Development of a Monoclonal Antibody Sandwich ELISA for the Determination of Antigen Content and Quality in Diphtheria Vaccines

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Abstract

At present, quality control of diphtheria vaccines by both manufacturers and national control laboratories relies heavily on *in vivo* assays to confirm potency. As part of the VAC2VAC project we have developed a monoclonal antibody (mAb) enzyme-linked immunosorbent assay (ELISA) to measure the relative amount and quality of diphtheria toxoid (DTxd) in diphtheria-tetanus based vaccines and believe this test has the potential to play a key role in a control strategy no longer including an *in vivo* potency test. The mAb ELISA is highly specific, has good dilutional linearity, and is suitable for detecting DTxd in a range of different human vaccine products. We demonstrate the ability of the assay to discriminate between batches of different content and quality using vaccine batches that were prepared to contain differing amounts of DTxd or were altered by exposure to heat or oxidative stress. We also demonstrate successful transfer of the method to other laboratories and show that different diphtheria antigen materials may be able to serve as a reference antigen for local standardization of the method. The assay is ideally suited for incorporation into a consistency approach for routine diphtheria vaccine quality control testing and may be suitable to serve as the stability indicating test in replacement of the current *in vivo* potency test.

Plain language summary

Diphtheria vaccines help to protect against diphtheria infection. Currently, animal tests are used to ensure the potency of such vaccines. Since these tests were first introduced, there have been improvements in non-animal technologies that can be used to ensure consistent production of potent vaccine batches. To demonstrate that a new batch of diphtheria vaccine is consistent with a previous batch of known potency, the quality and amount of the component that stimulates the immune response upon vaccination must be assessed in comparison. We have developed an assay that can measure the quality of a range of different diphtheria vaccine product types. The assay is very specific and reliable, and different laboratories obtained comparable results, showing that the assay is suited for routine use. Once validated by manufacturers and recognized by regulators, this assay will greatly reduce the number of animals needed for batch release of diphtheria vaccines.

1 Introduction

Diphtheria is a potentially fatal infection caused by toxigenic strains of corynebacteria, primarily *Corynebacterium diphtheriae*. Diphtheria is generally an acute respiratory infection, characterized by the formation of a pseudomembrane in the throat, but cutaneous infections are also possible. Diphtheria toxin (DTxn), an exotoxin that inhibits protein synthesis and causes cell death, is the most important virulence factor for disease-causing *C. diphtheriae* strains. As well as causing local tissue destruction, DTxn can enter the bloodstream and disseminate throughout the body causing complications such as myocarditis and neuropathy (Sharma et al., 2019). Vaccines protect against disease by stimulating

the production of toxin-neutralizing antibodies. They are among the most widely employed and successful human vaccines, and their use has significantly decreased the incidence of the disease worldwide (Clarke et al., 2019), although there can still be significant outbreaks where there is insufficient vaccination coverage.

Diphtheria vaccines consist of formaldehyde-inactivated preparations of DTxn (forming diphtheria toxoid, DTxd) adsorbed onto an aluminium adjuvant (aluminium hydroxide, $Al(OH)_3$, aluminium phosphate, $AlPO_4$, or a combination of both), and include additional non-diphtheria components such as tetanus toxoid, pertussis (acellular antigens or whole cell), inactivated poliomyelitis, hepatitis B surface antigen, and *Haemophilus influenzae* type b polysaccharide. The chemical inactivation of DTxn results in toxoids that consist of

a heterogeneous population of crosslinked toxin molecules (Rappuoli, 1997), and the presence of the adjuvant and additional antigens make the final composition of vaccines even more complex. Extensive quality control testing is therefore required on each final batch produced before it can be released on the market. At present, quality control of diphtheria vaccines by both manufacturers and national control laboratories relies on *in vivo* assays to confirm potency. The potency assays stipulated by the European Pharmacopoeia monographs and World Health Organization (WHO) guidelines involve multiple dilutions of a reference and test preparations and require large numbers of animals to fulfil the requirements of a valid assay (WHO, 1990, 2005; Council of Europe, 2008).

Over the last few decades there has been a strong drive to replace *in vivo* potency tests with *in vitro* methods (McFarland et al., 2011; Stickings et al., 2011; Schutte et al., 2017, Akkermans et al., 2020). However, despite recent advances in the development of methods that refine or reduce animal use for routine batch release evaluation of vaccines (Winsnes et al., 2002, 2004, 2006; Sesardic et al., 2004; Gupta et al., 1996), there have been fewer developments that have resulted in a complete replacement of animal potency tests (van den Biggelaar et al., 2021). Supported by the Innovative Medicines Initiative 2 (IMI2), the VAC2VAC project[1] was initiated in 2016 with the aim to develop and validate quality control testing approaches for both human and veterinary vaccines using non-animal methods. The overall goal of the project is to develop assays and approaches that will allow acceptance of the "consistency approach" for established vaccines by regulatory agencies, thereby significantly reducing the use of animals for routine vaccine production in the future.

The consistency approach shifts the focus of quality control testing from the final batch to the entire production process (De Mattia et al., 2011). Production processes have been significantly improved since diphtheria and tetanus vaccines were first developed, with extensive in-process controls, product monitoring using superior analytical tools, and adherence to robust quality management systems that provide assurance in production and testing. As such, rather than each new batch of vaccine being seen as unique, it can be considered as one of a series of batches sharing many of the same characteristics as a manufacturer-specific vaccine batch of proven clinical efficacy and safety. For application of this approach, a set of meaningful *in vitro* tests that can monitor the critical characteristics associated with the consistency of the manufacturing process and with safety and efficacy of the product is required.

The quantity and quality of antigen present in diphtheria vaccines determines product safety and effectiveness and is therefore a critical factor to be evaluated as part of consistency testing. In the course of the VAC2VAC project we have developed a monoclonal antibody (mAb) enzyme-linked immunosorbent assay (ELISA) to measure the relative amount and quality of DTxd in diphtheria-tetanus (DT) based vaccines and believe this test has the potential to play a key role in a control strategy no longer including an *in vivo* potency test. The ELISA uses a pair of well-characterized and relevant mAbs that recognize functional epitopes on the DTxd (Riches-Duit et al.,

2021). These were selected based on their binding and functional activity (including their ability to bind the native, detoxified and adsorbed antigen, and antigen that was altered following exposure to elevated temperature). Epitope competition studies were performed to identify a pair of high-affinity antibodies that could be used in a sandwich ELISA format. Here we describe the development and performance of the ELISA using these mAbs.

We had previously developed a sandwich ELISA for quantification and characterization of DTxd, which was found to be suitable for monitoring the consistency of DT-based vaccines in terms of antigen content and degree of adsorption (Coombes et al., 2009). However, this assay utilized a polyclonal antibody for detection of the antigen. Polyclonal antibodies are prone to batch-to-batch variability, such as differences in antibody reactivity, making them harder to implement as part of a control strategy. They also require the use of animals every time a new batch is produced. In contrast, the culture of B cell hybridomas offers a reproducible and potentially inexhaustive supply of antibody. We have therefore developed a new assay that utilizes two well-characterized mAbs and demonstrate proof of concept for the intended use of the assay by evaluating ELISA performance characteristics (within and between laboratories), suitability for use with different product types, and ability to discriminate between vaccine batches of different quality.

