.04	0.06	0.07	0.04	0.08	0.09	0.06	**0.06**
Vac F (DTaP HepB Hib IPV)	S21	1.79	2.18	1.82	1.18	1.41	1.91	1.91	0.93	n.p.	2.05	1.62	1.96	2.04	2.03	1.95	1.61	1.51	2.10	2.29	1.85	1.56	1.85	2.73	2.38	1.61	2.11	**1.91**
	S22	1.22	1.27	1.30	0.89	0.97	1.08	1.19	0.49	n.p.	1.14	0.98	1.19	1.14	1.14	1.03	0.79	0.83	1.34	1.29	0.94	0.87	1.27	1.39	1.30	1.08	1.20	**1.14**
	S23	0.52	0.63	0.60	1.73	0.54	0.56	0.59	0.21	n.p.	0.52	0.47	0.53	0.51	0.57	0.53	0.43	0.38	0.47	0.51	0.49	0.44	0.47	0.47	0.55	0.57	0.65	**0.52**
	S24	0.25	0.30	0.27	0.02	0.31	0.16	0.28	0.10	n.p.	0.25	0.18	0.23	0.22	0.28	0.25	0.17	0.17	0.23	0.20	0.21	0.17	0.23	0.17	0.18	0.25	0.29	**0.23**
Vac G (DTaP IPV Hib)	S25	0.75	0.83	0.94	1.45	0.76	0.46	0.86	0.46	n.p.	0.76	0.49	0.67	0.76	0.83	0.78	0.62	0.88	0.73	0.70	0.73	0.58	0.76	1.18	0.76	0.96	0.99	**0.76**
	S26	0.37	0.44	0.32	0.76	0.37	0.34	0.50	0.22	n.p.	0.45	0.30	0.37	0.43	0.53	0.43	0.33	0.44	0.56	0.35	0.33	0.32	0.45	0.55	0.38	0.45	0.48	**0.43**
	S27	0.16	0.19	0.16	0.43	0.13	0.15	0.19	0.10	n.p.	0.22	0.14	0.15	0.16	0.26	0.20	0.15	0.19	0.16	0.16	0.16	0.14	0.18	0.16	0.17	0.21	0.19	**0.16**
	S28	0.06	0.06	0.05	0.14	0.04	0.05	0.06	0.03	n.p.	0.06	0.05	0.05	0.06	0.09	0.07	0.05	0.06	0.05	0.05	0.05	0.04	0.05	0.04	0.04	0.11	0.06	**0.05**
Vac H (DT)	S29									n.p.	0.69	0.76	0.72	0.62	0.80	0.83	0.54	0.99	0.50	0.72	0.63	0.72	0.71	0.82	0.92	0.93	1.05	**0.72**
	S30									n.p.	0.46	0.57	0.38	0.45	0.56	0.49	0.40	0.73	0.40	0.43	0.35	0.48	0.45	0.53	0.65	0.56	0.59	**0.48**
	S31									n.p.	0.14	0.11	0.20	0.18	0.17	0.21	0.16	0.24	0.14	0.19	0.14	0.12	0.20	0.12	0.09	0.28	0.24	**0.17**
	S32									n.p.	0.08	0.06	0.14	0.10	0.12	0.12	0.10	0.53	0.11	0.13	0.10	0.07	0.14	0.07	0.05	0.19	0.16	**0.11**
Vac I (DTwP)	S33	1.19	1.38	0.97	3.65	1.17	1.42	1.56	0.95	n.p.	1.21	1.19	1.43	1.14	1.29	1.66	1.17	1.35	1.18	1.41	1.37	0.87	1.50	1.57	1.21	1.81	1.47	**1.35**
	S34	0.58	0.85	0.34	1.54	0.75	0.59	0.87	0.52	n.p.	0.54	0.58	0.44	0.61	0.67	0.86	0.58	0.67	0.57	0.65	0.64	0.42	0.75	0.71	0.53	0.82	0.89	**0.64**
	S35	0.12	0.15	0.09	0.34	0.12	0.11	0.18	0.08	n.p.	0.11	0.11	0.12	0.12	0.17	0.17	0.11	0.11	0.44	0.14	0.10	0.05	0.14	0.15	0.08	0.19	0.16	**0.12**
Vac J (DTaP)	S36	3.79	4.43	2.99	9.74	3.82	3.15	4.28	2.31	n.p.	3.80	2.10	2.99	3.25	3.40	3.11	3.67	3.89	3.58	4.54	2.90	2.57	4.18	5.17	2.27	4.71	4.35	**3.67**
	S37	1.17	1.20	0.92	1.89	0.92	0.85	1.21	0.42	n.p.	0.85	0.75	0.68	0.89	1.06	0.87	0.85	1.00	0.88	1.21	0.75	0.54	1.03	1.00	0.61	1.32	1.13	**0.92**
	S38	0.47	0.48	0.30	0.78	0.43	0.34	0.57	0.16	n.p.	0.37	0.29	0.29	0.36	0.44	0.36	0.39	0.42	0.48	0.50	0.29	0.17	0.45	0.51	0.20	0.52	0.44	**0.42**
	S39	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.01	n.p.	0.01	0.00	0.01	0.01	0.02	0.01	0.01	0.03	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.02	0.01	**0.01**
Vac K (DTaP IPV)	S40	2.95	2.58	2.56	2.93	3.28	2.75	3.27	2.11	n.p.	2.68	3.23	2.57	2.69	3.18	2.61	2.86	2.87	3.01	3.28	1.81	2.34	2.95	3.53	3.36	4.24	3.18	**2.93**
	S41	1.62	1.79	1.71	2.49	1.79	1.81	2.31	1.31	n.p.	2.26	1.81	1.76	1.90	1.87	1.61	1.74	1.64	1.88	1.88	1.53	1.30	1.90	1.97	2.60	2.72	2.06	**1.81**
	S42	0.35	0.39	0.33	0.64	0.36	0.29	0.49	0.28	n.p.	0.46	0.30	0.32	0.36	0.33	0.34	0.33	0.41	0.39	0.34	0.30	0.14	0.33	0.26	0.33	0.42	0.41	**0.34**
	S43	0.01	0.01	0.01	0.04	0.01	0.01	0.02	0.01	n.p.	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.03	0.01	0.02	0.00	0.00	0.01	0.00	0.00	0.02	0.01	**0.01**
Vac L (DTaP IPV)	S48	3.19	3.30	2.85	6.05	3.21	3.04	4.10	2.61	n.p.	3.81	2.48	2.24	2.54	2.93	2.68	2.56	2.92	2.39	3.11	2.60	2.17	3.00	2.79	3.92	3.84	4.19	**2.93**
	S49	1.43	1.63	1.45	2.76	1.76	1.29	1.83	0.96	n.p.	1.45	0.96	1.08	1.15	1.49	1.20	1.22	1.29	1.13	1.33	1.07	0.90	1.37	1.16	1.65	1.80	1.92	**1.33**
	S50	0.39	0.57	0.45	0.83	0.40	0.37	0.65	0.28	n.p.	0.48	0.31	0.34	0.38	0.46	0.38	0.37	0.36	0.39	0.37	0.27	0.28	0.45	0.26	0.49	0.60	0.55	**0.39**
	S51	0.06	0.05	0.08	0.08	0.07	0.05	0.08	0.03	n.p.	0.06	0.04	0.01	0.04	0.06	0.04	0.05	0.04	0.05	0.07	0.04	0.04	0.05	0.00	0.03	0.10	0.07	**0.05**
Vac M (DTaP IPV Hib)	S52	2.32	2.39	1.81	5.62	2.22	2.15	3.14	0.98	n.p.	2.79	1.78	1.66	1.66	2.10	1.97	1.84	2.64	1.68	2.03	1.34	1.58	2.21	1.77	0.90	3.67	2.75	**2.03**
	S53	1.54	1.30	1.43	2.44	1.31	1.38	1.79	1.11	n.p.	1.51	1.41	0.21	1.15	1.49	1.53	1.29	1.93	1.21	1.54	1.19	1.34	1.72	1.33	2.04	2.08	2.13	**1.43**
	S54	0.37	0.39	0.30	0.49	0.40	0.39	0.50	0.27	n.p.	0.44	0.46	0.36	0.33	0.41	0.45	0.31	0.50	0.15	0.42	0.31	0.33	0.45	0.33	0.41	0.56	0.52	**0.40**
	S55	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	n.p.	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.00	0.01	0.03	0.01	**0.01**
Ref N (R-D and R-T)	S56	1.12	1.08	0.92	1.56	1.01	0.64	1.09	0.66	n.p.	1.11	0.76	1.20	0.93	0.99	0.97	0.98	0.85	1.26	1.16	0.75	1.08	0.93	2.37	0.89	1.52	1.25	**1.01**
	S57	0.53	0.60	0.55	0.82	0.54	0.44	0.71	0.27	n.p.	0.52	0.36	0.54	0.51	0.53	0.52	0.49	0.68	0.60	0.63	0.43	0.51	0.47	0.83	0.34	0.71	0.65	**0.53**
	S58	0.29	0.30	0.23	0.40	0.28	0.18	0.39	0.18	n.p.	0.29	0.15	0.28	0.25	0.28	0.27	0.24	0.44	0.30	0.31	0.18	0.23	0.22	0.56	0.16	0.36	0.30	**0.28**
	S59	0.14	0.15	0.15	0.21	0.15	0.10	0.21	0.09	n.p.	0.14	0.06	0.14	0.14	0.14	0.13	0.12	0.31	0.15	0.14	0.08	0.11	0.10	0.26	0.08	0.23	0.14	**0.14**

