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

Extended Study: Correlation of Serology with *in vivo* Toxin Neutralisation

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SUMMARY

Phase I of BSP034 collaborative study was extended in two laboratories to include correlation of serology with in vivo toxin neutralisation test (TNT) using 2 separate sets of 20 serum pools, produced in-house. The study investigated the extent to which the in vitro methods for diphtheria antibodies, Vero cell assay and diphtheria enzyme-linked immunosorbent assay for diphtheria antitoxin (D-ELISA), can detect neutralising antibodies by comparison with TNT in guinea pigs. The study was also performed to compare the antibody neutralising potency obtained in relation to guinea pig (GP) or equine (DI) antitoxin standard. In addition, the study provided an opportunity to compare ELISA for tetanus antitoxin (T-ELISA) and TNT assay for detection of anti-tetanus antibodies, from the same set of serum pools. The data obtained show that antitoxin potency obtained by Vero cell assay, D-ELISA and T-ELISA using the same GP standard, highly correlated with neutralising potency as determined in respective TNT assays. Vero cell assay with DI provided estimates that also correlated with neutralising potency, but were of significantly lower titre. Since reference to DI standard is widely used in serodiagnosis, as well as in clinical studies where diphtheria antitoxin titres obtained in the Vero cell method are taken as surrogate markers for vaccine efficacy, it should be investigated if a similar difference is also observed for human serology.

KEYWORDS

Diphtheria vaccine; potency test; Vero cell assay; ELISA; toxin neutralisation; serology; biological standardisation; collaborative study; European Pharmacopoeia; Council of Europe.

ABBREVIATIONS

BRP: Biological Reference Preparation; D-ELISA: ELISA for diphtheria antitoxin; DI: Equine antitoxin standard; ECVAM: European Centre for Validation of Alternative Methods; EQ: Diphtheria antitoxin, equine 3rd British Standard; GP: Guinea pig antitoxin standard; IS: International Standard; IU: International Units; Lf dose: The quantity of toxin or toxoid that flocculates in the shortest time with 1 IU of antitoxin; PD_{50}: The statistically determined dose of a vaccine that in test conditions may be expected to protect 50 per cent of the animals against a challenge dose of the micro-organisms or toxins against which it is active; Ph. Eur.: Pharmacopée Européenne, European Pharmacopoeia; PRP: Polyribosylribitol phosphate; r: Correlation coefficient; T-ELISA: ELISA for tetanus antitoxin; TNT: Toxin neutralisation test; ToBI: Toxin or toxoid binding inhibition; VCA: Vero cell assay.

1. INTRODUCTION

A collaborative study on the evaluation of an alternative functional assay to the European Pharmacopoeia (Ph. Eur.) *in vivo* challenge procedure for potency determination of vaccines containing the diphtheria toxoid component was initiated in January 2001 [1]. This study was run under the aegis of the Biological Standardisation Programme, coordinated by the European Directorate for the Quality of Medicines (EDQM) with the project code BSP034.

The Ph. Eur. method for potency testing of diphtheria component in vaccines is performed by direct challenge of immunised guinea pigs with diphtheria toxin [2]. The main purpose of the validation study was to introduce a functional serological method as an alternative to the direct challenge procedure. Although the Ph. Eur. potency testing is not performed by *in vivo* toxin neutralisation test (TNT), it is generally accepted that protection to diphtheria is antibody mediated and that the presence of toxin neutralising antibodies contribute to protection. Furthermore, TNT forms the basis of the United States and WHO minimum requirements for potency test for diphtheria and tetanus toxoids [3,4].

The validation study was divided into 3 phases. Phase I study concluded that comparable diphtheria potency estimates were obtained in the Ph. Eur. direct challenge assay in guinea pigs and in Vero cell assay for 5 vaccines of different activities [5]. Phase II studies, confirmed that both Vero cell assay and ELISA were considered suitable models for replacement of the Ph. Eur. method for routine batch release purpose, and both were able to rank vaccines of different potencies [1]. Both were recommended for inclusion in Phase III of BSP034 i.e. the final validation study. The extended study, performed in 2 laboratories, examined the extent to which the 2 methods [Vero cell assay and ELISA for diphtheria antitoxin (D-ELISA)] can detect diphtheria toxin neutralising antibodies by comparison with TNT in guinea pigs.

