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In vitro antigen ELISA for quality control of tetanus vaccines

Laura Coombes [a], Rob Tierney [a], Peter Rigsby [b], Dorothea Sesardic [a], Paul Stickings [a,*]

[a] Division of Bacteriology, National Institute for Biological Standards and Control, Health Protection Agency, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK
[b] Biostatistics Section, National Institute for Biological Standards and Control, Health Protection Agency, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK

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ABSTRACT

Consistency of production is recognised as an important aspect of vaccine manufacture and suitably validated *in vitro* assays are required for quality control testing of these products. For the manufacture and batch release of tetanus vaccines, antigen content and integrity, and degree of adsorption of antigen to the adjuvant are critical parameters that should be monitored for consistency. Here we describe the development and use of an Enzyme Linked Immunosorbent Assay (ELISA) to quantify tetanus antigen in combined vaccine products and to measure the degree of adsorption of antigen to adjuvant. Whilst the antigen assay cannot be assumed to predict potency for different products, it can be used as part of a panel of *in vitro* methods to provide a more informative product profile and to monitor trends in production. The antigen assay is particularly valuable for providing quantitative information on every final lot when modifications of *in vivo* potency tests, such as single dilution assays, are used.

1. Introduction

Tetanus is caused by the action of the highly potent neurotoxin, tetanus toxin (TTxn), which is produced under anaerobic conditions by the bacterium *Clostridium tetani*. Immunity to tetanus is mediated by antibodies against the toxin and is conferred only by active or passive immunization since the pathogenic dose of TTxn is too low to confer protection against subsequent exposure. Active immunization programmes against tetanus are based on the use of a formaldehyde inactivated preparation of TTxn (tetanus toxoid, TTxd) to induce a protective antibody response. For immunization of children, tetanus is given as a combined diphtheria-tetanus-pertussis (DTP) vaccine or diphtheria-tetanus-acellular pertussis (DTaP) vaccine. These combination vaccines may also include inactivated polio virus (IPV), *Haemophilus influenzae* type B capsular polysaccharide (Hib) and hepatitis B surface antigen (HepB) components. Tetanus toxoid has been included (as part of the DTP combination) in the WHO Expanded Programme on Immunization since its inception in 1974. It is a highly effective vaccine and maternal and neonatal tetanus has largely been eliminated in populations with effective immunization programmes [1].

The classical tetanus toxoid vaccine is produced via a number of steps including cultivation of a suitable strain of C. tetani, toxin harvest by filtration, detoxification using formaldehyde, concentration and purification of the toxoid, and adsorption onto a mineral carrier [2]. Detoxification using formaldehyde results in intramolecular and intermolecular crosslinking between toxin molecules and may also form crosslinks with foreign proteins present in the culture medium, resulting in a complex heterogeneous product that is poorly defined [3]. Final products are further complicated by the addition of adjuvant, stabilizer and preservative, as well as by combination with other antigens in complex combination vaccines [2]. The effect of these interactions on tetanus immunogenicity is not well understood. As such, the quality control testing of these products, by both manufacturers and regulatory bodies, relies heavily on *in vivo* assays to ensure safety and potency [4,5].

The need for validated *in vitro* assays for quality control of toxoid vaccines is, however, also well recognized, and the availability of improved and emerging technologies means that toxoid vaccines can be well characterized using *in vitro* methods [6,7]. The application of these methods together with well defined manufacturing quality systems (Good Manufacturing Practice, GMP) forms the basis of the consistency approach [8] which could lead to a reduction in animal use for routine batch release of well characterized vaccines in the long term. Some regulatory guidelines (such as those published by the WHO and European Pharmacopoeia) already allow for a modification of *in vivo* potency tests once consistency of production has been demonstrated [4,5]. In such cases, there is an increased emphasis on *in vitro* methods to provide information on product quality particularly where there is a loss of quantitative information (that is suitable for trend analysis) on consecutive batches – for example where single dilution assays are

* Corresponding author. Tel.: +44 (0) 1707 641446; fax: +44 (0) 1707 641054.
E-mail address: Paul.Stickings@nibsc.hpa.org.uk (P. Stickings).

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used only to demonstrate that the potency of the batch significantly exceeds a certain threshold.

Antigen (toxoid) content and degree of adsorption to adjuvant are key factors to be evaluated as part of consistency testing of tetanus vaccines [2,9]. We have previously published details of an *in vitro* ELISA assay for monitoring the diphtheria antigen content and degree of adsorption in combination vaccines [10]. Similar ELISA methods have been described previously for the qualitative and quantitative characterization of TTxn and TTxd, both during production and in final preparations of tetanus vaccines [11–13]. Here we present the results of the in-house development of an ELISA assay that can be used to measure the antigen content and degree of adsorption in adsorbed tetanus vaccines. We examined the suitability and limitations of the assay for characterizing and monitoring tetanus vaccines and for measuring tetanus antigen content in polysaccharide conjugate vaccines where TTxd is used as a carrier protein. The antibodies used in the ELISA assay for capturing and detecting the tetanus antigen have been stabilized by freeze-drying, providing a stock of standard reagents that can be used by other control laboratories and vaccine manufacturers.