Explanations: Listed are the estimated activities per serum and per laboratory. Each value represesents the geometric mean of 2 assays.
Grey boxes indicate that the difference between the 2 assays was more than 2.38-fold (see text for explanations) and black boxes indicate that the difference was more than 3.36-fold.
n.p. = not performed.
Med = Median value of all laboratories.

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Table 4a - Estimated activities per serum and per laboratory by T-ELISA (in IU/ml)

		Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	Lab 10	Lab 11	Lab 12	Lab 13	Lab 14	Lab 15	Lab 16	Lab 17	Lab 18	Lab 19	Lab 20	Lab 21	Lab 22	Lab 23	Lab 24	Lab 25	Lab 26	Med
Vac A (DT)	S01	n.p.	19.77	23.17	19.21	19.56	16.36	20.71	19.97	n.p.	24.10	14.45	16.46	22.72	17.16	19.23	16.84	19.28	18.49	1.83	19.79	10.34	18.79	20.19	25.27	24.36	20.34	19.42
	S03	n.p.	12.24	11.04	12.57	11.72	11.05	11.82	10.26	n.p.	13.45	6.34	8.89	11.58	9.39	10.51	9.71	11.23	9.85	9.07	10.98	6.32	9.28	12.46	16.16	9.27	12.09	11.01
	S02	n.p.	5.01	5.03	6.33	5.02	3.67	6.40	4.63	n.p.	5.93	3.75	4.90	4.94	4.20	4.48	4.04	7.27	5.05	3.55	4.06	2.98	4.38	2.78	5.14	4.33	4.89	4.76
	S04	n.p.	1.30	1.39	1.56	1.29	0.87	1.34	1.13	n.p.	1.22	0.70	1.60	1.21	1.11	1.50	1.05	1.22	1.22	1.19	1.10	0.73	1.20	0.71	1.33	1.36	1.40	1.22
Vac B (DTaP HepB IPV)	S05	n.p.	13.51	18.36	19.57	13.30	13.13	12.76	18.62	n.p.	15.94	7.35	16.29	11.53	12.43	12.84	10.47	13.99	16.59	3.44	14.41	7.65	11.21	16.31	23.22	9.79	15.26	13.40
	S06	n.p.	8.26	8.38	8.57	8.71	8.21	8.13	8.15	n.p.	9.48	5.12	11.15	6.68	6.81	6.58	5.93	7.90	6.36	6.89	7.05	4.78	5.94	9.32	11.64	6.75	8.99	8.01
	S07	n.p.	2.97	3.54	4.61	3.44	2.99	3.55	3.31	n.p.	2.95	2.15	5.11	3.11	3.05	3.09	2.76	3.73	5.07	3.17	3.33	2.25	2.61	3.80	4.60	3.26	4.34	3.29
	S08	n.p.	0.82	0.97	1.23	0.83	0.48	1.12	0.94	n.p.	0.88	0.50	0.81	0.71	0.82	0.66	0.72	0.84	0.97	0.74	0.66	0.65	0.66	0.30	1.32	0.96	1.00	0.82
Vac C (dT)	S09	n.p.	25.40	23.32	26.14	22.44	23.82	23.21	20.49	n.p.	14.92	22.41	28.86	19.24	20.06	21.24	19.04	19.46	21.05	4.97	29.17	18.17	14.78	30.06	38.45	16.49	28.52	21.82
	S10	n.p.	21.20	18.53	22.68	19.20	20.21	25.04	22.96	n.p.	13.66	26.15	26.69	18.19	17.58	16.89	15.52	15.34	17.96	10.57	21.96	21.99	11.01	19.42	33.30	22.18	26.56	19.81
	S11	n.p.	14.60	12.72	11.26	12.61	10.76	11.02	10.98	n.p.	14.98	9.39	11.67	13.35	11.65	13.49	9.89	12.94	9.81	12.80	16.80	12.95	12.24	15.34	21.23	11.31	14.09	12.66
	S12	n.p.	7.61	6.97	7.32	6.46	6.28	6.83	5.80	n.p.	7.86	4.50	6.08	6.30	6.90	7.05	4.44	6.98	5.08	6.50	8.44	5.75	6.22	6.80	9.32	7.87	7.58	6.81
Vac D (DTaP HepB IPV Hib)	S13	n.p.	10.01	8.93	9.51	9.12	15.51	10.07	9.62	n.p.	10.21	7.33	8.16	8.67	8.33	11.71	6.04	10.28	7.90	1.75	12.07	6.74	9.64	7.99	17.37	6.86	12.31	9.32
	S14	n.p.	4.49	3.92	4.19	4.04	4.70	4.77	4.33	n.p.	4.83	2.99	3.73	3.56	3.98	4.39	3.31	5.49	4.06	3.58	4.52	3.10	4.07	3.15	8.34	4.61	5.37	4.13