The second purpose of the extended study was to compare the *in vivo* and Vero cell neutralising potency in relation to equine (DI) and guinea pig (GP) standard. These comparisons were studied because the results from the Phase I study have identified approximately 16-35 fold lower antibody potencies of individual serum samples when tested in Vero cell assay using DI rather than GP reference serum. The validation studies confirmed that antitoxin titres obtained in serology do not affect the potency of vaccines if either DI or GP standard is used [1]. Finally, the extended study provided an opportunity to compare tetanus ELISA (T–ELISA) and TNT assays for detection of anti-tetanus antibodies, from the same set of serum pools.

Two laboratories participated in the extended study and used a set of 20 guinea pig sample pools, which were derived

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from the in-house immunisation of 5 different vaccines at 4 concentrations. The sample pools (each derived from 14 animals) were tested by *in vitro* methods and confirmed to have antitoxin potency in the range of about 2 $\log_{10}$ units apart, for both antigens (range diphtheria toxoid: 0.1-10 IU/ml; tetanus toxoid: 0.2-30 IU/ml).

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Reference standards

Antitoxins

- *Diphtheria antitoxin, equine 1st WHO International Standard (IS) (DI),* batch code no. 00/462, with a defined activity of 10 International Units (IU)/ml.
- *Diphtheria antitoxin, equine 3rd British standard EQ,* (NIBSC code no. 66/153), stable freeze-dried formulation with a defined activity of 110 IU/ampoule.
- *Tetanus antitoxin Human IgG 1st International Standard (TE-3)* with a defined activity of 120 IU/ampoule.
- *Clostridium tetani guinea pig antiserum (for vaccines - human use) Biological Reference Preparation (BRP) batch 1,* the Ph. Eur. guinea pig anti-tetanus reference serum with a defined activity of 0.2 IU/vial (EDQM catalogue number C2424550) [6].
- *Guinea pig serum, Diphtheria and Tetanus antitoxin (GP)* (NIBSC Code no. 98/572). A homologous stable guinea pig antiserum prepared for the purpose of the collaborative study with a mean estimate of 3.1 IU/ampoule for diphtheria antitoxin and 3.0 IU/ampoule of tetanus antitoxin [1].

Toxins

- *Diphtheria toxin for use in Vero cell assay and TNT.* In Lab 1, STT toxin (NIBSC 0.9 Lf/vial, 900 STT units/vial, specific activity ca. 20,000 Lr/Lf) was used in Vero cell assay and diphtheria toxin D-79/1 from RIVM was used in TNT. In Lab 2, the same toxin provided by Aventis (FA016723, 400 Lf/ml, specific activity ca. 25,000 Lr/Lf) was used in Vero cell assay and in TNT.
- *Tetanus toxin for use in TNT assay.* Same lot of tetanus toxin (NIBSC: AWX 4664) was used in the two laboratories.

2.1.2. Serum samples

A total of 20 pools produced in each laboratory in phase I of BSP034 [1] were available for this study. These pools were made by combining equal volumes from 14 vaccinated animals immunised with the same vaccine dose. The pools were tested in TNT *in vivo* for diphtheria and tetanus and in Vero cell assay and D-ELISA for diphtheria as well as in T-ELISA for tetanus using methods outlined in BSP034 Phase II protocol [6].

2.2. Methods

2.2.1. Toxin neutralisation test (diphtheria)

TNT was performed according to the general protocol outlined in the Ph. Eur. for diphtheria antitoxin assay [7]. The potency for diphtheria antitoxin is determined by comparing the dose necessary to protect guinea pigs against the erythrogenic effects of a fixed dose of diphtheria toxin, with the dose of antitoxin calibrated in IU, necessary to give the same protection. Table 1 summarises information on the approximate concentration of toxin and antitoxin, at the end point.

Table 1 - *Summary information on toxin neutralisation assays: concentrations at end point*

Reference reagents	*In vivo* Lab 1	*In vivo* Lab 2	*In vitro* Lab 1	*In vitro* Lab 2
98/572 (GP) IU/ml	0.01	0.005	0.1	0.015
BS 66/153 (EQ) IU/ml	0.025	-	-	-
WHO 00/462 (DI) IU/ml	-	0.0025	0.008	0.001
Toxin Lf/ml*	0.00125	0.0025	0.001	0.00025
Ratio GP/EQ or GP/DI	0.4	2	12.5	15

* Different toxin for two methods in Lab 1 and between laboratories (see 2.1 Materials).

In both laboratories, toxin was diluted to 0.025 Lf/ml and reference antitoxin was diluted to 0.05 IU/ml for use in the assay. In the reaction mixture, toxin and reference antitoxin or test samples were diluted in a total volume of 2.0 ml of saline or saline with glucose.

Toxin neutralisation *in vitro* (Vero cell assay) was performed as previously published [5] and described in the study protocol [EDQM internal document]. Table 1 summarises information on concentration of toxin and antitoxin at the end point.