2 Materials and methods

2.1 Monoclonal antibodies
The two anti-diphtheria mAbs selected for ELISA development were DT05 (a rat IgG2a antibody provided by the National Institute for Biological Standards and Control, NIBSC, UK) and Dim9 (a mouse IgG1 antibody provided by Intravacc, The Netherlands) (Riches-Duit et al., 2021). The mAbs were produced by hybridoma culture and purified by protein A/G affinity chromatography and were buffer exchanged into phosphate-buffered saline (PBS) for storage. Stocks of these mAbs are available from the NIBSC catalogue[2].

2.2 Toxoid and vaccine samples
Pre-adsorbed and adsorbed drug substance samples used for vaccine formulation and a range of drug product (final lot) vaccines were obtained from two manufacturers in the VAC2VAC consortium (coded HuA and HuB). The final lot samples include primary dose (DT) and booster dose (reduced diphtheria antigen content) vaccines (dT) with or without acellular pertussis (aP), inactivated poliomyelitis virus (IPV), *Haemophilus influenzae* type b (Hib), and/or hepatitis B (HepB) components. The vaccine products all contain an aluminium adjuvant: either $AlPO_4$, $Al(OH)_3$ or a mixture of both. See Table 1 for a summary of drug substances and products tested.

Dropout samples without any diphtheria antigen were also received for 2 products from HuA (dTap, $AlPO_4$ adjuvant and DTaP-IPV-HepB-Hib, $Al(OH)_3$ adjuvant) and 2 products from HuB

Tab. 1: Summary of drug substances and drug products tested

Manufacturer	Site	Product / drug substance	Concentration of DTxd (Lf/mL)	Adjuvant
HuA	1	DTxd pre-adsorbed	3990	N/A
		dT-IPV	10	$Al(OH)_3$
		DTaP-IPV	60	$Al(OH)_3$
		DTaP-IPV-HepB-Hib	60	$Al(OH)_3$
	2	DTxd pre-adsorbed	3710	N/A
		DTxd adsorbed	600	$AlPO_4$
		dTaP	4	$AlPO_4$
		DTaP-IPV	30	$AlPO_4$
HuB	N/A	DTxd pre-adsorbed	4000	N/A
		DT adsorbed	167	$Al(OH)_3$
		DTaP	50	$Al(OH)_3$
		DTaP-IPV	50	$Al(OH)_3$
		DTaP-HepB-IPV	50	$Al(OH)_3 + AlPO_4$
		dTaP	5	$Al(OH)_3 + AlPO_4$
		dTaP-IPV	5	$Al(OH)_3 + AlPO_4$

(dTaP, $AlPO_4$ + $Al(OH)_3$ adjuvant and DTaP, $Al(OH)_3$ adjuvant) and were used to confirm assay specificity and to prepare graded dose samples.

The 2nd WHO International Standard (IS) for Diphtheria Toxoid for use in Flocculation test (NIBSC code 13/212, 1870 Lf/ampoule) and the 4th WHO IS for Diphtheria Toxoid (Adsorbed) (NIBSC code 07/216, ~100 Lf/ampoule) were included in some assays, either as a positive control or as part of studies to investigate suitability of different DTxd-containing materials to act as a reference preparation in the ELISA. Each ampoule of 13/212 and 07/216 was reconstituted in 1 mL ultrapure water to give a stock concentration of 1870 Lf/mL and approximately 100 Lf/mL respectively.

2.3 Desorption studies

Vaccine final lot samples were centrifuged (13,000 rpm for 10 min) to separate them into non-adsorbed (vaccine supernatant) and adsorbed (adjuvant pellet) fractions. The pellets were re-suspended in a sodium phosphate/EDTA solution (1 volume of 56 g/L EDTA: 49 volumes 90 g/L disodium hydrogen phosphate) and incubated for 16-20 h at +37°C to elute the adsorbed antigen. Finally, a second centrifugation step was performed to remove any remaining adjuvant. The desorbed samples were titrated in the ELISA alongside the non-adsorbed fraction and the whole (adjuvanted) vaccine sample. Relative antigen estimates for the adsorbed fraction and non-adsorbed fraction were calculated relative to the whole vaccine sample.

2.4 Graded dose samples and spiking assays

Graded dose samples containing 25%, 50% and 75% diphtheria an-tigen were prepared by mixing the dropout samples (0% DTxd) received from HuA and HuB with the normal vaccine sample (100% DTxd) in different ratios. To prepare over-formulated samples, one of the dropout samples from HuA was also spiked with adsorbed DTxd (330 Lf/mL) from the same manufacturer to give 25%, 50%, 75%, 100%, 200% and 400% diphtheria antigen.

2.5 Alteration of vaccine samples by heat stress

One vaccine product from HuA (dTaP, $AlPO_4$ adjuvant) and one product from HuB (DTaP, $Al(OH)_3$ adjuvant) were incubated for 8 weeks at elevated temperatures of +37°C and +45°C, with control samples held at the normal storage temperature of +4°C. The vaccine samples were tested in the ELISA with and without desorption (to determine if the adsorption profile also changed after incubation at elevated temperatures), as described in Section 2.3. The whole vaccine control sample at +4°C was included on every plate to act as a reference.

2.6 Alteration of vaccine samples by oxidative stress

The same vaccine products used in the heat treatment studies were also used in oxidative stress studies using hydrogen peroxide (H_2O_2) treatment. Samples were incubated for 1 week at +37°C with various concentrations of H_2O_2 (0.01 μg/mL, 0.1 μg/mL 1 μg/mL, 10 μg/mL, 100 μg/mL and 1 mg/mL). Stock solutions of H_2O_2 were prepared as 33.3x concentrate, and 30 μL was mixed with 970 μL of the vaccine sample to give the final concentrations required. Vaccine samples were also prepared with water instead of H_2O_2 as a control and incubated at +4°C or +37°C. As with the heat-stress study, samples were tested in the ELISA with and without desorption.

2.7 Sandwich ELISA

ELISA method

For the optimized ELISA, plates were coated overnight at +4°C with 100 µL/well anti-diphtheria mAb DT05 diluted to 2 µg/mL (rat IgG concentration) in carbonate buffer (0.05 M, pH 9.6). The plates were washed (3x) by immersion in PBS (pH 7.4) containing 0.05% (vol/vol) Tween 20 (PBST), then blocked with 150 µL/well PBST containing 5% (wt/vol) dried skimmed milk powder for 1 h at +37°C. Following a second wash in PBST, serial three-fold dilutions of the samples in PBST containing 0.5% (wt/vol) dried skimmed milk powder (sample buffer, SB) were prepared in the plate (final volume 100 µL), and the plates were incubated at +37°C for 2 h. Plates were washed as described previously, and 100 µL/well of mAb Dim9 diluted to 0.25 µg (mouse IgG concentration) in SB was added for 2 h at +37°C. After further washing, bound mAb Dim9 were detected using 100 µL/well of goat anti-mouse HRP conjugate (Abcam Ab97040) diluted 1/4000 in SB. After a further incubation for 1 h at +37°C and a final wash, 100 µL/well substrate solution containing 0.5 mg/mL 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) and 0.008% H_2O_2 in 0.05 M citric acid buffer (pH 4) was added. The reaction was allowed to develop at room temperature for up to 30 min, and the optical density (OD) was measured at 405 nm using a Molecular Devices microplate reader. When applicable, relative antigen estimates were calculated using sigmoidal curve analysis with a log transformation of the assay OD response using CombiStats software (version 6.1, EDQM).