2. Materials and methods

2.1. Production of monoclonal antibody

Anti-tetanus monoclonal antibody (TT10), which is used as the capture antibody in the antigen ELISA, was produced from rat hybridoma cell lines, provided by Wellcome Research Laboratories (Beckenham, U.K.). The hybridomas were prepared and the antibody characterized as described previously by Sheppard *et al* [14].

For production of the freeze-dried standard, the monoclonal antibody stock was purified by 50% ammonium sulphate precipitation, followed by dialysis overnight against three changes of phosphate buffered saline (PBS, pH 7.4). The antibody stock was filter sterilized (0.22 μM) and sodium azide was added to a final concentration of 0.02% w/v. Antibody stocks were stored at −20 °C prior to filling into ampoules. Before filling, the antibody was titrated in the capture ELISA to determine the optimum working dilution, and the material for filling was dialysed (as above) to remove sodium azide. The antibody was diluted in PBS (pH 7.4) containing 0.5% w/v human serum albumin (HSA) and 0.1% w/v trehalose (Sigma, U.K.) so that one filled ampoule would be sufficient for approximately 10 ELISA plates with a 1/200 working dilution. Filling (0.5 ml per 3 ml DIN ampoule) was performed on a Bausch and Strobel AFV 5090 filling line (Bausch & Strobel, Ilshofen, Germany) at +4 °C with constant stirring within NIBSC's Standards Processing Division. Filled ampoules were freeze-dried using a Sorial CS15 freeze-dryer (Sorial, Argentueil, France) and flame sealed. The finished product was coded 10/134 and stored at −20 °C.

2.2. Production of polyclonal antibody

The polyclonal antibody is used as the primary detecting antibody in the capture ELISA. Polyclonal antiserum against tetanus toxoid was prepared in-house by immunizing female guinea pigs (Dunkin Hartley strain, 250–350 g, from Harlan UK), with a monovalent tetanus vaccine diluted in normal saline (1 Lf/dose). Animals were boosted 6 weeks later with the same dose. Immunization was performed by subcutaneous (s.c.) injection in a total volume of 1 ml. All animals were bled 3 weeks after the booster dose and the serum collected from the clotted blood.

For production of the freeze-dried polyclonal antibody standard, the serum stock was titrated in the capture ELISA to determine the optimum working dilution. The antibody stock was diluted in PBS (pH 7.4) containing 0.5% w/v HSA and 0.1% w/v trehalose so that one filled ampoule would be sufficient for approximately 10 ELISA plates with a 1/200 working dilution. Filling was performed as described previously for the monoclonal antibody. Filled ampoules were freeze-dried using a Sorial CS100 freeze-dryer and flame sealed. The finished product was coded 10/132 (tetanus polyclonal antibody) and stored at −20 °C.

2.3. Post-fill characterization of freeze-dried antibodies

Freeze-dried antibodies were examined for appearance, residual moisture content and oxygen head space. The precision of fill was determined by weighing representative ampoules throughout the production run. Measurement of the mean oxygen head space after sealing served as a measure of ampoule integrity. The mean oxygen head space was measured using a Headspace Oxygen Analyzer Model FMS-760 (Lighthouse Instruments LCC, Charlottesville, USA). Residual moisture was measured using the coulometric Karl Fischer method (Mitsubishi CA-100, A1-Enviroscienses, Luton, UK) and expressed as a percentage of the mean dry weight of the ampoule contents.

2.4. Vaccine products and sample preparation

A range of TTxd containing vaccines (TT, Td, DT, DTP, DTP-HepB, DTP-HepB-Hib, DTaP-IPV-Hib, DTaP-IPV-HepB) from 7 different manufacturers (coded A–G) were tested for total and non-adsorbed tetanus antigen content. To measure the total antigen content, vaccines were desorbed using 10% w/v tri-sodium citrate as described previously [10]. Non-adsorbed antigen was measured in the supernatant of the adsorbed vaccine following centrifugation to pellet the adjuvant. Combination products where the Hib component (conjugated to TTxd, Hib-TT) was provided separately were tested both with Hib-TT (single dose reconstituted in 0.5 ml of tetanus combination vaccine) and without.

Polysaccharide conjugate bulk concentrates (non-adsorbed) containing TTxd as the carrier protein were also tested for tetanus antigen content. Samples of Hib-TT, meningococcal group A (MenA-TT), C (MenC-TT), W (MenW-TT) and Y (MenY-TT), and pneumococcal serotype 18C (pneumo 18C-TT) were assayed in the capture ELISA to determine if the tetanus antigen could be detected after conjugation with polysaccharide. The analogous, un-conjugated bulk TTxd was also assayed for the Hib product.

2.5. Reference toxoid for capture ELISA

The 2nd WHO International Standard for tetanus toxoid for use in flocculation test (NIBSC code 04/150, 690 flocculation units [Lf] per ampoule [15]) was used as the reference toxoid in the antigen assay. Each ampoule of 04/150 was reconstituted in 1 ml H_2O to give a stock concentration of 690 Lf/ml.