	S15	n.p.	1.93	1.84	2.34	1.94	1.73	2.13	1.53	n.p.	2.25	0.82	1.81	1.66	1.69	1.49	1.39	1.99	1.55	1.75	1.67	1.31	2.07	0.69	3.05	2.20	2.56	1.78
	S16	n.p.	0.44	0.42	0.65	0.45	0.38	0.45	0.35	n.p.	0.63	0.25	0.48	0.37	0.39	0.38	0.33	0.47	0.51	0.51	0.47	0.36	0.46	0.13	0.49	0.88	0.54	0.45
Vac E (DT)	S17									n.p.	14.27	14.44	14.67	14.12	13.03	17.73	12.03	15.97	11.58	4.64	19.49	11.21	14.79	17.04	19.68	12.25	24.35	14.44
	S18									n.p.	5.74	6.10	5.31	4.42	4.81	6.25	4.63	6.59	3.50	6.07	6.10	4.59	6.34	4.90	8.43	6.73	8.56	6.07
	S19									n.p.	1.48	2.36	2.37	1.69	2.01	2.35	1.81	2.86	1.82	2.41	2.14	1.79	2.46	1.00	3.33	2.51	3.13	2.35
	S20									n.p.	0.37	0.41	0.63	0.37	0.53	0.45	0.42	0.55	0.36	0.53	0.33	0.47	0.55	0.07	0.73	0.75	0.70	0.47
Vac F (DTaP HepB Hib IPV)	S21	n.p.	34.33	27.98	51.98	33.24	47.56	27.92	35.51	n.p.	32.97	30.35	31.76	31.23	24.36	42.43	19.94	33.45	22.87	12.97	41.72	22.70	30.84	24.52	50.43	24.85	43.11	31.50
	S22	n.p.	23.32	20.70	35.03	22.14	23.42	20.30	24.71	n.p.	25.60	21.89	21.51	19.24	16.47	31.94	14.08	23.64	16.71	25.37	31.30	16.40	29.66	11.35	34.00	21.34	32.45	22.73
	S23	n.p.	16.12	9.77	22.66	10.95	17.42	10.20	8.96	n.p.	13.13	8.18	13.04	9.87	8.73	15.19	8.12	11.16	7.15	11.00	15.94	6.79	12.35	8.44	15.58	11.14	16.94	11.07
	S24	n.p.	5.55	4.09	13.07	5.24	5.79	5.37	3.81	n.p.	4.95	3.85	6.52	4.45	4.37	7.18	3.05	5.21	4.17	5.65	6.30	3.54	5.88	2.91	7.64	5.47	6.71	5.30
Vac G (DTaP IPV Hib)	S25	n.p.	11.61	14.54	24.32	11.10	11.62	11.62	12.79	n.p.	13.96	9.11	14.52	10.10	9.20	14.67	7.55	13.36	6.93	6.41	15.26	7.23	13.92	8.96	16.57	10.73	16.13	11.62
	S26	n.p.	5.80	5.13	12.35	5.98	8.02	6.32	6.99	n.p.	7.16	4.23	7.94	4.87	5.18	7.96	3.53	6.53	3.42	5.92	7.11	3.67	7.47	3.29	10.78	7.42	8.22	6.43
	S27	n.p.	3.75	3.84	6.06	3.39	5.12	3.68	4.00	n.p.	3.65	2.65	4.84	3.34	3.11	4.50	2.46	4.04	1.96	3.76	4.35	2.19	4.28	2.19	4.78	4.60	4.77	3.80
	S28	n.p.	2.28	2.61	5.96	1.87	3.32	2.35	2.46	n.p.	2.13	1.81	3.29	1.98	1.95	3.54	1.48	2.34	1.32	2.51	2.90	1.38	2.54	1.34	2.96	2.88	2.85	2.40
Vac H (DT)	S29									n.p.	22.68	26.27	29.67	18.34	16.80	31.77	13.74	24.16	8.78	8.73	31.38	15.07	22.57	18.38	34.34	18.96	31.80	22.57
	S30									n.p.	14.89	29.24	22.10	14.12	12.97	18.23	8.63	17.76	6.74	18.17	22.30	14.00	21.33	24.66	27.49	16.46	21.92	18.17
	S31									n.p.	6.80	4.96	5.13	5.99	5.36	8.06	5.66	7.02	4.83	7.78	7.25	5.85	6.15	3.95	6.81	6.56	8.65	6.15
	S32									n.p.	5.56	4.52	5.32	4.22	4.52	5.69	4.03	8.91	4.45	4.71	5.28	4.56	4.84	1.71	6.66	5.03	6.64	4.84
Vac I (DTwP)	S33	n.p.	7.55	6.87	18.95	6.18	9.89	7.89	6.11	n.p.	14.33	6.12	8.00	7.15	5.97	9.29	5.52	7.46	9.61	4.47	7.84	7.31	7.49	5.90	7.81	8.01	9.54	7.52
	S34	n.p.	6.05	4.61	11.29	4.27	6.98	5.71	5.33	n.p.	7.53	4.53	5.61	5.22	4.28	6.90	3.81	5.33	3.01	4.65	5.64	4.97	4.93	4.02	7.35	5.57	7.12	5.33
	S35	n.p.	1.08	0.92	2.43	1.07	1.28	1.28	1.13	n.p.	1.17	0.67	1.21	0.91	1.01	1.30	0.89	1.22	1.02	0.93	1.10	0.87	1.18	0.49	1.37	1.42	1.44	1.12
Vac J (DTaP)	S36	n.p.	24.12	23.07	48.58	17.79	21.93	28.12	19.46	n.p.	26.79	12.90	21.38	24.28	20.45	22.69	20.64	20.80	12.41	22.84	19.65	12.36	22.36	21.68	25.20	22.68	29.12	22.14