2.2.2. Toxin neutralisation test (tetanus)

TNT was performed in mice and the neutralising potency of each serum pool was determined by comparing its ability to neutralise a standardised dose of tetanus toxin with that of a reference antitoxin. The method is outlined in Ph. Eur. monograph *tetanus antitoxin for human use* and is based on the protection of mice against toxin-induced paralysis after sub-cutaneous injection [8].

Both laboratories used the same tetanus toxin. Lab 1 used toxin diluted to 80 PD_{50}/ml and reference antitoxin (Ph. Eur. BRP) diluted to 0.0025 IU/ml in the assay. Lab 2, used toxin diluted to 1000 PD_{50} and the same reference antitoxin as well as IS for tetanus antitoxin diluted to 0.025 IU/ml. In the reaction mixture, toxin and reference antitoxin or test samples were diluted in a total volume of 2.5 ml with phosphate buffered saline (PBS), stabilised with either peptone or gelatine. The mice were injected sub-cutaneously with 0.5 ml of the reaction mixtures and observed for 4 days.

2.2.3. ELISA

D-ELISA and T-ELISA for diphtheria and tetanus antitoxins were performed as outlined in the protocol for the validation study (EDQM unpublished document).

2.2.4. Statistical analysis

Pearson's correlation coefficients between *in vivo* TNT potencies and *in vitro* serology potencies for individual serum pools were calculated for the two sets of data independently generated in both laboratories. For correlation of the methods testing diphtheria antibodies, the 4th dose of vaccine was excluded in both laboratories and doses C3 and D3 were also excluded from Lab 1 (Tables 2 and 3). For correlation of methods in testing of tetanus antibodies, the 4th dose was also excluded for all vaccines in both laboratories, except for vaccine F in Lab 1 where the 1st dose was excluded from analyses (Table 4).

3. RESULTS AND DISCUSSION

In vivo neutralising potencies for diphtheria antibodies, estimated for serum pools from Phase I study as determined in Lab 1 and 2 are summarised in Tables 2 and 3, respectively. Each sample was assayed by TNT and antitoxin potency expressed in relation to GP or in relation to equine British (EQ) or DI standards as indicated. Potencies determined by

Table 2 - *Diphtheria potency results from Laboratory 1*

Vaccine code	Vaccine dose (IU/ml)	Vero cell assay potency* (IU/ml)		ELISA potency* (IU/ml)	*In vivo* potency (TNT) (IU/ml)		TNT/VCA**	TNT/ELISA***
		(Ref: GP)	(Ref: DI)		(Ref: GP)	(Ref: EQ)		
R1	16.0	6.8	0.42	2.6	3.11	0.78	46 %	120 %
R2	6.4	2.4	0.15	1.3	1.87	0.47	78 %	144 %
R3	2.6	0.7	0.04	0.6	0.64	0.16	91 %	107 %
R4	1.0	-	-	0.1	-	-	-	-
C1	16.2	7.7	0.80	2.1	2.92	0.73	38 %	139 %
C2	6.5	3.9	0.40	1.8	1.72	0.43	44 %	96 %
C3	2.6	0.3	0.03	0.5	0.25	0.06	-	-
C4	1.0	0.2	0.02	0.3	-	-	-	-
D1	16.0	7.5	0.47	2.2	1.76	0.88	23 %	80 %
D2	6.4	1.9	0.12	1.0	0.60	0.30	32 %	60 %
D3	2.6	0.4	0.03	0.8	0.4	0.1	-	-
D4	1.0	0.3	0.02	0.3	-	-	-	-
E1	16.0	7.7	0.54	1.4	1.80	0.45	23 %	129 %
E2	6.4	5.5	0.34	1.0	1.20	0.30	22 %	120 %
E3	2.6	0.4	0.03	0.4	0.11	0.03	28 %	28 %
E4	1.0	-	-	0.1	-	-	-	-
F1	80.0	30.9	2.73	7.6	11.72	2.93	38 %	154 %
F2	32.0	19.2	1.70	7.0	12.84	3.21	67 %	183 %
F3	12.6	9.6	0.85	4.6	3.48	0.87	36 %	76 %
F4	6.1	1.7	0.15	1.8	1.30	0.33	76 %	72 %

** calculated at EDQM (taken from Phase I report)*

*** in vivo potency (ref: GP) as per cent of Vero cell assay potency (ref: GP)*

**** in vivo potency (ref: GP) as per cent of ELISA potency (ref: GP)*

- not calculated or below detection limit

VCA: Vero cell assay

Pearson's correlation coefficients (r):