2.6. Capture (sandwich) ELISA for tetanus toxoid

The capture ELISA was essentially performed as described previously [16,17]. Test samples and reference toxoid were titrated (in the range of approximately 0.1–0.0005 Lf/ml, using serial two-fold dilutions) on plates coated with the anti-tetanus monoclonal antibody (1/200 dilution of NIBSC working standard 10/134 reconstituted in 0.5 ml H_2O). The amount of antigen bound to the monoclonal antibody was visualised by successive incubations with polyclonal antibody against tetanus toxoid (1/200 dilution of NIBSC working standard 10/132 reconstituted in 0.5 ml H_2O), HRP-labelled antibody, and substrate. Following colour development, optical density was measured at 405 nm. Antigen content was calculated relative to the reference toxoid by parallel line analysis (log optical density vs. log dose), using a minimum of 3 sequential

points from the linear section of the dose–response curve for the reference and test sample, and expressed in Lf/ml. Analysis of variance was used to test for any significant deviation from linearity or parallelism of the dose response relationship ($p < 0.01$). The degree of adsorption was calculated by subtracting the estimate for non-adsorbed antigen content from the estimate for total antigen content, and expressed as a percentage of the total.

2.6.1. ELISA specificity and sensitivity

Other antigens commonly found in combination vaccines together with TTxd were assayed by ELISA to confirm specificity for the tetanus antigen. Purified diphtheria toxoid (DT), whole cell pertussis (wP), acellular pertussis antigens (pertussis toxoid (PT), filamentous haemagglutinin (FHA), pertactin (PRN), fimbriae 2/3 (FIM)), HepB antigen and trivalent IPV concentrates were assayed at concentrations similar to that found in final vaccines: DT (30 Lf/ml), wP (8 IU/ml), PT (40 μg/ml), FHA (40 μg/ml), PRN (6 μg/ml), FIM (10 μg/ml), HepB (25 μg/ml) and IPV (80, 16 and 64 D-antigen units/ml of type-1, type-2 and type-3, respectively). The sensitivity of the assay was established from the lowest point on the linear section of the standard curve for the reference toxoid.

2.6.2. Assay precision and linearity

The intra-assay (within-plate and between-plate) variability and inter-assay variability were established to assess the repeatability of the ELISA. Within-plate variability was determined by assaying 10 replicates of the same non-adsorbed toxoid sample on one plate (along with the reference toxoid in duplicate). The variation between samples was calculated using the standard deviation (SD) of $\log_{10}$ antigen values and was expressed as the geometric coefficient of variation (GCV, defined as $[10^{SD}-1] \times 100\%$). 6 plates were tested per day and the assay was repeated on 4 consecutive days. The data for all 24 plates was pooled to obtain a single estimate of within-plate variance. The GCV between-plates and between-days were determined similarly, comparing the log geometric mean antigen concentration obtained for each plate on the same day ($n = 6$, data pooled from the 4 days), and the log geometric mean antigen concentration obtained for each day ($n = 4$), respectively.

Repeatability of the desorption step was also assessed using a TT vaccine ($AlPO_4$ adjuvant), a DT vaccine ($Al(OH)_3$ adjuvant) and a DTaP-IPV-Hib vaccine ($AlPO_4$ adjuvant). A pool of each vaccine was prepared to provide enough material for 24 replicates each to be desorbed independently. 8 replicates were tested per day on one plate (outer columns excluded) and the assay was repeated on 3 separate days. The intra-assay (within-plate) and inter-assay GCV was assessed as described above. Linearity analysis was also carried out for these vaccines, tested on 3 separate days, using five target concentration levels (in the range of 2.2–35.9 Lf/ml for TT, 0.2–3.8 Lf/ml for DT and 0.7–11.0 Lf/ml for DTaP-IPV-Hib).

2.7. In vivo potency

Vaccine potency was determined using established *in vivo* methods [4,5] and expressed in International Units (IU) per single human dose (SHD), where units are defined by the International Standard for Tetanus Toxoid Adsorbed [18]. All animal procedures were subject to ethical review and performed in accordance with local rules and UK Home Office regulations.

3. Results

3.1. Post-fill characterization of freeze-dried antibody reagents

The lyophilized product for the monoclonal and polyclonal working standards was of good appearance, with a robust and homogenous cake. For 10/134 (tetanus monoclonal antibody) the mean fill mass was 0.509 g with a coefficient of variation (CV) of 0.22% ($n = 84$), and for 10/132 (tetanus polyclonal antibody) the mean fill mass was 0.511 g with a CV of 0.18% ($n = 473$). The mean oxygen head space was determined as 0.25% for 10/134 ($n = 6$) and 0.40% for 10/132 ($n = 12$). The mean residual moisture content was determined to be 0.53% for 10/134 ($n = 6$) and 0.49% for 10/132 ($n = 12$). The freeze-dried material was tested in the capture ELISA (by titration of test samples against the reference toxoid) to confirm that biological activity was recovered relative to the liquid antibody stocks (not shown).

3.2. Assay performance

The tetanus ELISA as described here was found to be highly sensitive, with a minimum value of 0.001 Lf/ml observed on the linear section of the standard curve (linear section ranging from 0.001 to 0.017 Lf/ml). Other relevant antigens (DT, wP, PT, FHA, PRN, FIM, HepB and IPV), tested at concentrations commonly found in combination vaccines, were all below this minimum value in the capture ELISA confirming the specificity of the assay for tetanus antigen (not shown).