	S37	n.p.	5.97	6.83	10.30	5.17	4.64	7.28	5.45	n.p.	7.41	3.81	6.39	5.32	6.18	4.68	6.05	5.71	3.67	5.21	4.51	6.10	4.92	3.00	9.59	6.07	8.50	5.84
	S38	n.p.	3.29	2.92	5.11	2.17	2.39	4.20	3.42	n.p.	3.76	1.83	2.96	2.56	2.97	2.56	3.20	2.74	2.01	3.24	1.86	2.07	2.70	1.16	4.32	3.03	3.58	2.94
	S39	n.p.	0.22	0.28	0.25	0.20	0.16	0.27	0.28	n.p.	0.27	0.13	0.27	0.19	0.26	0.19	0.30	0.27	0.42	0.32	0.14	0.20	0.13	0.05	0.23	0.32	0.29	0.25
Vac K (DTaP IPV)	S40	n.p.	22.68	20.66	35.10	17.39	20.31	22.71	17.10	n.p.	17.38	21.01	20.42	21.00	19.50	18.56	20.26	17.21	25.42	20.34	14.44	12.26	23.13	16.18	24.76	2.34	28.72	20.32
	S41	n.p.	14.04	14.29	28.06	10.82	14.42	13.84	13.34	n.p.	15.61	8.48	12.54	13.44	11.87	14.59	11.40	13.82	10.72	13.77	11.33	7.94	13.78	9.33	23.31	12.32	18.51	13.60
	S42	n.p.	2.77	3.51	3.65	2.48	2.37	2.68	2.76	n.p.	3.58	2.28	3.04	2.81	2.48	2.40	2.94	2.68	3.02	3.28	1.84	1.29	3.28	1.16	4.25	3.29	3.59	2.79
	S43	n.p.	0.17	0.21	0.22	0.16	0.13	0.17	0.15	n.p.	0.20	0.12	0.20	0.14	0.18	0.16	0.19	0.13	0.20	0.38	0.16	0.13	0.17	0.05	0.19	0.27	0.22	0.17
Vac L (DTaP IPV)	S48	n.p.	21.27	16.51	33.24	12.46	16.20	15.07	16.60	n.p.	23.95	10.80	18.10	14.84	12.99	16.16	13.63	15.64	10.26	15.38	14.02	7.89	14.16	12.69	33.13	13.14	19.65	15.23
	S49	n.p.	4.53	5.03	8.78	3.14	3.43	4.36	4.15	n.p.	5.03	3.38	4.80	4.12	3.70	3.34	4.49	3.72	3.37	4.29	3.34	3.69	4.46	2.89	6.79	4.45	5.13	4.22
	S50	n.p.	0.86	1.07	1.34	0.68	0.52	1.06	0.83	n.p.	1.26	0.64	1.03	0.66	0.82	0.55	0.90	0.66	1.01	0.99	0.36	0.73	0.90	0.22	1.48	1.18	1.13	0.88
	S51	n.p.	0.15	0.19	0.26	0.12	0.10	0.15	0.11	n.p.	0.17	0.09	0.04	0.13	0.18	0.13	0.17	0.10	0.16	0.24	0.13	0.11	0.17	0.03	0.14	0.23	0.16	0.14
Vac M (DTaP IPV Hib)	S52	n.p.	30.45	23.16	96.21	17.99	39.01	34.57	21.57	n.p.	19.62	23.52	24.95	24.43	21.24	36.06	21.81	22.29	16.42	27.42	29.05	8.94	57.44	14.86	51.71	31.09	38.12	24.69
	S53	n.p.	20.19	17.73	33.55	14.93	23.28	19.86	28.88	n.p.	13.42	17.21	2.42	15.92	15.97	22.94	15.59	14.96	7.94	17.20	19.86	11.77	36.79	9.28	47.70	20.18	26.96	17.47
	S54	n.p.	9.72	8.57	20.00	7.98	12.50	9.72	11.33	n.p.	7.06	9.36	9.00	8.81	6.10	14.08	7.67	6.96	5.76	9.01	8.73	6.09	11.53	3.64	26.26	11.00	14.40	9.00
	S55	n.p.	3.57	3.76	8.56	3.10	5.54	3.34	1.95	n.p.	3.25	2.47	1.14	3.22	2.85	5.65	2.68	2.88	3.15	3.43	4.48	2.40	3.16	1.51	7.37	3.62	4.48	3.24
Ref N (R-D and R-T)	S56	n.p.	1.71	1.93	2.85	1.38	2.07	1.44	1.76	n.p.	1.81	1.53	1.42	1.49	1.40	1.77	1.50	1.24	1.95	2.27	1.80	1.42	1.40	1.07	2.88	2.14	1.90	1.73
	S57	n.p.	0.90	0.86	1.44	0.76	0.80	0.91	0.71	n.p.	0.78	0.78	0.73	0.75	0.74	0.93	0.75	1.46	0.88	1.22	0.80	0.60	0.67	0.52	1.37	1.14	1.00	0.80
	S58	n.p.	0.44	0.42	0.41	0.37	0.44	0.42	0.32	n.p.	0.41	0.38	0.38	0.38	0.38	0.46	0.40	1.13	0.45	0.69	0.40	0.30	0.35	0.22	0.44	0.58	0.50	0.41
	S59	n.p.	0.22	0.28	0.43	0.23	0.22	0.22	0.27	n.p.	0.20	0.15	0.20	0.21	0.23	0.24	0.21	0.83	0.25	0.34	0.19	0.13	0.17	0.10	0.21	0.32	0.25	0.22