ELISA development and optimization

Checkerboard titrations of the coating mAb (4 - 0.03125 µg/mL rat IgG) and the detecting mAb (0.5 - 0.0156 µg/mL mouse IgG) were performed to identify optimal concentrations to use in the ELISA. The mAbs were titrated with a fixed concentration of DTxd (0.276 Lf/mL) to give the signal and no DTxd (SB only) to give the background. Several anti-mouse HRP conjugate antibodies, selected for their minimal cross-reactivity to the opposite species, were tested. Different blocking and sample buffer conditions were also investigated to optimize the signal:noise ratio, including the use of bovine serum albumin (BSA) or different concentrations of skimmed milk powder as an irrelevant protein.

ELISA qualification

A representative human vaccine product (HuB, DTaP, Al(OH)₃ adjuvant) was used to determine the intermediate precision of the assay and to define suitable assay validity criteria. Two batches of the product were selected, one assigned as the reference and the other assigned as the test vaccine. Samples were titrated in duplicate on a plate with two replicate plates included per assay. A column containing SB only was also included on each plate to measure the background signal. The assay was repeated on four separate days with two operators performing two assays each. Individual relative estimates for the test batch against the reference batch on the same plate were calculated. From this, geomean relative estimates were calculated, and the assay precision was expressed using geometric coefficients of variation (GCV = $\{10^s - 1\} \times 100\%$, where "s" is the standard deviation of the $\log_{10}$ transformed estimates). The geomean background OD, correlation coefficient |R|, confidence intervals for the slope ratio, and 95% confidence limits of the relative estimates were recorded for each plate.

2.8 Consistency testing

The suitability of the ELISA for monitoring the batch-to-batch consistency of different lots of the same product was assessed by testing multiple lots of one vaccine product from HuA (DTaP-IPV-HepB-Hib, Al(OH)₃ adjuvant) and multiple lots of two vaccine products from HuB (dTaP and dTaP-IPV both containing an Al(OH)₃ and AlPO₄ adjuvant). All vaccine lots were titrated in duplicate in a single assay. For each product, one lot was selected at random to be used as a product-specific reference and assigned an arbitrary "potency" value of 1. Results for all other batches were expressed relative to this product-specific reference. For the HuB vaccines, results for the dTaP-IPV product were also expressed relative to the dTaP reference batch (also assigned an arbitrary "potency" value of 1). From this, a geomean relative estimate was calculated for each product, and the variability between individual estimates was determined (expressed as GCV, as described above).

2.9 Transfer studies

Transfer studies were arranged with HuA (two sites) and HuB (one site). Protocols (including plate layouts) and critical reagents were provided by NIBSC, and the same vaccine samples were tested at both the receiving site (industry partner) and donating site (NIBSC). For each product selected, two batches were tested (one assigned as the reference and the other as the test sample) so that relative antigen estimates could be calculated. Each lab performed three independent assays (two plates per assay), and all raw data was returned to NIBSC for analysis and comparison.

2.10 Curve analysis

Curve analysis was performed for seven different products (five from HuA and two from HuB) against different diphtheria antigen-containing materials that could potentially serve as a reference preparation in the ELISA: the materials evaluated included WHO standards 13/212 (non-adsorbed toxoid) and 07/216 (adsorbed toxoid), manufacturer-matched drug substance (pre-adsorbed or adsorbed DTxd), and a range of manufacturer-matched drug products. All samples were titrated in duplicate, and assays were repeated on three different days. Individual curves were analyzed using a sigmoidal curve model with a log transformation of the response. The slope and asymptotes for each sample were extracted from CombiStats, and the values for the final vaccine products were compared to the different reference materials on the same plate (ratio calculated for the slopes and difference calculated for the asymptotes).

3 Results

3.1 ELISA optimization

Suitable concentrations of the coating and detecting mAbs were identified from checkerboard titration assays with a fixed concentration of toxoid. A concentration of 2 µg/mL for DT05 and 0.25 µg/mL for Dim9 was selected, which consistently gave a background OD < 0.2, a signal OD > 1, and was in excess (showed
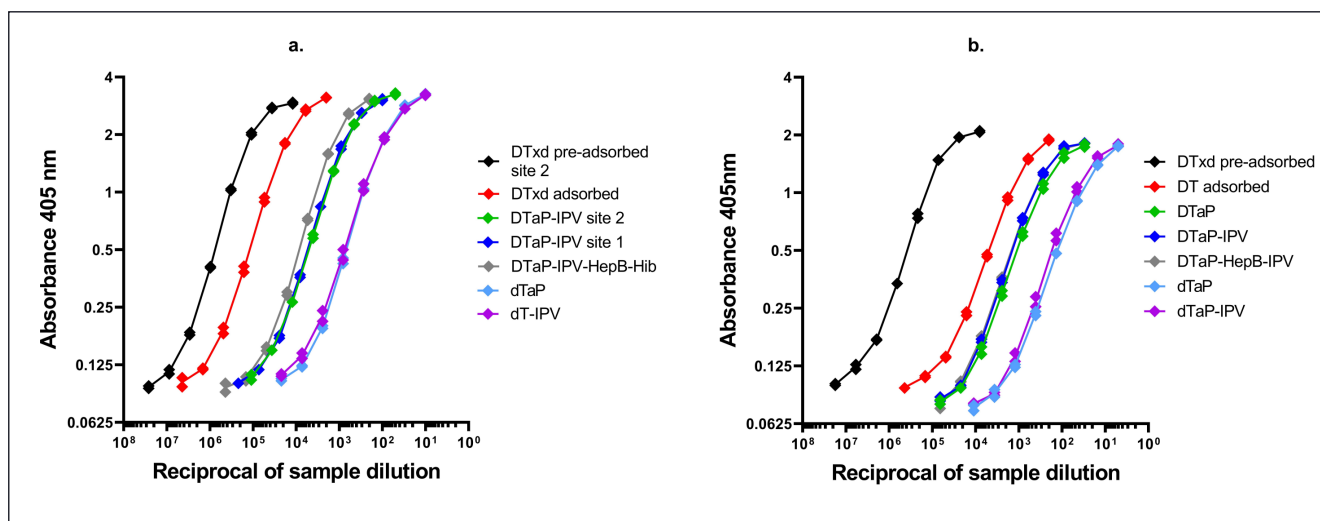
Fig. 1: Dose response curves for drug substances and drug products from HuA (a) and HuB (b)
Samples were tested in duplicate on a plate. Graphs show the individual data points for each replicate, with the connecting line through the mean, for a single representative assay.

a saturating response) to help ensure a robust assay (data not shown). A conjugate antibody (Abcam, Ab97040) that had been depleted using rat immunosorbents gave a significantly lower background OD and a higher signal:noise ratio than other similar reagents tested and was therefore chosen as the secondary detecting antibody. Use of PBST-containing 1% (wt/vol) BSA for the blocking buffer and sample buffer significantly increased the background OD and reduced the signal:noise ratio compared to using PBST-containing skimmed milk powder (data not shown). Use of PBST + 5% (wt/vol) milk powder for the blocking buffer and PBST + 0.5% milk powder for the sample buffer were therefore chosen to give the lowest background without impacting the signal.