ELISA repeatability was determined by assaying the same non-adsorbed TTxd across several plates on consecutive days. The GCV of the estimate for tetanus antigen content was calculated within-plates and between-plates to assess the intra-assay variability and was found to be 6.4% and 2.7%, respectively. The variability observed between assays was similar to that observed between-plates tested on the same day, with an inter-assay GCV of 2.2%. From this data, an edge effect was observed across columns within a plate, with outer columns returning higher antigen estimates than columns in the centre of the plate (not shown). The within-plate GCV was improved to 4.5% when the results for samples in the outer columns (1 and 12) were excluded from the analysis. The between-plate and inter-assay GCV remained low (2.9% and 1.9%, respectively). To determine the effect of the desorption process on assay repeatability, the within-plate and inter-assay variability for 3 different adsorbed vaccine products was compared to that obtained for the non-adsorbed TTxd (outer columns excluded). All replicates for the adsorbed vaccine products were desorbed independently. Results show that the desorption process was highly reproducible, with the GCV for the adsorbed products being within 2% of that obtained for the non-adsorbed toxoid (Table 1). Linearity analysis was also performed for these 3 vaccine products and found to be acceptable (Table 2 and Fig. 1). Agreement between the observed and target values was good for all three vaccines, with fitted slopes close to 1.0, intercepts close to 0 and high values of r^2 (>0.99).

3.3. Applications for the capture ELISA antigen assay

3.3.1. Routine consistency monitoring/product profiling

The ELISA was used to determine the tetanus antigen content and degree of adsorption for a range of vaccine products from 7 different manufacturers, as summarised in Table 3. Valid estimates for antigen content were obtained for all products tested, with the dose response lines of the test samples and reference toxoid satisfying the validity criteria for regression, parallelism and linearity. The total amount of tetanus antigen measured (Lf/ml) and the degree of adsorption to adjuvant was product-specific. The amount of tetanus antigen measured using the ELISA ranged from 4.6 Lf/ml to 17.8 Lf/ml and was different from the labelled Lf content for most of the products tested. Some products adsorbed onto aluminium phosphate had as little as 11% of this antigen adsorbed (DTP-HepB-Hib, manufacturer E), whereas the vaccine products adsorbed onto aluminium hydroxide adjuvant were 98–100% adsorbed (Table 3).

Table 1
Precision of antigen estimates for adsorbed vaccine products compared to non-adsorbed TTxd.

Sample	Geometric mean antigen (GM, Lf/ml) and within-plate GCV (%)								Pooled within-plate GCV (%)	Inter-assay GCV (%)
	Day 1		Day 2		Day 3		Day 4			
	GM	GCV	GM	GCV	GM	GCV	GM	GCV		
TTxd	7266.3	4.4	7353.2	4.9	7040.6	3.4	7149.8	5.3	4.5	1.9
TT vaccine[a]	35.4	5.0	35.7	5.5	36.5	4.3	–	–	4.9	1.6
DT vaccine[b]	3.7	4.3	3.9	3.2	3.7	5.2	–	–	4.3	2.8
DTaP-IPV-Hib vaccine[a]	10.7	5.6	11.3	5.1	11.0	6.4	–	–	5.7	2.8

Within-plate GCV for the adsorbed vaccine products represents variability of desorption as well as variability across plates. TTxd results for within-plate GCV on individual days are based on pooled values from 6 plates.
[a] Vaccine adsorbed onto $AlPO_4$ adjuvant.
[b] Vaccine adsorbed onto $Al(OH)_3$ adjuvant.

Linear regression trend analysis was used to determine consistency of production for a pentavalent combined vaccine product tested over a four year period. Data shows consistent antigen content and degree of adsorption between batches ($n = 57$), with no significant increasing or decreasing trend detected (Fig. 2). A total of twenty batches of this product were tested *in vivo* to determine potency and the variability of these potency estimates (GCV) was 78.8%. The variability of estimates for total antigen content and degree of adsorption for the same batches tested in the *in vitro* antigen ELISA was 8.4% and 13.1%, respectively.

3.3.2. Relationship between antigen content, degree of adsorption and potency

There was no clear correlation between antigen content measured *in vitro* (or the degree of adsorption) and the potency determined *in vivo*. For products where the tetanus antigen was almost completely adsorbed to the adjuvant (notably the vaccines adsorbed to aluminium hydroxide), the *in vivo* potency was comparable to other products where the degree of adsorption was much lower (Table 3). The DTP vaccines from manufacturer A and C had a 2-fold difference in antigen content measured *in vitro*, but comparable *in vivo* potency. Comparison of two TT vaccines shows that the product with the higher tetanus antigen content (measured *in vitro*) had a lower *in vivo* potency (unpaired *t*-test, $p = 0.007$). Both products showed a similar degree of adsorption (54.2 and 57.1%). For the all-in-one products containing a Hib-TT component (Hib polysaccharide conjugated to TTxd) the relationship between antigen content (measured by the capture ELISA) and potency was even less clear. The DTP-HepB-Hib product from manufacturer A had the highest *in vivo* potency of all the products tested (geometric mean 624 IU/SHD) but the (equal) lowest *in vitro* antigen content of 4.6 Lf/ml. A comparable product from manufacturer D had the same *in vitro* antigen content (4.6 Lf/ml), but a significantly lower geometric mean potency *in vivo* of 197 IU/SHD (unpaired *t*-test, $p = 0.026$).