Explanations: Listed are the estimated activities per serum and per laboratory. Each value represesents the geometric mean of 2 assays.
Grey boxes indicate that the difference between the 2 assays was more than 2.38-fold (see text for explanations) and black boxes indicate that the difference was more than 3.36-fold.
n.p. = not performed.
Med = Median value of all laboratories.

Table 4b - *Estimated activities per serum and per laboratory by T-ToBI (in IU/ml)*

		Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Med
Vac A (DT)	S01	13.88		10.52	8.94	10.84	9.59*			10.52
	S03	7.27		5.07	6.43	4.61	6.20*			6.20
	S02	2.35		1.49	2.00	1.33	1.99*			1.99
	S04	0.58		0.24	0.35	0.32	0.40*			0.35
Vac B (DTaP HepB IPV)	S05	13.92		10.90	8.45	16.69	13.93*			13.92
	S06	6.74		5.86	3.41	7.24	7.14*			6.74
	S07	1.83		2.42	2.91	2.56	2.56*			2.56
	S08	0.44		0.39	0.60	0.39	0.45*			0.44
Vac C (dT)	S09	42.11		18.63	23.08	36.50	21.08*			23.08
	S10	26.80		12.34	13.59	23.25	14.57*			14.57
	S11	17.65		9.43	10.54	15.56	9.24*			10.54
	S12	5.69		3.32	5.07	4.59	4.09*			4.59
Vac D (DTaP HepB IPV Hib)	S13	5.37		5.04	4.54	5.10	5.16*			5.10
	S14	2.08		1.79	0.83	2.12	2.04*			2.04
	S15	0.61		0.63	0.43	0.59	0.85*			0.61
	S16	0.14		0.13	0.09	0.10	0.21*			0.13
Vac E (DT)	S17									
	S18									
	S19									
	S20									
Vac F (DTaP HepB Hib IPV)	S21	13.74		11.03	7.89	14.79	11.60*			11.60
	S22	8.64		4.63	4.83	5.64	6.17*			5.64
	S23	4.42		3.44	2.54	2.58	2.89*			2.89
	S24	1.63		1.02	0.73	0.67	0.77*			0.77
Vac G (DTaP IPV Hib)	S25	6.67		6.13	4.11	5.42	4.54*			5.42
	S26	2.02		1.85	1.61	1.77	1.90*			1.85
	S27	3.41		1.07	0.86	1.16	1.16*			1.16
	S28	1.41		0.56	0.40	0.47	0.62*			0.56
Vac H (DT)	S29									
	S30									
	S31									
	S32									
Vac I (DTwP)	S33	7.05		5.55	5.43	6.71	5.77*			5.77
	S34	5.40		3.34	2.83	3.79	3.49*			3.49
	S35	0.97		0.50	0.06	0.48	0.55*			0.50
Vac J (DTaP)	S36	19.82		16.29	14.76	26.27	19.56*			19.56
	S37	5.97		3.11	2.71	3.65	3.53*			3.53
	S38	1.60		0.99	0.68	1.11	1.36*			1.11
	S39	0.22		0.06	0.04*	0.05	0.08*			0.06
Vac K (DTaP IPV)	S40	34.92		15.86	26.18	13.44	15.64*			15.86
	S41	17.91		10.98	12.66	13.32	9.78*			12.66
	S42	2.68		1.16	0.10	1.53	1.66*			1.53
	S43	0.09		0.05	0.00	0.03	0.07*			0.05
Vac L (DTaP IPV)	S48	14.96		8.16	9.15	10.79	9.00*			9.15
	S49	3.73		1.64	.	1.68	1.79*			1.74
	S50	0.30		0.15	.	0.13	0.19*			0.17
	S51	0.03		0.02	.	0.02	0.03*			0.02
Vac M (DTaP IPV Hib)	S52	20.68		9.62	.	14.86	8.21*			12.24
	S53	12.29		5.48	.	7.86	6.24*			7.05
	S54	3.77		1.86	.	1.77	2.08*			1.97
	S55	0.37*		0.28	.	0.48	0.55*			0.42
Ref N (R-D and R-T)	S56	1.60*		1.14	.	1.22	1.14*			1.18
	S57	0.82*		0.56	.	0.66	0.69*			0.67
	S58	0.47*		0.27	.	0.36	0.38*			0.37
	S59	0.12*		0.14	.	0.15	0.17*			0.15

Explanations: Listed are the estimated activities per serum and per laboratory. Each value represesents the geometric mean of 2 assays.

Values that represent only one assay: Laboratory 6 performed only one assay. Other cases where only one result was available are marked with a *.

Grey boxes indicate that the difference between the 2 assays was more than 2.38-fold (see text for explanations) and black boxes indicate that the difference was more than 3.36-fold.

n.p. = not performed.

Med = Median value of all laboratories.