3.2 Assay performance characteristics
Use of the ELISA with different drug substances and products from different manufacturers
Suitable sigmoidal dose response curves were obtained in the ELISA for all toxoid and vaccine samples tested (Fig. 1). Vaccine samples were tested in the presence of their aluminium adjuvant (which we have referred to as testing "whole vaccine"). The magnitude of the response correlated well with the type of sample titrated, with the highest response seen for the drug substance samples (which are concentrated), followed by the pediatric (primary) dose vaccines, and then the booster dose (reduced antigen content) vaccines.

Proportion of antigen detected in the whole vaccine compared to after a desorption step
To assess the proportion of antigen detected in final vaccine products in the presence of the adjuvant compared to after a desorption step, two products from HuA (dTaP and DTaP-IPV-HepB-Hib) and HuB (dTaP and DTaP) were desorbed and compared to the sample with no pre-treatment (whole vaccine). The proportion of antigen detected in the whole vaccine was product- and adjuvant-specific with 43-67% of the antigen detected in the presence of adjuvant compared to after desorption (Tab. 2).

Assay linearity and specificity
Dilutional linearity was assessed using data from the spiking study where the dropout dTaP vaccine from HuA was spiked with adsorbed DTxd to give 25%, 50%, 75%, 100%, 200% and 400% diphtheria antigen (Fig. 2). Estimates for the spiked samples were calculated relative to the normal vaccine sample. There was good agreement between the observed and target values, with a fit-

Tab. 2: Proportion of DTxd detected in the presence of adjuvant compared to after a desorption step
The degree of adsorption measured for each product is also shown. Data is the result of a single assay.

Manufacturer	Product	Adjuvant	Proportion of antigen detected (%)[a]	Degree of adsorption (%)[b]
HuA	dTaP	$AlPO_4$	67	89
	DTaP-IPV-HepB-Hib	$Al(OH)_3$	56	58
HuB	dTaP	$Al(OH)_3 + AlPO_4$	63	100
	DTaP	$Al(OH)_3$	43	100

[a] (whole vaccine / (adsorbed fraction + non-adsorbed fraction)) x 100; [b] (adsorbed fraction / (adsorbed fraction + non-adsorbed fraction)) x 100

ted slope not significantly different to 1.0 (1.02 with 95% CI of 0.97-1.07), intercept not significantly different to 0 (-0.03 with 95% CI -0.13 to 0.07), and a high r^2 value (> 0.99).

No signal was observed for the dropout samples, which contained adjuvant and all other components of the vaccine product except the diphtheria antigen, confirming the specificity of the assay for DTxd (Fig. 3).

Assay precision

The intermediate precision of the ELISA method was determined by testing a representative product (one reference batch and one test batch) across duplicate plates on several days (*n* = 8). The GCV of the relative antigen estimate was 10.1%. Based on this study, validity criteria for sigmoidal curve analysis of relative antigen content were defined as follows: a weighted correlation coefficient |R| greater than 0.975, 90% confidence intervals for slope ratio within 0.85-1.18 to confirm parallelism, and 95% confidence limits for the relative antigen estimate within 80-125% of the estimate. An acceptable intermediate precision (GCV) for the transfer studies to other laboratories was defined as 15% or less.
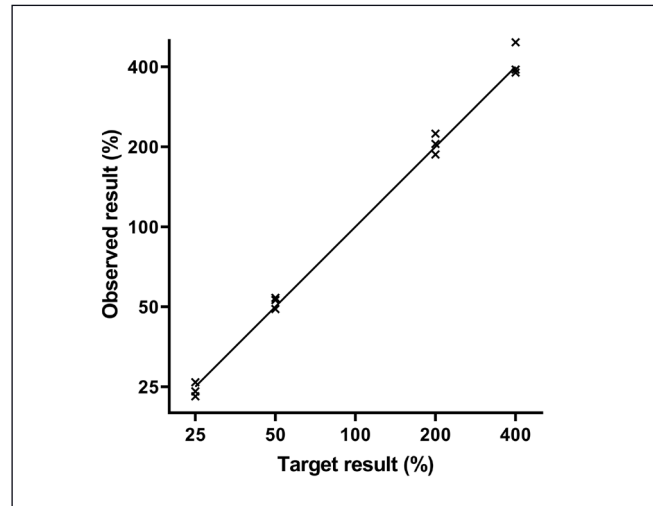
Fig. 2: Linearity analysis results for spiked samples
Plot includes line of identity (slope = 1.0, intercept = 0);
n = 3 assays at each target concentration level.
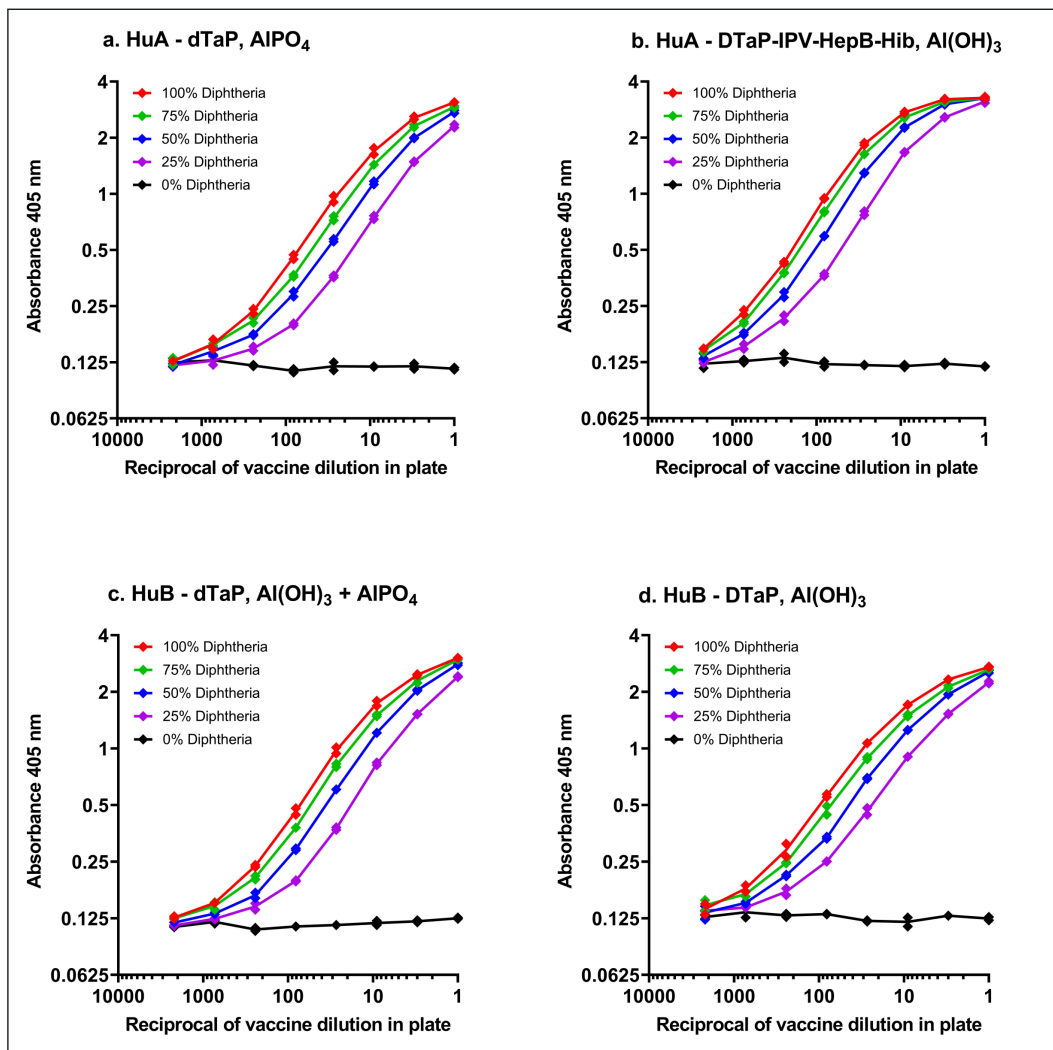
Fig. 3: Titration of graded dose samples from HuA (a and b) and HuB (c and d) formulated by mixing dropout samples (0% diphtheria) with the normal vaccine (100% diphtheria) in different ratios
Samples were tested in duplicate on a plate. Graphs show the individual data points for each replicate, with the connecting line through the mean, obtained in 1 of 2 replicate assays.
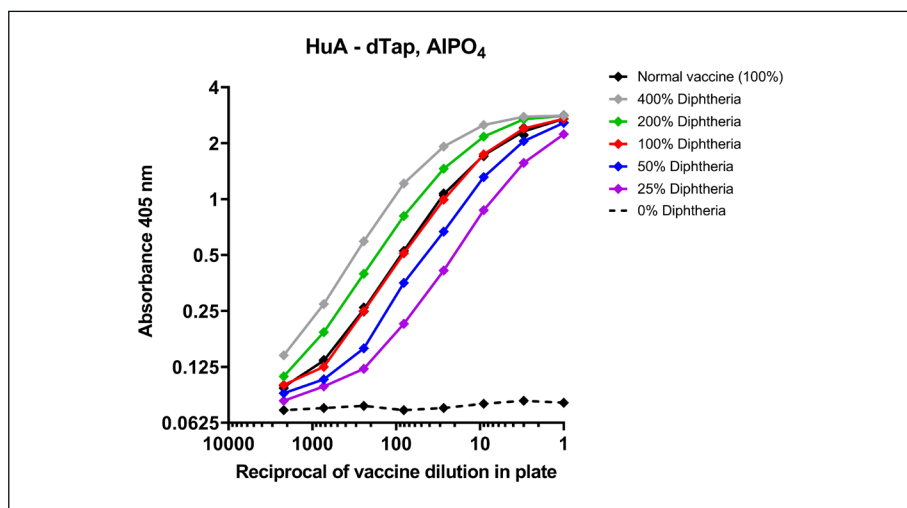
Fig. 4: Titration of spiked samples from HuA alongside a normal dTaP vaccine sample (100%)
Samples were tested in a single column except for the normal control sample, which was tested in duplicate. Graph shows the individual data points for each replicate (where applicable), with the connecting line through the mean, obtained in 1 of 3 replicate assays.