To directly investigate the effect of a Hib-TT component on the measurement of *in vitro* antigen content and *in vivo* potency, the DTP and DTP-HepB vaccines from manufacturer A were tested with and without the Hib-TT component that is provided in a separate container. The Hib-TT component is lyophilised and was reconstituted (using the appropriate combined vaccine product) immediately prior to use. Fig. 3 shows that when the vaccines were tested together with the Hib component, the *in vivo* potency for three different batches was increased by an average of 116% (DTP + Hib vs. DTP, paired *t*-test $p = 0.0859$) and 139% (DTP-HepB + Hib vs. DTP-HepB, paired *t*-test $p = 0.0015$). However, the amount of tetanus antigen detected in the same batches using the *in vitro* capture ELISA remained unchanged (Fig. 3).

3.3.3. Detection of tetanus antigen in polysaccharide conjugate bulks

Meningococcal (MenA, MenC, MenW, MenY), pneumococcal (serotype 18C) and Hib polysaccharide conjugate bulk concentrates, all conjugated to TTxd, were tested in the capture ELISA for detection of the tetanus carrier protein. Low levels of tetanus antigen were detected for all bulk conjugates (Table 4). For the Hib-TT bulk the corresponding un-conjugated bulk TTxd was also tested in the ELISA assay. The amount of tetanus antigen detected in the ELISA assay for the un-conjugated bulk (diluted to the same protein concentration as in the Hib-TT bulk conjugate) was 100-fold higher compared to the conjugated bulk (Table 4).

Table 2
Linearity analysis for adsorbed vaccine products.

Vaccine type	Slope (*p*-value)	Intercept (*p*-value)	r^2	Residual sum of squares (df)
TT	1.001 (<0.001)	−0.013 (0.348)	0.998	0.006 (13)
DT	0.999 (<0.001)	0.007 (0.187)	0.998	0.005 (13)
DTaP-IPV-Hib	0.981 (<0.001)	0.011 (0.330)	0.996	0.010 (13)

Values shown are calculated from linear regression of observed estimates (log Lf/ml) against target values.

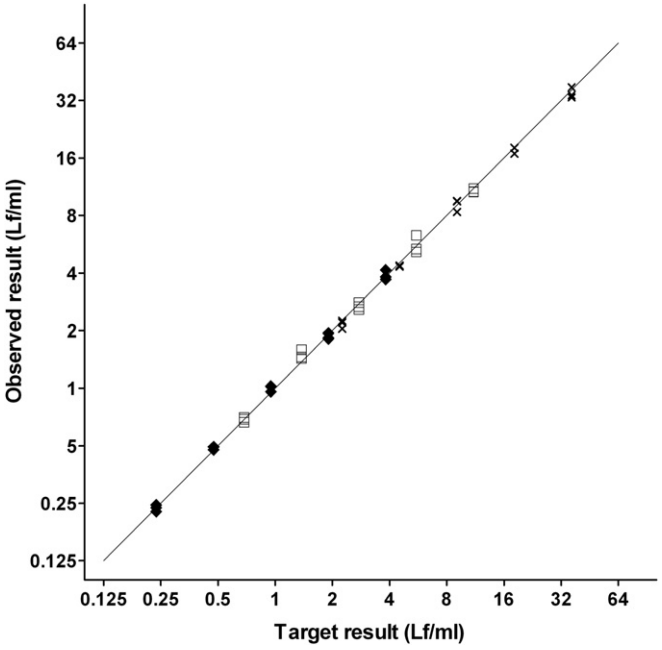

Fig. 1. Linearity analysis results for adsorbed vaccine products TT (cross), DT (solid black diamond) and DTaP-IPV-Hib (clear square); $n = 3$ assays at each target concentration level. Plot includes line of identity (slope = 1.0, intercept = 0).

Table 3
Profile for the tetanus component in combination vaccines.

