Biologicals 69 (2021) 38–48

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Research paper

Characterisation of diphtheria monoclonal antibodies as a first step towards the development of an *in vitro* vaccine potency immunoassay

Rebecca Riches-Duit [a,1], Laura Hassall [a,1], Amy Kogelman [b], Janny Westdijk [b], Alexandre Dobly [c], Antoine Francotte [c], Paul Stickings [a,*]

[a] National Institute for Biological Standards and Control, Division of Bacteriology, South Mimms, Potters Bar, EN6 3QG, UK
[b] Institute for Translational Vaccinology, P.O. Box 450, 3720, AL, Bilthoven, the Netherlands
[c] Sciensano, Quality of Vaccines and Blood Products, Rue Juliette Wytsmanstraat 14, 1050, Brussels, Belgium

ARTICLE INFO

Keywords:
Diphtheria
Vaccine
Monoclonal
Antibody
Immunoassay
Potency

ABSTRACT

Immunoassays are used for routine potency assessment of several vaccines, in some cases having been specifically developed as alternatives to *in vivo* potency tests. These methods require at least one well characterised monoclonal antibody (mAb) that is specific for the target antigen. In this paper we report the results of the comprehensive characterisation of a panel of mAbs against diphtheria with a view to select antibodies that can be used for development of an *in vitro* potency immunoassay for diphtheria vaccines. We have assessed binding of the antibodies to native antigen (toxin), detoxified antigen (toxoid), adsorbed antigen and heat-altered antigen. Antibody function was determined by a cell-based toxin neutralisation test and diphtheria toxin-domain recognition was determined by Western blotting. In addition, antibody affinity was measured, and epitope competition analysis was performed to identify pairs of antibodies that could be deployed in a sandwich immunoassay format. Not all characterisation tests provided evidence of "superiority" of one mAb over another, but together the results from all characterisation studies allowed for selection of an antibody pair to be taken forward to assay development.

1. Introduction

The VAC2VAC project is public-private consortium of 22 partners funded by the Innovative Medicines Initiative 2 (IMI2). The main objective for the project is the development of *in vitro* assays that will support regulatory acceptance of a consistency approach [1] for established vaccines where potency and/or safety testing in animals is currently required, ultimately reducing the use of animals for batch testing as part of routine vaccine production in the future. More information on the VAC2VAC project can be found on the project website [2].

One objective in the project is to develop an immunoassay for the diphtheria (D), tetanus (T) and acellular pertussis (aP) antigens that are used in the manufacture of combined vaccines (and monovalent vaccines in the case of tetanus). Combined vaccines based on diphtheria, tetanus and pertussis antigens (either acellular or whole cell pertussis vaccine) are among the most widely used vaccines with around 86% of infants globally receiving three vaccine doses in 2018 according to WHO Global Health Observatory data. Although the test methodology varies in different regulatory jurisdictions, all potency tests for D, T and aP vaccines currently require the use of animals [3,4]. Development of a non-animal potency test for these vaccines could have a significant impact in terms of a reduction in animal use during product manufacturing and release, because of the large number of vaccine batches produced, and the requirement to use animals for quality control testing.

We have previously developed ELISA methods for detection and quantification of diphtheria and tetanus antigen in combination vaccines and showed that this method could be applied to a range of different vaccines licensed for use in humans [5,6]. This work was a precursor to the further development and validation of ELISA-based approaches for the quality control of DTaP vaccines that is now being explored by the VAC2VAC consortium. We recently evaluated this capture antigen ELISA for the characterisation of tetanus vaccines for veterinary use [7]. However, these capture ELISAs use polyclonal detection antibodies, which are harder to implement as part of a control strategy because of variability between different production batches of

* Corresponding author.
E-mail address: paul.stickings@nibsc.org (P. Stickings).
[1] These authors contributed equally: Rebecca Riches-Duit and Laura Hassall.

https://doi.org/10.1016/j.biologicals.2020.12.002
Received 15 September 2020; Received in revised form 15 December 2020; Accepted 18 December 2020
Available online 13 January 2021

polyclonal antisera and difficulties identifying specific antibodies in a polyclonal population that are targeting functionally relevant epitopes. We are therefore developing ELISAs using fully characterised monoclonal antibodies for both capture and detection of the target antigen.

Immunoassays are used routinely in the quality control of many vaccines and in some cases have been specifically developed as alternatives to *in vivo* potency tests [3,8–15]. As immunity to diphtheria is antibody mediated [16], an approach to potency testing of diphtheria vaccines that relies on the use of specific, functional antibodies targeting protective epitopes is scientifically relevant.

In this study we report the results of an extensive characterisation of a panel of eight monoclonal antibodies against diphtheria with a view to select antibodies which will be used for the development of an *in vitro* potency immunoassay. To do this we have assessed binding of the antibodies to native antigen (toxin, DTxn), detoxified antigen (toxoid, DTxd), adsorbed antigen and heat-altered antigen. Where possible, we have used representative antigen samples from two different vaccine manufacturers to assess mAb binding. Antibody function was determined using a cell-based toxin neutralisation test and diphtheria toxin-domain recognition was determined by Western blotting. In addition, antibody affinity was measured, and epitope competition analysis was performed (using toxin) to identify pairs of antibodies that could be deployed in a sandwich immunoassay format.