Figure 1 - Box-and-whisker plots of results from Diphtheria Verocell assays

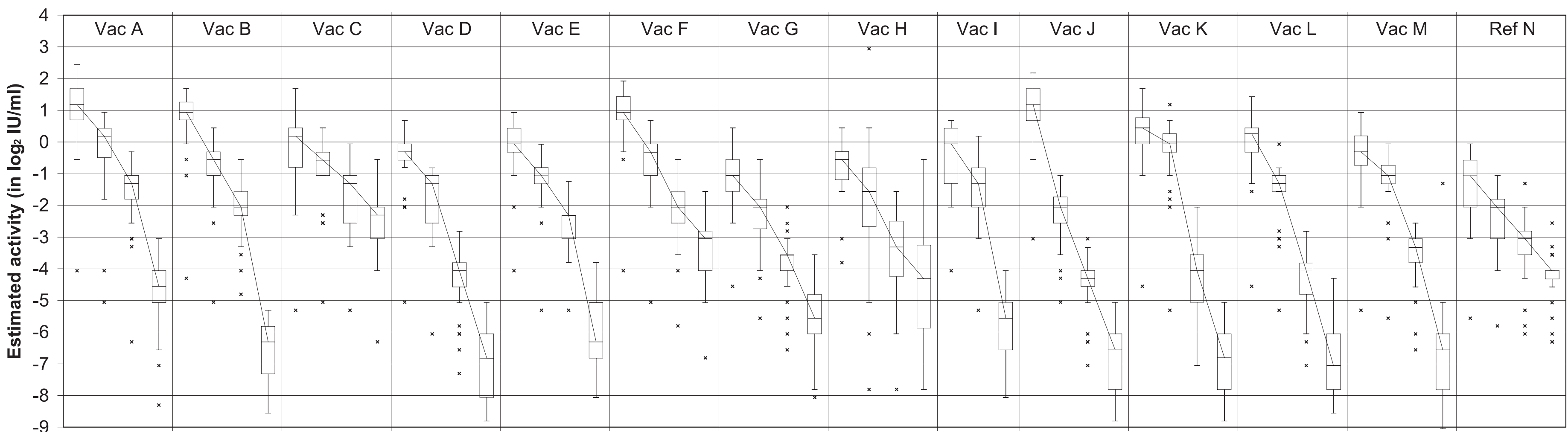
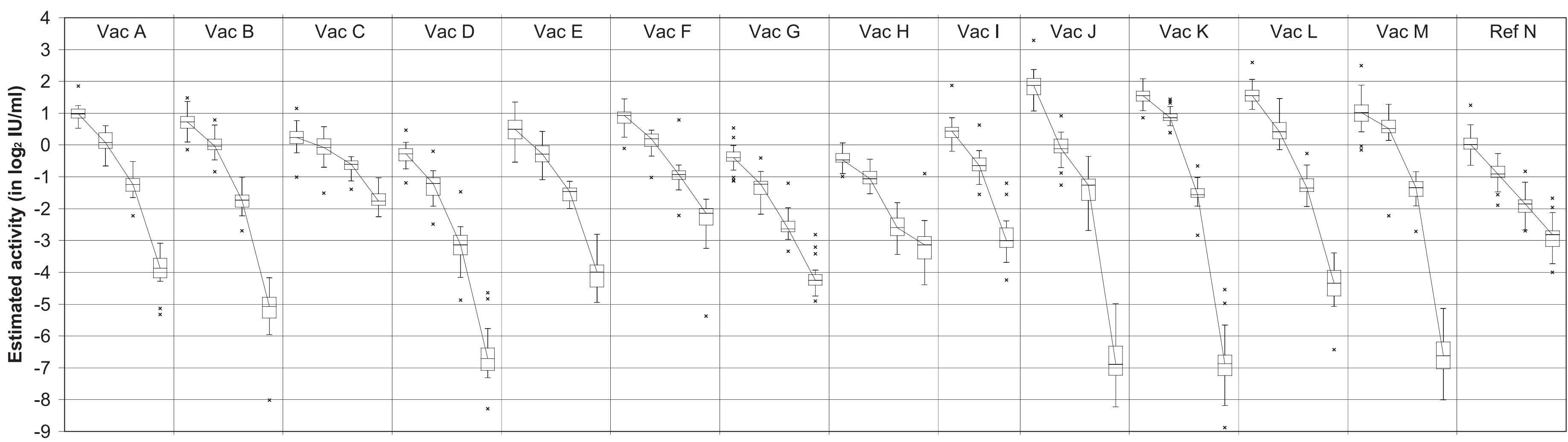


Figure 2 - Box-and-whisker plots of results from Diphtheria ELISA assays



Explanations: Each box-and-whisker plot represents the distribution of the laboratory means of a specific serum. The boxes are the interquartile range, with the median as a horizontal bar. The medians are connected by straight lines.
The whiskers represent values within 1.5 times the interquartile range from the boxes. Values outside that range are symbolised by crossmarks.
Each unit on the y-axis represents a 2-fold step. The sera are shown in descending order of activity, grouped per vaccine.