Detection of under- and overformulation of vaccine samples
The graded dose samples formulated by mixing the DTxd dropout vaccine samples with the normal vaccine showed a shift in the dose response curves that corresponded to the amount of diphtheria antigen present (Fig. 3). Similarly, results from spiking studies with the dTaP vaccine from HuA showed a decrease in signal for the 25% and 50% samples, and an increase in signal for the 200% and 400% samples, equivalent to the DTxd concentration (Fig. 4).

*Detection of temperature-induced alterations in vaccine samples*
One final vaccine product from HuA (dTaP) and one from HuB (DTaP) that had been incubated at elevated temperatures of +37°C and +45°C were tested in the ELISA with and without desorption. A desorption step was included to determine whether the adsorption profile had changed and contributed to any differences in responses observed. The DTaP vaccine from HuB is fully adsorbed, hence no DTxd was detected in the non-adsorbed (supernatant) samples from this product. Estimates for the samples stored at elevated temperatures were expressed as a percentage of the equivalent +4°C sample (whole vaccine, adsorbed antigen fraction or non-adsorbed antigen fraction) as shown in Figure 5. The results demonstrate a loss of antigen content in both vaccine products after incubation at elevated temperatures (to varying degrees).

Detection of chemical-induced alterations in vaccine samples
The effect of H_2O_2 (1 week at +37°C) was also assessed for one final vaccine product from HuA (dTap) and one from HuB (DTaP). As with the heat stress study, vaccines were tested in the ELISA with and without desorption. Incubating the vaccine at +37°C for a week with no H_2O_2 caused small changes in the antigen compared to the control sample that was stored at +4°C (data not shown). These changes are in line with those observed in the vaccine heat treatment studies, albeit to a lesser extent due to the shorter time-period of incubation (1 week compared to 8 weeks).