2. Materials and methods

2.1. Monoclonal antibodies

Eight diphtheria mAbs were characterised in this study, which were raised in either rat or mouse, from three providers in the VAC2VAC consortium (Table 1).

2.2. Antigen and vaccine samples

2.2.1. Compliant samples

Non-adsorbed diphtheria toxoid (DTxd) and bulk adsorbed diphtheria toxoid (Ad-DTxd) from two vaccine manufacturers (coded HuA and HuB) were used to assess binding of the mAbs to detoxified antigen. The Ad-DTxd samples contained either an aluminium hydroxide adjuvant or an aluminium phosphate adjuvant. These samples were compliant with all quality requirements for manufacturing, and representative of DTxd used to produce batches that are efficacious in clinical studies. Details of the DTxd samples are shown in Table 2. Diphtheria toxin (DTxn, product code #150, List Biologicals) was used to assess mAb binding to native toxin.

2.2.2. Altered samples

Non-adsorbed DTxd samples were deliberately altered to determine the impact on mAb binding. Samples were diluted in 0.9% NaCl to a concentration representative of the final vaccine products (60 Lf/ml and 50 Lf/ml for HuA and HuB respectively). Samples were then 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.

Table 1
Overview of diphtheria mAbs characterised in this study.

Reagent Provider	Species	mAb ID	IgG Concentration (mg/ml)
Intravacc	Mouse	Dim9	0.39
Intravacc	Mouse	Dim25	0.23
Intravacc	Mouse	Dim27	1.70
Intravacc	Mouse	Dim33	0.57
NIBSC	Rat	DT05	1.10
Sanofi Pasteur	Mouse	2–25	2.41
Sanofi Pasteur	Mouse	1–49	2.83
Sanofi Pasteur	Mouse	2–18	3.23

Table 2
Adsorbed and non-adsorbed DTxd samples used for mAb binding ELISA assays.

Manufacturer	Sample description	DTxd content (Lf/ml)	Adjuvant	Adjuvant concentration (Al^{3+} mg/ml)
HuA	DTxd non-adsorbed	3990	N/A	N/A
HuB	DTxd non-adsorbed	4200	N/A	N/A
HuA	DTxd adsorbed	300	Aluminium phosphate	1.38
HuB	DTxd adsorbed	167	Aluminium hydroxide	2.35

2.3. Measurement of IgG concentration

The mAbs used in this study were from different providers, so commercial ELISA kits were used to measure the IgG concentration (Mouse-IgG ELISA, Roche) or rat IgG (Immunoglobulin G Rat SimpleStep ELISA® Kit, abcam). For the Mouse-IgG ELISA, plates were coated for 1 h at room temperature, with shaking, with a sheep anti-mouse-Fcγ polyclonal antibody. Following washing and blocking, a dilution series of the IgG standard (in the range 6.25–200 ng/ml) and the diluted test samples in duplicate were added to the wells and plates incubated as above. Plates were washed again, and bound IgG was detected using a POD-labelled mixture of anti-mouse-κ and anti-mouse-λ antibodies (1 h at room temperature, with shaking). Following a final wash, plates were developed with an ABTS-perborate substrate solution and plates read at 405 nm. For the Immunoglobulin G Rat SimpleStep ELISA, a dilution series of the standard (in the range 0.31–20 ng/ml) and the diluted test samples were added, in duplicate, to 96 well plate strips pre-coated with an anti-tag antibody. An antibody cocktail consisting of capture and detector antibodies was then added and the plate incubated for 1 h at room temperature. Following washing, TMB substrate was added to each well and the plate incubated for 3 min in the dark on a plate shaker before stop solution was added and the plates read at 450 nm. For both kits, IgG concentrations for the mAb samples were extrapolated from the linear portion of the standard curves. Dilution of all mAbs for functional or binding assays was based on the measured IgG concentration (Table 1).

2.4. Binding to non-adsorbed antigen

Direct ELISAs were performed using plates coated overnight at +4 °C with 100 µl/well of diphtheria toxin (DTxn) diluted to 1 µg/ml or DTxd diluted to approximately 2 Lf/ml (based on labelled values) in carbonate buffer. Following coating, plates were washed (3x) by immersion in phosphate buffered saline containing 0.05% (vol/vol) Tween 20 (PBST), then blocked with 150 µl/well of PBST containing 2.5% (wt/vol) dried skimmed milk powder (PBSTM) for 1 h at +37 °C. Following a second wash in PBST, serial three-fold dilutions of the mAb samples in PBSTM were prepared in the plate (final volume 100 µl) from a starting concentration of 10 µg/ml, and the plates were incubated at +37 °C for 2 h. After further washing, bound mAb was detected using 100 µl/well of the relevant HRP-conjugated IgG diluted 1/2000 in PBSTM (rabbit anti-rat for mAb DT05 and rabbit anti-mouse for the other mAbs). After a further incubation of 1 h at +37 °C and a final wash, 100 µl/well of substrate solution containing 0.5 mg/ml ABTS and 0.008% hydrogen peroxide in 0.05 M citric acid buffer was added. The reaction was allowed to develop at room temperature for up to 30 min and the optical density was then measured at 405 nm (Molecular Devices, Wokingham, UK).