Figure 3 - Box-and-whisker plots of results from Tetanus ELISA assays

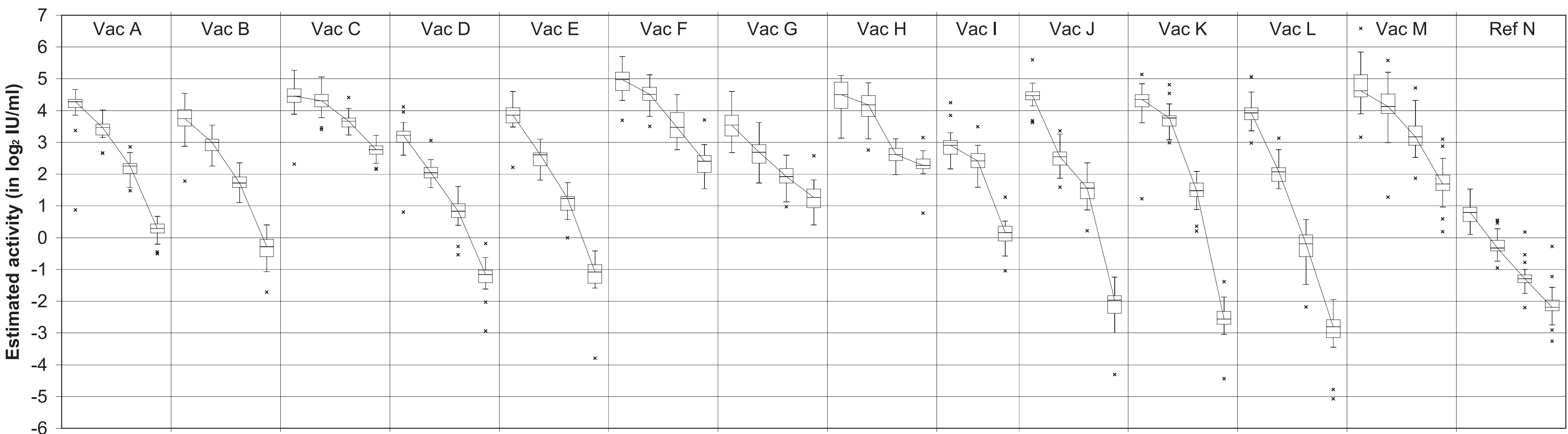
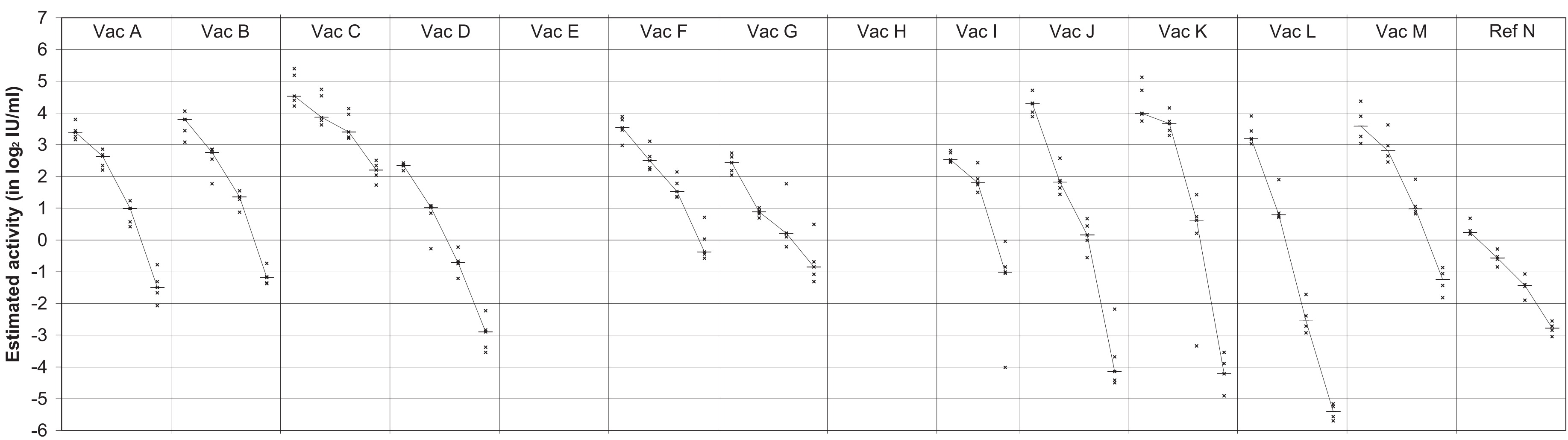


Figure 4 - Individual results and medians from Tetanus ToBI assays



Explanations: Each box-and-whisker plot represents the distribution of the laboratory means of a specific serum. The boxes are the interquartile range, with the median as a horizontal bar. The medians are connected by straight lines.
The whiskers represent values within 1.5 times the interquartile range from the boxes. Values outside that range are symbolised by crossmarks.
Because of the small number of laboratories having performed the ToBI assays no boxes or whiskers are shown, but only the individual values and their median.
Each unit on the y-axis represents a 2-fold step. The sera are shown in descending order of activity, grouped per vaccine.

Figure 5 - Two way plot Diphtheria methods

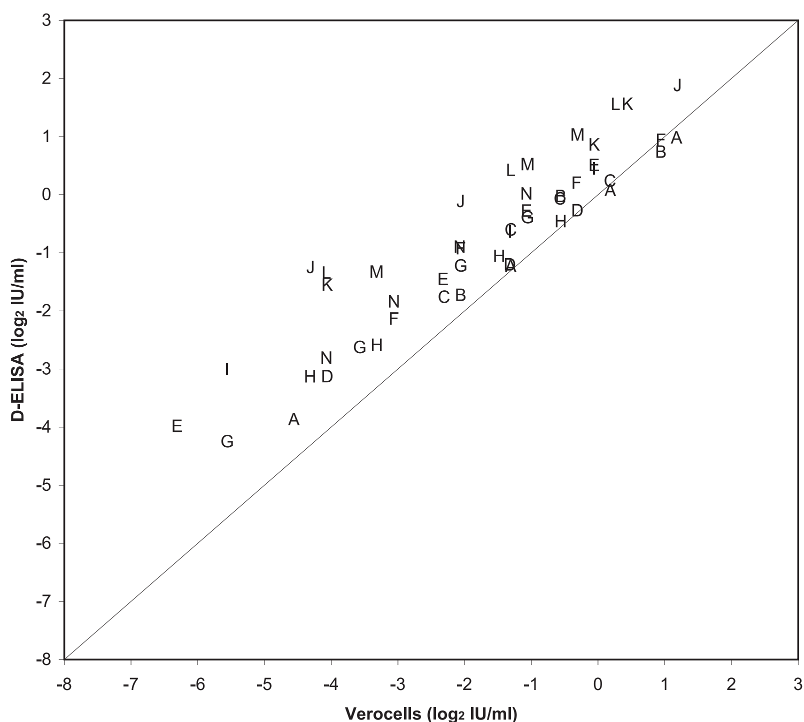


Table 5 - Order of closest agreement

Diphtheria methods			
Vac	Factor	Man	Components
A	1.10	I	D T
B	1.30	II	D T aP HepB IPV
H	1.40	IV	D T
C	1.41	III	d T
D	1.45	II	D T aP HepB IPV Hib
F	1.64	III	D T aP HepB Hib IPV
E	1.68	IV	D T
G	1.76	III	D T aP IPV Hib
N	2.23		
I	2.93	IIa	D T wP
M	3.14	IIIa	D T aP IPV Hib
K	3.17	IIa	D T aP IPV
L	4.01	IIIa	D T aP IPV
J	4.36	IIa	D T aP