Estimates for the samples incubated with H_2O_2 were expressed as a percentage of the equivalent +37°C no H_2O_2 control sample (whole vaccine, adsorbed antigen fraction or non-adsorbed antigen fraction) and are shown in Figure 6. A concentration of 1 mg/mL
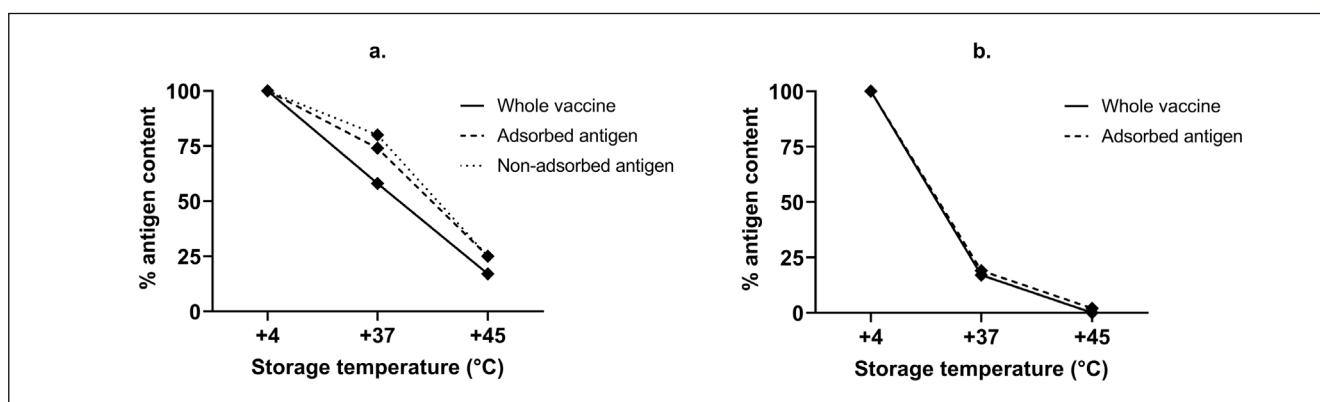
Fig. 5: Effect of storage temperature on the amount of DTxd detected in a dTaP vaccine from HuA (a) and a DTaP vaccine from HuB (b)
Estimates for the samples stored at +37°C and +45°C are expressed as a percentage of the equivalent +4°C sample (whole vaccine, adsorbed antigen fraction or non-adsorbed antigen fraction). Data is the result from a single assay.
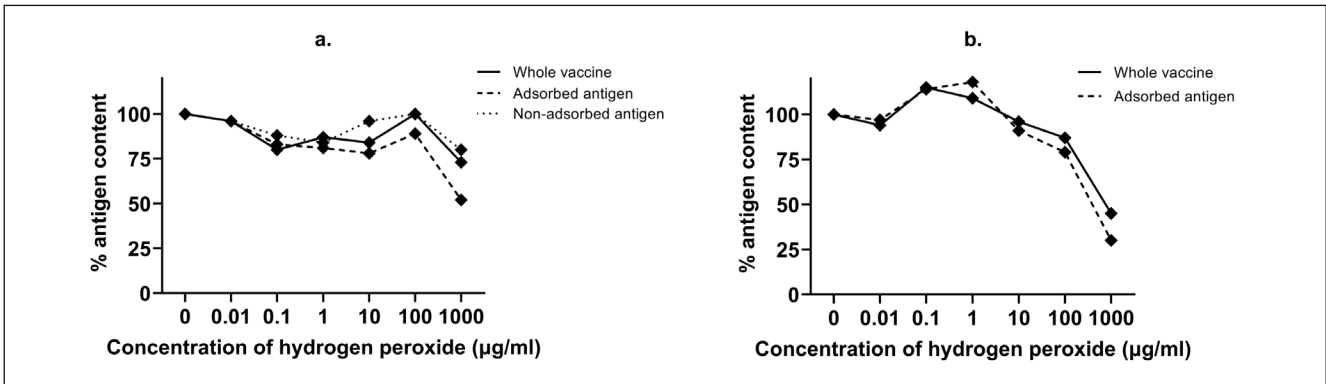
Fig. 6: Effect of H$_2$O$_2$ on the amount of DTxd detected in a dTaP vaccine from HuA (a) and a DTaP vaccine from HuB (b)
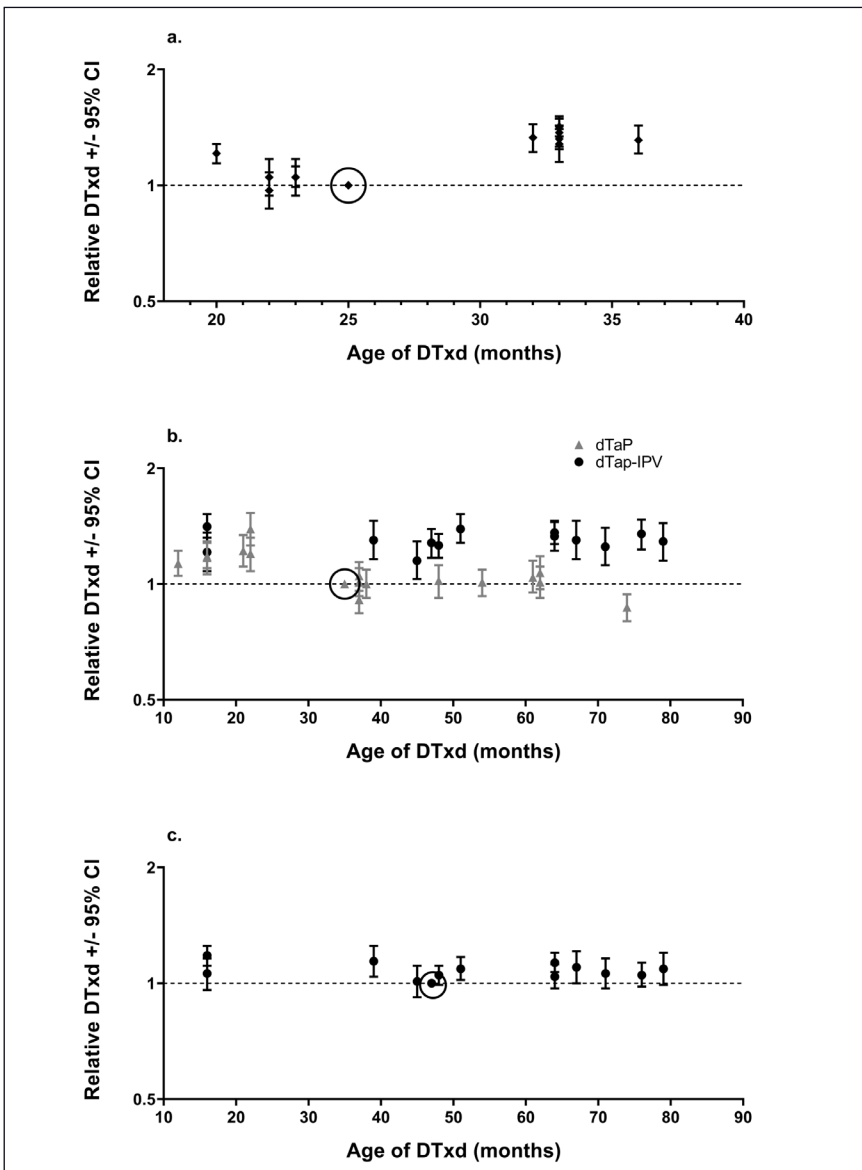Estimates for the samples incubated with H$_2$O$_2$ are expressed as a percentage of the equivalent +37°C no H$_2$O$_2$ control sample (whole vaccine, adsorbed antigen fraction or non-adsorbed antigen fraction). Data is the result from a single assay.

Fig. 7: Batch-to-batch variability observed for a DTaP-IPV-HepB-Hib product from HuA (a, black diamonds) and a dTaP (b, grey triangles) and dTaP-IPV (b and c, black circles) product from HuB
Graphs show relative estimates with 95% confidence intervals (CI) calculated against a reference batch (highlighted with a circle). Results for dTaP-IPV batches against a homologous reference are shown in graph c, and results for the same batches against a heterologous dTaP reference are shown in graph b. Data is the result from a single assay.

Tab. 3: Batch-to-batch consistency monitoring of 3 different products
Results show relative estimates calculated against a product-specific homologous reference, or against a "product-type" heterologous reference (dTaP-IPV against dTaP only). Data is the result of a single assay.

Manufacturer	Product	Reference type	No. of batches	Geomean (95% CI)	GCV (%)
HuA	DTaP-IPV-HepB-Hib	DTaP-IPV-HepB-Hib	11	1.21 (1.10-1.32)	14.2
HuB	dTaP	dTaP	16	1.07 (1.01-1.14)	12.4
	dTaP-IPV	dTaP	13	1.30 (1.25-1.34)	5.8
	dTaP-IPV	dTaP-IPV	12	1.08 (1.05-1.11)	4.5

H_2O_2 was required to cause a significant change in the amount of antigen detected. The changes were larger for the adsorbed antigen fraction (decrease of 48% for HuA and 70% for HuB) compared to the whole vaccine (decrease of 27% for HuA and 55% for HuB) and non-adsorbed fraction (decrease of 20% for HuA).