Between *in vivo* potencies (ref: GP) and Vero cell assay potencies (ref: GP), *r = 0.921*

Between *in vivo* potencies (ref: GP) and ELISA potencies (ref: GP), *r = 0.955*

Between *in vivo* potencies (ref: EQ) and Vero cell assay potencies (ref: DI), *r = 0.925*

(Calculation of these correlation coefficients excludes results for R4, C3, C4, D3, D4, E4 & F4)

in vitro assays are also shown for comparison. These were essentially as calculated previously and reported in Phase I final report [1]. Table 4 summarises the *in vivo* neutralising potency for tetanus antibodies, estimated on the same serum pools, as determined in Lab 1 and Lab 2. Each sample was assayed by TNT in mice and potency expressed in relation to guinea pig anti-tetanus serum BRP standard. Potencies determined by T-ELISA assay are also shown for comparison and these are as previously reported for sample pools [1].

3.1. Diphtheria TNT and Vero cell assay with GP standard

Plots of TNT potencies, expressed as per cent of the *in vitro* potencies are shown in Figure 1(a) (Lab 1) and Figure 1(b) (Lab 2). Although Pearson's correlation coefficients are very high, r = 0.921 and r = 0.993 for Lab 1 and Lab 2, respectively, in both sets of data, the potency values determined *in vitro* were higher that those determined by TNT assay. Data from Lab 2, shown in Figure 1(b) and Table 3, indicate that there is a near-constant ratio between the two values for all the samples and all dilutions, with *in vivo* potencies being approximately 60 per cent of those calculated by Vero cell assay using the same standard. Data from Lab 1 (Figure 1(a)) show a similar trend, but this set of data is more variable as the *in vivo* potencies were in the range of 20-90 per cent of the potencies determined by Vero cell assay. This variability can be either method - or vaccine - related since the guinea pigs were over-immunised with vaccine F and vaccine D was only used for immunisation of guinea pigs and serological assays in Lab 1. However, this variability will not affect the potency estimates of vaccines. Although it would need similar information from different laboratories to make firmer conclusions, accurate and precise estimates in the two assays (i.e. Vero cell assay and TNT) could be achieved by using method specific units for the GP reference standard.

3.2. Diphtheria-TNT and Vero cell assay with DI standard

In both laboratories, and as confirmed in the Phase I study for Vero cell assay [5], antitoxin potencies expressed in relation to the DI standard were considerably smaller than those expressed in relation to the GP standard. This was also observed in TNT *in vivo*. But, whereas the ratios between potencies obtained with GP and equine British or DI standards in TNT were close to 4 in both laboratories, these were 12.5 to 15-fold different in Vero cell assay (Tables 2, 3). Although the laboratories used different equine standards

Table 3 - *Diphtheria potency results from Laboratory 2*

Vaccine code	Vaccine dose (IU/ml)	Vero cell assay potency* (IU/ml)		ELISA potency* (IU/ml)	*In vivo* potency (TNT) (IU/ml)		TNT/VCA**	TNT/ELISA***
		(Ref: GP)	(Ref: DI)		(Ref: GP)	(Ref: DI)		
R1	16.0	3.3	0.12	2.4	2.07	0.52	63 %	86 %
R2	6.4	1.6	0.06	1.0	0.96	0.24	60 %	96 %
R3	2.6	0.5	0.02	0.3	0.28	0.07	56 %	93 %
R4	1.0	-	-	-	-	-	-	-
C1	16.3	4.1	0.11	1.9	2.42	0.60	59 %	127 %
C2	6.5	1.9	0.05	1.3	1.31	0.33	69 %	101 %
C3	2.6	0.8	0.02	0.4	0.48	0.06	60 %	120 %
C4	1.0	0.1	-	-	-	-	-	-
E1	16.6	6.6	0.17	1.6	3.84	0.96	58 %	240 %
E2	6.6	2.3	0.06	1.2	0.96	0.24	42 %	80 %
E3	2.7	0.6	0.02	0.4	0.38	0.09	63 %	95 %
E4	1.1	-	-	0.1	-	-	-	-
F1	16.0	7.7	0.20	2.8	4.84	1.21	63 %	173 %
F2	6.2	3.8	0.10	1.7	2.42	0.60	64 %	142 %
F3	2.5	1.4	0.04	0.8	1.04	0.26	74 %	130 %
F4	1.0	0.1	-	0.2	0.05	-	50 %	25 %
G1	16.0	6.6	0.25	3.3	3.84	3.08	58 %	116 %
G2	6.4	3.3	0.12	2.0	1.92	0.48	58 %	96 %
G3	2.6	1.6	0.06	1.0	0.96	0.24	60 %	96 %
G4	1.0	0.1	-	0.3	0.1	0.09	100 %	33 %