Vaccine type	Hib-TT component Y/N	Manufacturer (no. of batches)	Adjuvant	TTxd[a] (Lf/ml)	Total antigen Lf/ml	% adsorbed	*In vivo* potency IU/SHD
TT	N	A (5)	$AlPO_4$	20	10.7 (9.2–12.4)	54.2	236 (175–318)[b]
		B (5)	$AlPO_4$	20	15.6 (13.1–18.5)	57.1	127 (89–181)
Td	N	B (3)	$AlPO_4$	15	9.0 (7.3–11.1)	55.6	115 (66–200)
DT	N	C (1)	$Al(OH)_3$	20	5.9	98.3	204
DTP	N	A (3)	$AlPO_4$	15	9.3 (6.7–12.9)	37.6	257 (107–618)
		B (1)	$AlPO_4$	15	11.4	39.5	152
		C (3)	$Al(OH)_3$	20	17.8 (15.4–20.5)	100	259 (234–287)
DTP-HepB	N	A (3)	$AlPO_4$	15	9.0 (8.5–9.6)	34.4	263 (208–333)
DTP-HepB-Hib	Y	A (3)	$AlPO_4$	8	4.6 (3.9–5.5)	28.3	624 (317–1230)
		D (3)	$AlPO_4$	10	4.6 (3.2–6.7)	28.3	197 (55–702)
		E (3)	$AlPO_4$	15	14.0 (11.4–17.3)	10.7	222 (94-523)[c]
DTaP-IPV-Hib	Y	F (20)	$AlPO_4$	10	11.8 (11.4–12.3)	45.8	337 (257–443)
DTaP-IPV-HepB	N	G (1)	$Al(OH)_3$	20	12.2	99.7	141

Total antigen content, expressed in Lf/ml, and percent adsorbed were determined by in-house antigen assay. Percent adsorbed was calculated using geometric mean values obtained for total and non-adsorbed antigen. Tetanus potency in IU/SHD was determined using European Pharmacopoeia method B or method C (DTaP-IPV-Hib vaccine only) [4]. Antigen content and potency data are the geometric mean of the number of batches indicated with 95% confidence intervals (CI), with two exceptions.[b,c]

[a] The TTxd content of the tetanus vaccine component for each product, as stated in the Product Licence.
[b] Geometric mean of 4 batches.
[c] Geometric mean of 2 batches.

4. Discussion

Tetanus vaccines are extensively characterized during all stages of production, including measurement of antigen (TTxd) content and purity, together with measurement of adjuvant content and the degree of adsorption. ELISA assays have been shown to be suitable for the detection and quantification of tetanus antigen [11–13], and we report here the development of an in-house capture ELISA assay and its suitability for use in monitoring trends in production of tetanus vaccines. As part of these studies we prepared a stock of stable freeze-dried antibody reagents used for capture and detection of TTxd and confirmed their suitability for use in the assay. The capture monoclonal antibody used for production of the freeze-dried product (NIBSC code 10/134) was characterized in studies published previously [14,16]. This antibody is directed against the Hc fragment of TTxn and neutralizes TTxn *in vivo*. The monoclonal antibody has comparable binding characteristics for TTxn and TTxd, suggesting that the epitope to which it is directed is not destroyed by treatment with formaldehyde during the detoxification process used for production of toxoid [16]. Related standards (NIBSC 10/128 and 10/130) were also prepared for use in the assay used for diphtheria vaccines which was published previously [10].

The assay presented here is specific for TTxd, highly sensitive and reproducible. The assay has a detection limit of 0.001 Lf/ml (as

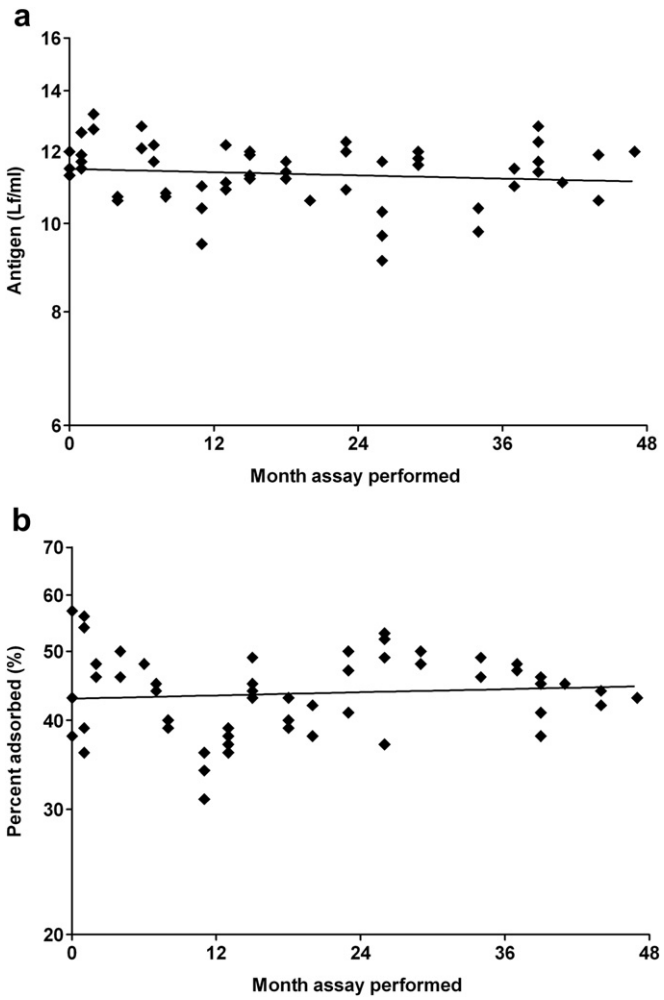

Fig. 2. Trend data for the tetanus component of a DTaP-IPV-Hib combined vaccine. Data shows total tetanus antigen content (a) and percent adsorption (b) for individual final bulks tested over a four year period with a linear regression trendline.