Consistency testing
The individual potency estimates for the different batches tested were precise with confidence intervals all within 89-113% of the estimate. A summary of the batch-to-batch variability observed is shown in Figure 7 and in Table 3. The variability between estimates (GCV) was comparable to that observed during the ELISA qualification, suggesting that the ELISA will perform well with real world samples.

More antigen was detected in the dTaP-IPV product when using a heterologous reference (dTaP) compared to a homologous reference even though they are formulated with the same amount of DTxd. This is likely due to the different adsorption profiles in the two products, which affect the accessibility of the antigen to the mAbs. The lot-to-lot variability, however, was comparable, suggesting that use of a product-type (as opposed to product-specific) reference preparation may be suitable for the calculation of relative antigen estimates.

3.3 Transfer studies
The ELISA was successfully transferred to two industry partners (across three different laboratory sites). All plates except one (out of 30 plates in total) met the required validity criteria for sigmoidal curve analysis (as specified in Section 3.2). The one plate that failed to meet the validity criteria was excluded from the transfer study analysis. Target acceptance criteria for a successful transfer were defined as 1) an intermediate precision (GCV) < 15%,

2) GCV for partner lab < GCV for NIBSC + 5%, and 3) geomean relative potency estimate for partner lab within 10% of the estimate obtained at NIBSC. The intermediate assay precision was less than 15% for all products tested, and the geomean relative estimates for the partner labs were within 10% of the geomean relative estimate obtained at NIBSC. For one product (HuB, DTaP) the GCV obtained by the partner was not within 5% of that obtained by NIBSC, however the precision (%) obtained at NIBSC was lower than would normally be expected, making the comparison hard to achieve for the partner lab. Results for all studies are summarized in Table 4.

3.4 Curve analysis
Suitability of different materials to act as a reference preparation in the ELISA was assessed for 7 different products by examining the similarity of the dose-response curve shapes. Test sample and candidate reference sample curves were considered to be similar if the difference between the upper or lower asymptotes fell within -0.05 and 0.05 and if the ratio of the slopes was between 0.9 and 1.11. In the first study, 2 batches of each product were tested against NIBSC standards 13/212 and 07/216, and drug substances from the same manufacturer (pre-adsorbed DTxd and adsorbed DTxd) on the same plate. The geomean slope ratio and average asymptote differences for 3 replicate assays are summarized in Table 5. Small differences were observed in the upper asymptotes and slopes for most of the products compared to the non-adsorbed materials (13/212 and matched pre-adsorbed DTxd), but not against the adsorbed materials tested (07/216 and matched adsorbed DTxd). In the second study different products from the same manufacturer were tested against each other. Products from HuA were split into 2 different "families" from different manufacturing sites, and only products in the same family were tested

Tab. 4: Results of transfer studies with industry partners
Geomean relative estimates and GCVs for each lab were calculated from results obtained in 3 independent assays (n = 2 per assay).

Manufacturer	Product	Geomean relative estimate difference (%)	Assay precision, GCV (%)		
			Partner	NIBSC	Difference
HuA site 1	dTap	1.1	3.0	3.6	0.6
HuA site 2	DTaP-IPV-HepB-Hib	5.7	3.2	1.8	-1.4
HuB	dTaP	0.9	4.4	1.7	-2.7
	DTaP	0.9	9.2	1.7	-7.5

Tab. 5: Comparison of curve shapes for different products against NIBSC standards and drug substances
Any differences observed in the slope ratios (as indicated by a slope ratio < 0.9 or > 1.11) or in the asymptotes (as indicated by an asymptote difference of < -0.05 or > 0.05) are highlighted in grey. Data shows the geomean slope ratio and average asymptote differences from 3 independent assays.

Manufacturer	Product	Batch	Slope ratio				Lower asymptote difference				Upper asymptote difference			
			13/212	07/216	Pre-adsorbed DTxd	Adsorbed DTxd	13/212	07/216	Pre-adsorbed DTxd	Adsorbed	13/212 DTxd	07/216	Pre-adsorbed DTxd	Adsorbed DTxd
HuA	DTaP-IPV-HepB-Hib	1	0.925	1.062	0.929	1.041	-0.022	-0.001	-0.001	-0.004	0.051	0.006	0.053	0.000
		2	0.950	1.091	0.954	1.070	0.002	0.023	0.023	0.020	0.043	-0.002	0.044	-0.009
	DTaP-IPV	1	0.888	1.031	0.903	0.981	-0.048	-0.021	-0.025	-0.022	0.034	-0.012	0.037	-0.011
		2	0.857	0.994	0.872	0.947	-0.058	-0.030	-0.035	-0.031	0.054	0.008	0.057	0.009
	dT-IPV	1	0.946	1.106	0.946	1.044	-0.015	0.023	-0.002	0.000	0.012	-0.032	0.019	-0.036
		2	0.909	1.062	0.908	1.003	-0.025	0.012	-0.012	-0.010	0.029	-0.015	0.037	-0.019
	dTaP	1	0.844	0.973	0.905	0.948	-0.028	-0.007	-0.015	-0.019	0.049	0.008	0.020	-0.004
		2	0.814	0.939	0.873	0.915	-0.045	-0.024	-0.032	-0.036	0.060	0.020	0.031	0.008
	DTaP-IPV	1	0.870	1.012	0.929	1.039	-0.030	-0.003	-0.021	0.014	0.055	0.009	0.027	-0.015
		2	0.863	1.004	0.922	1.031	-0.029	-0.002	-0.020	0.015	0.064	0.017	0.036	-0.006
HuB	dTaP	1	0.870	0.969	0.795	0.942	-0.050	-0.028	-0.011	-0.014	-0.007	-0.031	0.057	0.011
		2	0.890	0.991	0.813	0.963	-0.027	-0.004	0.013	0.010	-0.004	-0.028	0.060	0.014
	DTaP	1	0.876	0.998	0.836	0.976	-0.044	-0.049	-0.013	-0.002	0.014	-0.032	0.066	0.026
		2	0.883	1.007	0.843	0.985	-0.043	-0.048	-0.011	-0.001	-0.004	-0.050	0.048	0.008

against each other. No differences in the curve shapes were observed for any of the products assessed (data not shown), suggesting that products from the same manufacturer would be suitable to act as references for each other.

4 Discussion

The implementation of a consistency approach for quality control of diphtheria vaccines should be encouraged scientifically as well as from an animal welfare perspective. Although the *in vivo* potency tests for diphtheria vaccines have proven their value over the past decades in ensuring safety and potency of final vaccine products, they require animals and are time-consuming, expensive, and have many shortcomings including poor precision and relevance (Stalpers et al., 2021). Rather than a one-to-one replacement, which is not always possible or necessary (Council of Europe, 2018; Schutte et al., 2017), the consistency approach aims at substituting an existing *in vivo* method with a panel of *in vitro* methods that control the key qualitative and quantitative attributes of a product. For diphtheria vaccines, antigen quantity and integrity as well as the degree of adsorption to adjuvant are critical quality attributes to be monitored. Within the VAC2VAC project we have developed a capture ELISA, utilizing mAbs that recognize functional epitopes on the DTxd (Riches-Duit et al., 2021), to measure

the relative amount and quality of DTxd in DT-based vaccines. Also, a multiplex-based immunoassay has been developed by another partner in the VAC2VAC consortium for the characterization of DT-based vaccines using the same mAbs to detect the DTxd (Vermeulen et al., 2023).