** calculated at EDQM (taken from report on Phase I)*

*** in vivo potency (ref: GP) as per cent of Vero cell assay potency (ref: GP)*

**** in vivo potency (ref: GP) as per cent of ELISA potency (ref: GP)*

- values are < 0.01

Pearson's correlation coefficients (r):

Between *in vivo* potencies (ref: GP) and Vero cell assay potencies (ref: GP), *r = 0.993*

Between *in vivo* potencies (ref: GP) and ELISA potencies (ref: GP), *r = 0.862*

Between *in vivo* potencies (ref: DI) and Vero cell potencies (ref: DI), *r = 0.971*

(Calculation of these correlation coefficients excludes results for R4, C4, E4, F4 & G4)

for TNT, very similar estimates for serum pools were obtained from immunisations with the same vaccines. This, however was not the case in the Vero cell assay with the equine standard, where estimates obtained in Lab 1 were generally higher than those obtained in Lab 2 (Tables 2, 3). There are several possible reasons that may have contributed to this observation, e.g. different toxin and Vero cell methods or different concentrations of bovine serum used in the two methods. Potency estimates determined by TNT and in Vero cell assay with equine standard were not similar, although correlation was high r = 0.925 (Lab 1) and r = 0.971 (Lab 2). Generally in both laboratories, estimates by TNT were higher than those obtained in Vero cell assay, and this was more pronounced in Lab 2 even though identical standard and toxin were used in the two methods. As concluded in Phase I study [5], estimates obtained in Vero cell assay, with the DI standard, were not predictive of the TNT. In fact, when Lab 1 used *in vitro* information, it failed to accurately predict neutralising potency in some samples (C3 and D3 in Table 2). These results are consistent with those previously observed [5], indicating that lower antitoxin potency estimates are obtained *in vitro* with hyperimmune horse antitoxin as standard.

Previous studies [9,10] have identified the need to use the same ratio of toxin to antitoxin in the Vero cell assay and TNT in order to obtain comparable information. Only Lab 2 used the same toxin in TNT *in vivo* and in Vero cell assay, but in both laboratories the ratio of toxin to antitoxin *in vivo* was higher than in Vero cell assay. Although increasing the toxin dose in order to minimise the ratio between toxin and antitoxin may provide data with higher accuracy in TNT, it will however, also compromise the sensitivity of detection. A lower sensitivity assay will not be satisfactory for the purpose for which this assay is proposed. It must be emphasised that previous studies aimed at replacement of the method for the USA minimum potency test [3] are not suitable for this application. In those studies, antitoxin potencies of guinea pig sera derived from animals immunised with 0.75 ml of undiluted vaccines are used. In the present study, guinea pigs are immunised with approximately $1/10^{th}$ of the concentration used in the USA minimum potency test [3]. Therefore, the highest dose and antitoxins from immunised animals have lower titres.