Figure 6 - Two way plot Tetanus methods

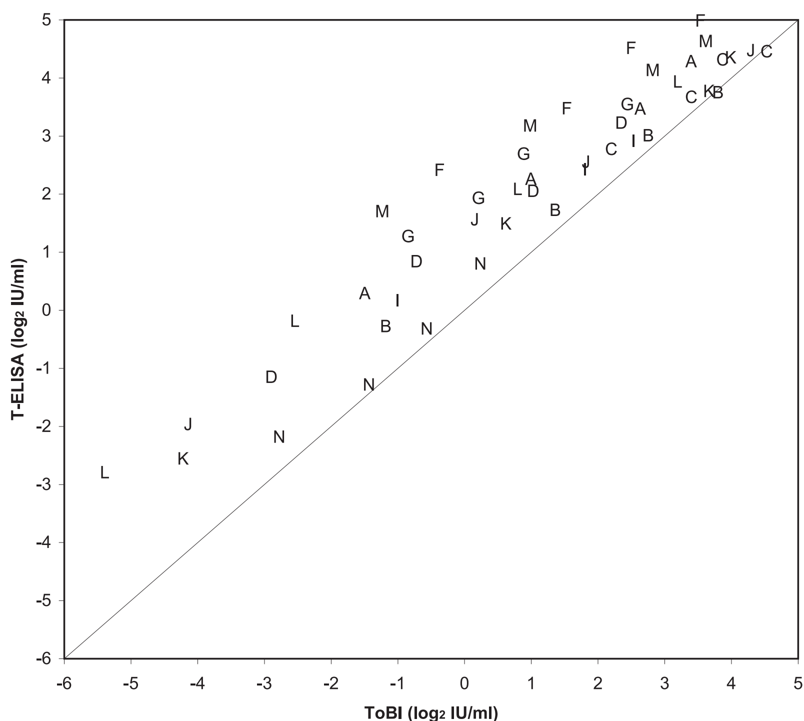


Table 6 - Order of closest agreement

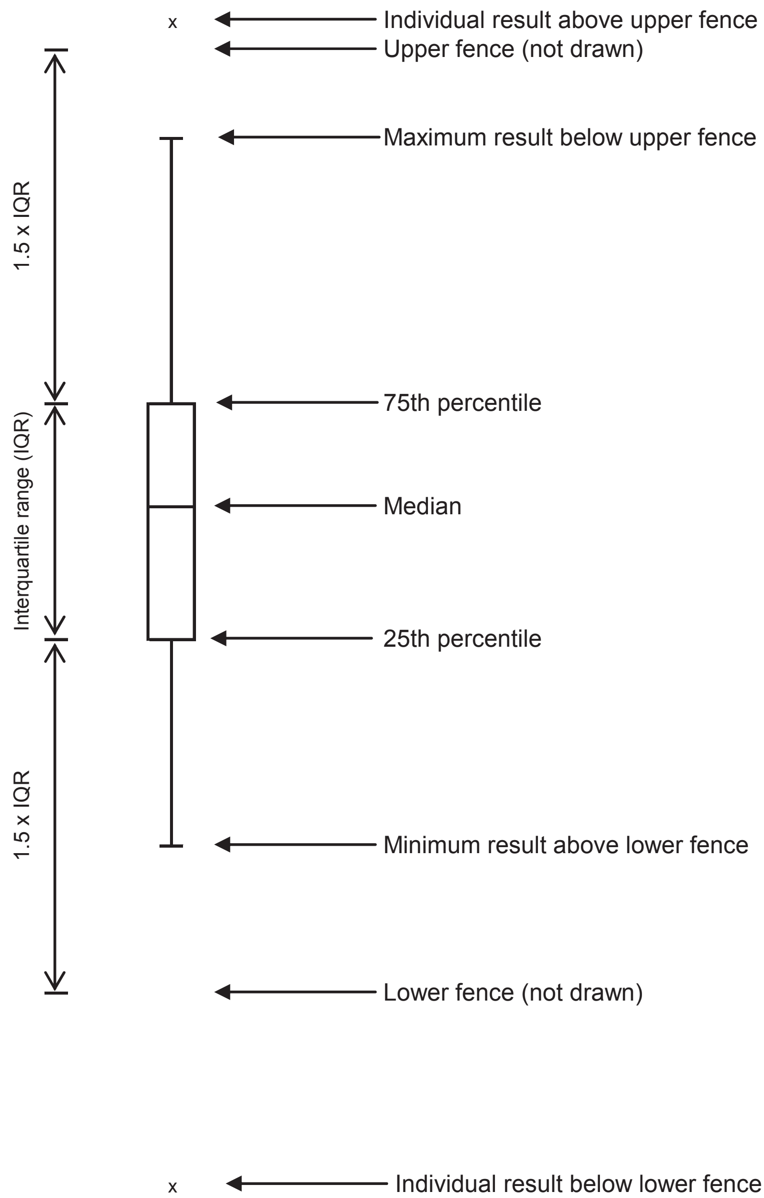
Tetanus methods			
Vac	Factor	Man	Components
B	1.19	II	D T aP HepB IPV
C	1.23	III	d T
N	1.28		
K	1.46	IIa	D T aP IPV
I	1.73	IIa	D T wP
J	1.89	IIa	D T aP
A	2.01	I	D T
D	2.27	II	D T aP HepB IPV Hib
G	2.97	III	D T aP IPV Hib
M	3.00	IIIa	D T aP IPV Hib
L	3.04	IIIa	D T aP IPV
F	3.52	III	D T aP HepB Hib IPV

The position of each letter represents the median outcome of all laboratories for a serum. The letter itself represents the vaccine code.
The diagonal line represents the line of agreement.
The tables list the vaccines in order of closest agreement between the methods.
The factor is the average difference between the methods calculated over the highest 3 doses for a specific vaccine.

ANNEX

Explanation: Box-and-whisker plots

The boxes show the interquartile range (the middle 50 per cent of the results) with the median as a horizontal bar. The medians are connected with straight lines. The whiskers represent the range of values within 1.5 times the interquartile range from the boxes. Values outside that range are symbolised by crossmarks.



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