The ELISA method presented here is highly specific, has good dilutional linearity (covering at least the range of 25-400% of a booster dose vaccine), and is suitable for detecting DTxd in a range of different human vaccine products. Studies using graded dose vaccine batches show that the ELISA can detect 25% changes in antigen content – something that, given the poor precision of *in vivo* potency assays, would be difficult to see using an animal assay.

We applied the ELISA to whole vaccine samples (i.e., in the presence of the aluminium adjuvant) and demonstrated that a desorption step is not required to produce suitable and reproducible sigmoidal curves. However, to understand what proportion of the antigen we were detecting in the whole vaccine sample by ELISA, we undertook studies to compare the amount detected before and after desorption. We identified that, depending on the adjuvant and the degree of adsorption, between 40-70% of the antigen is detected without a desorption step compared to with such a step. It should be noted that the desorption step may not be 100% efficient, and that the effect of the desorption process on the antigen is not fully understood. However, we have demonstrated that the desorption conditions themselves have no impact on the detection

of non-adsorbed antigen by ELISA (data not shown). In terms of monitoring consistency of production, it may not be necessary for 100% of the antigen to be detected as long as the proportion that is being detected is consistent and representative of the quality of the vaccine as a whole. Validation of a desorption step as part of the ELISA may be required in some cases; this will increase complexity of the method but at the same time provide additional information with regards to the degree of adsorption which can and should be monitored on a batch-to-batch basis. Where desorption is not strictly required, the adsorption profile of a product could still be assessed by simply centrifuging the vaccine sample and including the supernatant in the ELISA to monitor the relative amount of non-adsorbed DTxd present in each lot.

The superiority of an antigen ELISA method over an *in vivo* potency test for monitoring the consistency of production and providing information on lot-to-lot variation in the final product has previously been demonstrated (Coombes et al., 2009). Studies assessing the variability of diphtheria *in vivo* potency tests have shown large lot-to-lot variations with coefficients of variance (CVs) ranging from 20-40% (Stalpers et al., 2021). In contrast, results obtained in this study demonstrated consistently lower lot-to-lot variations with GCVs in the range of 5-20% for the 4 different products tested. The vaccine samples used to assess lot-to-lot variability covered a range of different "ages" (from the date of manufacture of the final bulk to the date of testing) from a few months to several years, providing a realistic indication of how the ELISA may perform, i.e., we did not simply test consecutive production batches that were all produced from the same drug substance over a short period of time.

It is important to ensure that the use of a consistency approach for vaccine quality control includes an assay that is stability indicating and capable of detecting changes in the product characteristics and/or quality attributes that are relevant for immune protection. We demonstrated the capacity for the mAb ELISA assay to discriminate between batches of different content and quality using vaccine batches that had been altered by exposure to heat or oxidative stress. The ELISA was readily able to distinguish between normal and stressed samples, suggesting that it may be able to serve as the stability indicating test in a consistency approach. The samples exposed to heat and H_2O_2 were tested in the ELISA with and without desorption to confirm that any loss in signal we observed was not just due to a change in the adsorption profile affecting the accessibility of the mAb epitopes on the toxoid. A reduction in signal for whole vaccine samples as well as the adsorbed and non-adsorbed fractions was detected after exposure to elevated temperatures and high concentrations of H_2O_2. However, the decrease in signal observed after incubating vaccine samples with H_2O_2 was larger for the adsorbed fraction in both products compared to the non-adsorbed fraction and whole vaccine sample. This could be because oxidation makes the DTxd harder to desorb from the adjuvant.

For the ELISA to be successfully applied as a consistency test for vaccine quality control, a reference preparation needs to be identified for calculation of relative antigen estimates and, for the analysis to be valid, the dose response curves for the test sample and reference should be equivalent. For example, for sigmoidal curve analysis they should share common functional parameters (i.e., hill slope, upper and lower asymptotes) and ideally would only differ by a horizontal displacement. If sufficient parallelism is not demonstrated, then the relative calculation obtained from the two curves cannot be confidently interpreted. Here the suitability of different materials (including ISs and manufacturer-specific drug substances and drug products) to act as a reference preparation for monitoring antigen content in different vaccine drug products was assessed. The adsorbed WHO IS (07/216) and adsorbed DTxds had curve shapes that were more comparable to final drug products than the non-adsorbed WHO IS (13/212) and the pre-adsorbed DTxds; however, the differences we highlighted between the non-adsorbed materials and final products were very small. Manufacturer-specific drug products were also found to be suitable to act as a reference preparation for other products produced by the same manufacturer. This suggests that vaccine manufacturers or other laboratories wishing to implement and validate this ELISA method will have different options in terms of the material selected to serve as a reference preparation. In most regions, potency assays for diphtheria vaccine are standardized by expression of potency estimates relative to a reference vaccine that is traceable to the WHO IS for Diphtheria Vaccine, Adsorbed (Stickings et al., 2010). This has enabled minimum criteria for vaccine potency (in international units) to be defined in regulatory guidelines and monographs (WHO, 2005; Council of Europe, 2008). A move away from these *in vivo* potency assays to the use of validated *in vitro* alternatives as part of a consistency approach will almost certainly require product-specific acceptance criteria that allow manufacturers to demonstrate production of vaccine batches that are consistent with clinical batches (or, for products already on the market, consistent with batches shown to be safe and effective in routine use).

The diphtheria vaccine ELISA presented here has excellent performance characteristics and is applicable to a wide range of diphtheria vaccines. We intend to widen the scope of applicability by testing diphtheria vaccines containing whole-cell pertussis components and have preliminary in-house data suggesting that the assay will be suitable for such vaccines (not shown). Successful transfer of the method was demonstrated to three other laboratory sites, and we have shown that different diphtheria antigen materials may be able to serve as a reference antigen for local standardization of the method. The assay is ideally suited for incorporation into a consistency approach for routine diphtheria vaccine quality control testing and may be suitable to serve as the stability indicating test in replacement of the current *in vivo* potency test.

The benefit of this assay over the one we previously described for the quantification and characterization of DTxd (Coombes et al., 2009) is the replacement of the polyclonal antibody used for detection of the antigen with a second mAb. Polyclonal antibodies require the use of animals every time a new antibody batch is produced and are prone to batch-to-batch variability, whereas mAbs can be generated as a constant and renewable resource. The move away from a polyclonal antibody also provides the option to make the assay truly "non-animal" via use of recombinant mAbs in future as well as direct detection with a labelled mAb instead of a conjugate antibody. The mAbs used in the ELISA (DT05 and Dim9) are available from nibsc.org[2] to facilitate the validation and implementation of the method in other laboratories.

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Conflict of interest

Authors have no conflicts of interest.

Data availability

Data described in the manuscript can be obtained from the corresponding author upon request.

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