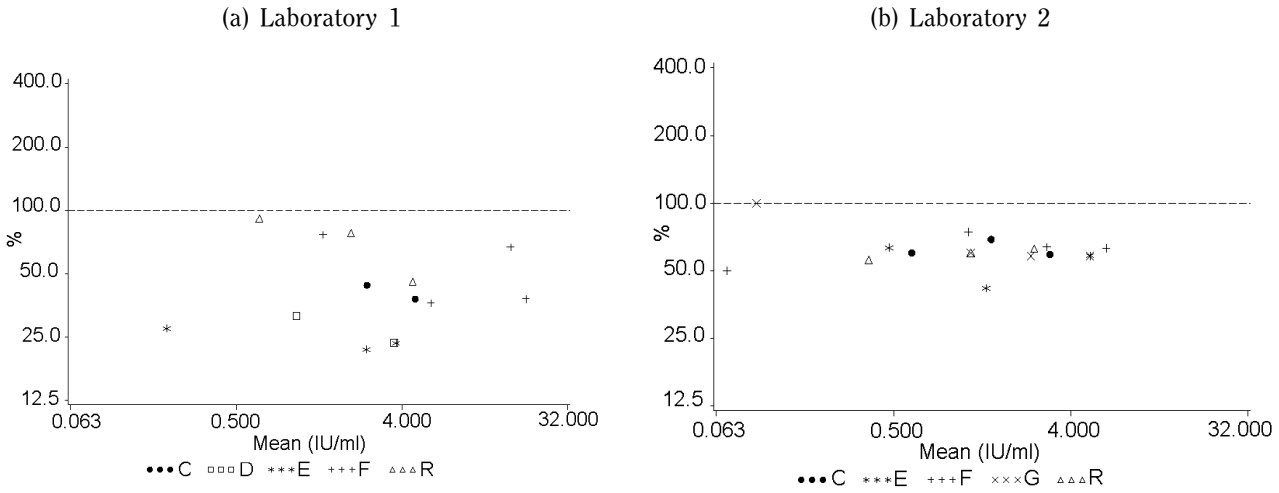

Figure 1 - *Diphtheria TNT antitoxin potencies as per cent of Vero cell assay potencies - Plots of TNT diphtheria antitoxin potencies expressed as per cent of Vero cell potencies for 16 serum pools. Results are expressed in relation to GP reference (98/572) for Lab 1 (a) and Lab 2 (b).*

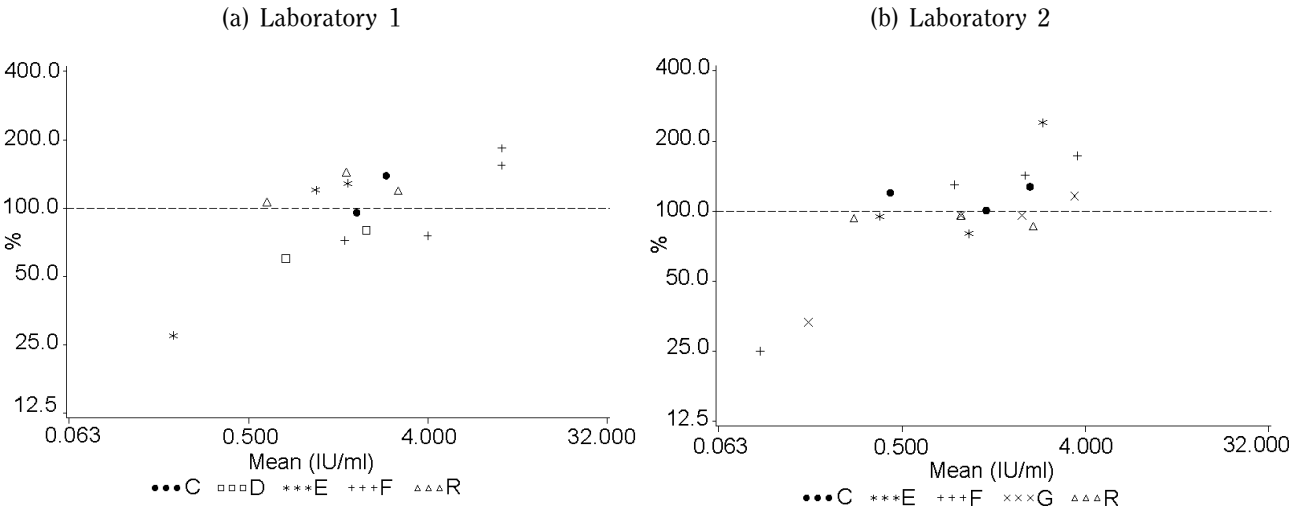

Figure 2 - *Diphtheria TNT antitoxin potencies as per cent of ELISA potencies - Plots of TNT diphtheria antitoxin potencies expressed as per cent of D-ELISA potencies for 16 serum pools. Results are expressed in relation to GP reference (98/572) for Lab 1 (a) and Lab 2 (b).*