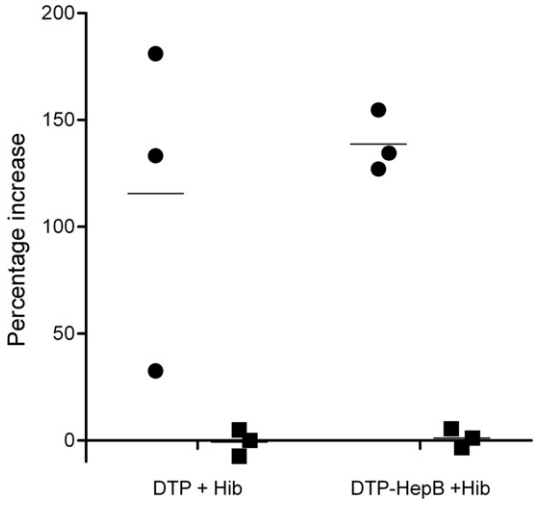

Fig. 3. The effect of Hib-TT on the tetanus potency (black circles) and tetanus antigen content (black squares) of DTP and DTP-HepB vaccines. Data shows the percentage increase compared to the same vaccine lots ($n = 3$) tested without Hib-TT (line at the mean).

Table 4
Detection of tetanus carrier protein in polysaccharide conjugate bulk concentrates.

Polysaccharide conjugate	Manufacturer	Protein (μg/ml)	Tetanus antigen Lf/ml (95% CI)	
			Conjugated bulk	Un-conjugated bulk[a]
Hib-TT	1	432	2.0 (1.9–2.1)	208.4 (197.2–220.2)
MenA-TT	2	300	4.5 (4.1–4.9)	ND
MenC-TT	3	300	3.3 (3–3.6)	ND
MenW-TT	3	300	1.8 (1.7–2.0)	ND
MenY-TT	3	300	0.20 (0.19–0.21)	ND
Pneumo 18C-TT	3	300	0.42 (0.39–0.45)	ND

Protein (μg/ml) was measured using absorption spectroscopy, except for Hib-TT which was obtained from the manufacturer's protocol.

[a] Un-conjugated bulk TTxd was diluted to an equivalent protein concentration as in the Hib-TT bulk conjugate based on the blending protocol.

defined by the linear range of the standard curve), suggesting that it will be suitable for detection of low levels of non-adsorbed antigen in final lot vaccines. This may not be possible with other immunochemical methods such as single radial diffusion (SRD), which has a limit of detection of approximately 4 Lf/ml [19] and flocculation assays where the optimal working range is 30–100 Lf/ml [15]. The variability of the assay (as defined by the geometric coefficient of variation) was low and was reduced further by excluding outer columns on the microtitre plate from the assay. These results are comparable to those obtained for the assay to detect diphtheria toxoid [10]. Because this assay is intended for use with adsorbed tetanus vaccines we investigated whether desorption of antigen from adjuvant (performed prior to detection of antigen in the ELISA) contributed to assay variability. Our results suggest that this desorption step is highly reproducible and the GCV remained below 6% within-plates and below 3% between assays for the adsorbed tetanus vaccines included in the study. The ELISA also showed an acceptable degree of linearity across the range of concentrations tested for the three vaccine products investigated (TT, DT and DTaP-IPV-Hib vaccine).

The assay described here is intended for use in monitoring batch to batch consistency (using the parameters of antigen content and degree of adsorption) and we show that the assay is suitable for testing a variety of adsorbed tetanus vaccines – including combined vaccines containing IPV, acellular or whole cell pertussis components, and vaccines containing HepB and/or Hib components. The total amount of tetanus antigen measured using the antigen ELISA did not always compare closely to the labelled Lf content values for the different products tested. Vaccine manufacturers use flocculation assays to determine antigen content and this is done at the bulk purified toxoid stage where antigen content typically exceeds 2000 Lf/ml. The labelled antigen content of the final product is based on extrapolation from this flocculation value and the total dilution factor of bulk toxoid used for preparation of the final lot – therefore the labelled antigen content is not determined from a direct measurement of antigen content in the final product. The most commonly used method for the flocculation assay (Ramon method) is dependent on a visual (i.e. subjective) measurement of the end point and results are typically reported without any estimate of uncertainty. Therefore any comparison of the results obtained for the final product using the antigen assay and Lf values reported by the vaccine manufacturer should be viewed in this context. The antigen assay described here may be suitable for antigen quantification during vaccine production subject to validation against the flocculation test for a particular product (performed at the same stage of production – i.e. on the bulk purified toxoid). It should also be noted that the values obtained for antigen content using the antigen ELISA are likely to be dependent on the reference toxoid used (NIBSC unpublished observations) and any validation against the flocculation test should therefore be done using the same reference toxoid in both assay methods. For consistency monitoring a non-adsorbed toxoid with defined stability is likely to be suitable as the reference preparation.