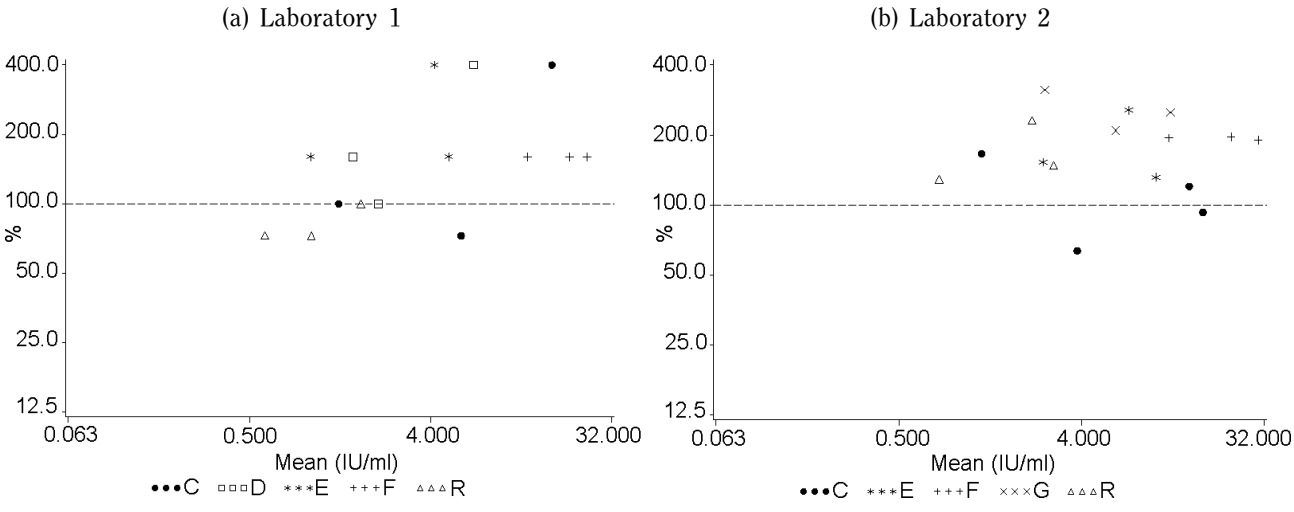

Figure 3 - *Tetanus TNT antitoxin potencies as per cent of ELISA potencies - Plots of TNT tetanus antitoxin potencies expressed as per cent of T-ELISA potencies for 16 serum pools. Results are expressed in relation to Ph. Eur. BRP guinea pig anti-tetanus reference for Lab 1 (a) and Lab 2 (b).*

Table 4 – *Tetanus results from Laboratories 1 and 2*

Vaccine code	Potency (IU/ml)				*In vivo TNT potency as % of ELISA potency*	
	Lab 1		Lab 2			
	TNT	ELISA*	TNT	ELISA*	*Lab 1*	*Lab 2*
C1	32.4	8.1	15.45	16.6	*400*	*93*
C2	4.87	6.7	15.03	12.5	*73*	*120*
C3	1.4	1.4	3.05	4.8	*100*	*64*
C4	·	·	1.66	1.0	·	*166*
D1	13.2	3.3	·	·	*400*	·
D2	2.2	2.2	·	·	*100*	·
D3	2.08	1.3	·	·	*160*	·
E1	6.24	3.9	10.8	8.2	*160*	*132*
E2	8.4	2.1	11	4.3	*400*	*256*
E3	1.28	0.8	3.21	2.1	*160*	*153*
F1	·	·	41.4	21.8	·	*190*
F2	30.72	19.2	30.9	15.7	*160*	*197*
F3	25.12	15.7	15.15	7.8	*160*	*194*
F4	15.52	9.7	·	·	*160*	·
G1	·	·	17.54	7.0	·	*251*
G2	·	·	8.57	4.1	·	*209*
G3	·	·	4.69	1.5	·	*313*
R1	1.8	1.8	3.55	2.4	*100*	*148*
R2	0.87	1.2	3.47	1.5	*73*	*231*
R3	0.51	0.7	0.9	0.7	*73*	*129*

** calculated at EDQM (taken from Phase I report)*

Ph. Eur. BRP [8], was used as a reference standard in both assays

Pearson's correlation coefficients (r):

Between *in vivo* potencies and ELISA potencies:

Lab 1: *r = 0.855*

Lab 2: *r = 0.910*

(Calculation of these correlation coefficients excludes results for the 4th dose for all vaccines except for vaccine F in laboratory 1 where F1 was excluded).

3.3. Diphtheria-TNT and D-ELISA

Plots of TNT potencies, expressed as per cent of *in vitro* potencies obtained by D-ELISA are shown in Figure 2(a) (Lab 1) and Figure 2(b) (Lab 2). Pearson's correlation coefficients are r = 0.955 and r = 0.862 for Lab 1 and Lab 2 respectively, and in this case potency estimates were not different from each other. *In vivo* potencies calculated with GP standard were close to 100 per cent of ELISA potencies in many cases. ELISA appears to be more accurate in predicting potency determined by TNT with the same GP standard. Data from Tables 2 and 3 confirm that antitoxin titres calculated by ELISA were very similar for the same vaccines and doses in both laboratories, although the sera were derived from separate in-house studies. Differences between potencies obtained by TNT and Vero cell assay were not observed in ELISA. This observation would suggest that the ELISA assay is more robust and less sensitive to the ratio of toxin to antitoxin and avidity/affinity profile of antibodies. However, good correlation is highly dependent on use of "equivalent" species-specific reference antitoxin standard. Recent studies [13] have also confirmed high correlation with toxoid binding inhibition test (ToBI) and TNT for serum samples of guinea pigs immunised with different lots of diphtheria tetanus or diphtheria tetanus pertussis combination vaccines (TD and DTP), confirming that *in vitro* serological assays using diphtheria toxoid as the antigen can provide information comparable to that of TNT. It should be pointed out that, although the study of Marcovistz et al., [11] confirmed good correlation between TNT and diphtheria toxoid ToBI assay, the range of antitoxin titre was less than 2-fold apart. In this study antitoxin titres were close to 100-fold apart (Figures 1 and 2).