The low variability of this assay makes it extremely useful for within-product consistency monitoring. Results from routine monitoring of a pentavalent combined vaccine shows that the tetanus antigen content and degree of adsorption have remained consistent over a four year period with no trends observed. For routine batch release of this product NIBSC has introduced a modified potency assay (a single dilution assay) according to the procedures outlined in the European Pharmacopoeia for the assay of tetanus vaccines [4]. This modified *in vivo* assay provides assurance that the potency of each batch of the product is higher than the minimum requirement, but the data generated from this modified potency test is not useful in terms of monitoring batch to batch consistency (since the test is designed to show that the potency exceeds a threshold and does not provide a point estimate of potency). The use of the *in vitro* antigen assay is therefore important for generating quantitative information that can be used to monitor consistency and identify trends. In addition, the superiority of the *in vitro* assay over the multiple dilution *in vivo* assay for consistency monitoring is evident from results obtained for those batches where potency was determined using a multiple dilution assay where the variability between batches (GCV) was 78.8%. Although the *in vivo* assay confirms the ability of the vaccine to induce a tetanus antibody response it is far less suited for monitoring trends or batch to batch consistency compared to the *in vitro* assay because of the inherent variability associated with animal assays.

The results obtained for the different tetanus vaccines highlight the fact that there is no clear correlation between the amount of tetanus antigen measured (or the degree of adsorption) and the biological activity (potency) of the tetanus component. This was most clearly evident in the results obtained for a DTP-HepB-Hib vaccine from two different manufacturers where the *in vivo* potency was significantly different (>3-fold difference) despite the two products having the same antigen content and degree of adsorption measured using the *in vitro* antigen assay. Factors other than the antigen content and degree of adsorption to adjuvant are likely to influence the potency of the tetanus component measured using a bioassay. Such factors may include the type (e.g. $AlPO_4$ or $Al(OH)_3$) and physical state (e.g. preformed precipitate or formation of precipitate *in situ* with the toxoid) of adjuvant [20], the interaction between one or more components in a combined vaccine [21–23], and the choice of assay system used to determine potency *in vivo* [18,24,25]. The situation is complicated further in that TTxd is used as a carrier protein in several polysaccharide conjugate vaccines, and Hib polysaccharide conjugated to TTxd is present in some combined vaccine products that also contain conventional (i.e. un-conjugated) TTxd. The tetanus potency of these products may be extremely high [23] and we confirmed the direct effect of a Hib-TT conjugate (present in a separate container) on the tetanus potency measured for a DTP vaccine and DTP-HepB vaccine (from the same manufacturer), where potency was increased by more than 100% for both vaccine products in the presence of the conjugate. However, the amount of antigen detected in these vaccines did not change when the Hib-TT conjugate was added. Enhancement of tetanus immune responses measured *in vivo* may not always be observed in the presence of a TTxd-conjugate vaccine [26], but where enhancement is observed our data suggest that the effect of the TTxd-conjugate may not be predicted by measurement of antigen content *in vitro* in the final product.

We further investigated the ability of the antigen ELISA to measure the TTxd content in polysaccharide conjugate vaccine components. Bulk conjugates from a range of different vaccine components (including meningococcal A, C, W and Y serotypes, pneumococcal serotype 18C and Hib polysaccharide) – all conjugated to TTxd – were tested in the assay for detection of the carrier protein. As a control, the un-conjugated bulk carrier protein from the Hib component was tested after dilution to the same protein concentration as that found in the bulk Hib-TT. The un-conjugated carrier protein had a tetanus antigen estimate of 208 Lf/ml (as determined by the antigen assay) and, at the same protein concentration, the amount of tetanus antigen detected in the Hib-TT bulk was less than 1% of this amount. The amount of tetanus antigen measured in the bulk conjugates from the meningococcal and pneumococcal components was also low – ranging from 0.2 to 4.5 Lf/ml. Un-conjugated carrier protein samples were not available for these products, but based on comparison of the total protein content of these samples (300 μg/ml) and the protein content of the Hib component (432 μg/ml) it can be estimated that the recovery of tetanus antigen for these components is between 0.1 and 3% (assuming equivalent purity of the different toxoid samples). These data support our results obtained with the DTP and DTP-HepB vaccines where no increase in antigen was detected on addition of the Hib component (despite an increase of >100% in tetanus potency). Similar findings have been reported elsewhere for a Hib-TT conjugate using a similar assay [12]. The biochemical reasons for this are unclear and could be due to steric hindrance and/or masking of the antibody binding site(s) on the carrier protein after conjugation. It should be noted however that the extent of detection of tetanus carrier protein in all-in-one products containing a polysaccharide component (conjugated to TTxd) will not adversely affect the use of the assay as a within-product consistency tool.

In this paper we have described a simple and reproducible *in vitro* assay that can be used to monitor the critical parameters of antigen content and degree of adsorption in adsorbed tetanus vaccines. The assay is ideally suited for within-product consistency monitoring and can be applied at different stages of the production process – including the final lot. The production of freeze-dried antibodies provides a resource of stable reagents that can be used by other laboratories that may wish to introduce this assay and may help in the standardization of the method. The assay may be useful for investigating the complex relationship between antigen content/degree of adsorption and immunizing potency in different tetanus vaccines, and is particularly valuable when there is a loss of quantitative information (that is suitable for use in trend analysis) as a result of potency assay modifications or a reduced frequency of *in vivo* testing.

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