3.4. Tetanus-TNT and T-ELISA

Summary results of the correlation between potencies obtained *in vivo* TNT and in T-ELISA, using BRP guinea pig standard, are shown in Table 4. The data is presented graphically in Figure 3, where *in vivo* potency is expressed as per cent of T-ELISA potency. Pearson's correlation coefficients are high with r = 0.86 and 0.91 for Lab 1 and Lab 2 respectively (Table 4). Antibody titres, which were in the range of approximately 1.0 to 30 IU/ml, were mostly higher when determined by TNT than by ELISA, in both laboratories. The difference between TNT and ELISA titres were approximately 2-fold in most cases, but up to 4-fold higher values were calculated by TNT for few samples. The variability of estimates however, did not relate to vaccine type or vaccine immunising dose. TNT assays of different sensitivities were used in Lab 1 and Lab 2, but this did not appear to affect the results in any way. Lab 2 included the IS for tetanus antitoxin, and calibration values were comparable to those obtained with BRP (data not shown). It is also worth noting that a good comparison between TNT and ELISA was obtained for vaccine C (DTaP IPV- Hib) in both laboratories, but only Lab 1 included Hib component Polyribosylribitol phosphate (PRP) conjugated to tetanus toxoid (TT) to the combination. The presence of Hib in vaccine C did not appear to result in higher anti-tetanus antibodies in Lab 1.

The observations made in the present studies thus confirm what was found in previous tetanus serology validation studies organised by the EDQM and supported by ECVAM [12] where high correlation between TNT and ELISA was reported, particularly for serum pools. The current study analysed a wider range of vaccines at several doses and at suitable concentrations for use in potency testing, thereby confirming the adequacy of serology for batch potency testing of tetanus vaccines for human use.

3.5. Calibration of diphtheria guinea pig reference standard (98/572)

From the *in vivo* TNT studies, performed in guinea pigs in the two laboratories, it can be concluded that, in relation to equine British (66/153) and DI (00/462) standards, the activity of the GP reference (98/572) is closer to 1.0 IU/ampoule (range 0.75-1.5). The activity of 3.1 IU/ampoule for diphtheria antitoxin content of GP was based on TNT calibration in 4 laboratories in relation to WHO IS lot no. 97/762. The discrepancy in estimates may have resulted from inaccurate activity determination in the previous lot of IS at the time of calibration. Another possible reason for higher estimates in the previous study could have resulted from the method of detection, as some laboratories used the lethal challenge procedure. This appears to be less likely, as the range of determined potency was 2.5-3.7 IU/ampoule for both methods. However, re-calibration of the guinea pig reference reagent will not have any impact on its use in expression of potency of vaccines, as concluded from Phase II validation study [1].

4. CONCLUSIONS

Guinea pig serum pools derived from 5 vaccines, representing combinations of diphtheria, tetanus, pertussis (cellular or acellular) poliomyelitis (inactivated) and/or haemophilus type b conjugate components (Td, DTaP, DTaP IPV Hib and

DTwP), obtained by immunisation at 4 doses were used in the study.

Vero cell assay, with the GP standard, provided diphtheria antitoxin potencies that highly correlated to TNT in guinea pig. Vero cell assay, with the DI, provided antibody potencies that correlated to TNT, but were of lower titre. These results were confirmed in two laboratories with 2 different sets of 20 serum samples.

D-ELISA with the GP standard and diphtheria toxoid as a coating antigen, also provided antitoxin potencies that were highly correlated to TNT and these were in agreement with those determined by TNT, in both laboratories.

T-ELISA with the guinea pig anti-tetanus serum BRP standard, and tetanus toxoid as a coating antigen, provided potency estimates for tetanus antibodies, which were highly correlated to neutralising potency as determined by TNT in mice, in both laboratories. However, unlike diphtheria, the estimates were generally higher in TNT assay.

The study confirmed that, in order to obtain accurate prediction values between ELISA and TNT, a species-specific reference standard is required. However, calibration of a guinea pig reference for use in vaccine potency by serology is not critical because the potency of a vaccine is expressed in relation to the units assigned by comparison to the relevant reference vaccine.

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