2.5. Binding to adsorbed antigen

A modified version of the Direct Alhydrogel Formulation Immunoassay (DAFIA), established by Zhu et al. [17] and Westdijk et al. [18],

was performed to assess binding of the mAbs to Ad-DTxd. The assay was performed as described previously using a colorimetric readout with an HRP-labelled secondary antibody, instead of a fluorometric readout. Briefly, Ad-DTxd was diluted to 1 Lf/ml in PBST containing 5% BSA (sample buffer, SB) and titrated using two-fold dilutions in the wells of a 96 well plate (final volume 100 μl). The concentration of aluminium was kept constant by performing the titration in the related aluminium adjuvant diluted in SB. An adjuvant-only control was also included in the plate. Plates were centrifuged at 1000 g for 4 min and the supernatant was gently removed using a pipette. Plates were washed (3x) by adding 200 μl/well of PBST containing 0.2% BSA, centrifuging and removing the supernatant as before. Following washing plates were blocked with 200 μl/well SB at room temperature with agitation for 1.5 h. Plates were centrifuged and washed as described previously and mAb diluted to 1 μg/ml in SB (100 μl/well) was added for a further 1 h at room temperature with agitation. Following another centrifuge and wash step, bound mAb was detected using 100 μl/well of the relevant HRP-conjugated IgG diluted 1/2000 in SB (rabbit anti-rat for mAb DT05 and rabbit anti-mouse for the other mAbs). After a further incubation of 1 h at room temperature with agitation and a final wash step, 100 μl/well of TMB substrate was added and the plates were incubated in the dark for 5 min for the colour to develop. The reaction was stopped by the addition of an equal volume of 1 M H_2SO_4. To read the plates, 100 μl of the supernatant from each well was transferred to a clean flat bottom plate and the absorbance was measured at 450 nm.

2.6. Biosensor analysis

2.6.1. Calibration free concentration analysis (CFCA)

The active concentration of the mAbs for affinity measurements was determined on the Biacore system (Biacore T200, GE Healthcare, US). Briefly, a goat anti-mouse IgG Fc-specific antibody (Thermo Scientific) or a goat anti-rat antibody (Southern Biotech) was immobilised onto a CM5-sensorchip with a target level of 10000 RU by primary amine coupling. A second flow cell which had been treated chemically without any anti-serum was used as the reference cell for determining non-specific binding. The mAbs were diluted to 0.5–2 μg/ml (based on IgG measurements) in HBS-P buffer, containing 0.1 M HEPES, 1.5 M NaCl and 0.5% v/v Surfactant P20 (GE Healthcare, US) and injected during 36 s at two different flow rates (5 and 100 μl/min). The sensor chip was regenerated with 10 mM glycine-HCl, pH 1.5. The active concentration was calculated from the slope of the binding curve (assuming a 1:1 interaction model and using the molecular mass of 150 kDa for the antibody).

2.6.2. Affinity measurements

Affinity was determined using a protein G sensorchip. The mAbs were injected over the active flow cell resulting in a specific response of 10–20 RU. DTxn (Intravacc) was diluted to concentrations of 0.03, 0.08, 0.23, 0.7 and 2.1 μg/ml and injected in a single cycle for 3 min per concentration (flow rate 30 μl/min). The dissociation time was 30 min after the injection of the highest concentration of DTxn. All the mAbs underwent single cycle kinetic analysis, and Dim27 was also determined with multi cycle kinetics (each analyte concentration was injected in a separate cycle and the analyte was allowed to dissociate fully or was removed by regeneration) because of the relatively fast dissociation behaviour. The kinetics were determined by direct curve fitting of the sensorgram to a 1:1 model interaction.

2.6.3. Epitope competition analysis

Epitope mapping was performed using biosensor analysis with a CM5-sensorchip coupled to the relevant anti-mouse or anti-rat Fc-specific antibody (target level of 3500 RU) as described above. Subsequently, one of the mAbs was captured by the relevant immobilised antibody resulting in a response of 100–400 RU. Blocking was performed using a non-specific monoclonal antibody (ImmunoPure Mouse IgG, Whole Molecule, Thermo Fisher Scientific, US). DTxn (60 μg/mL) was then injected until a plateau level was obtained (typically in 2 min at a flow rate of 5 μl/min). The binding of a second monoclonal antibody was analysed after 2 min (flow rate 10 μl/min).

2.7. Domain mapping by Western blot

Purified recombinant DTxn domains (A and B fragments as well as single receptor binding (R) and translocation (T) domains of the B fragment) were kindly provided by the Technical University of Braunschweig [19]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the DTxn fragments according to their electrophoretic mobility. The DTxn fragments were mixed with LDS sample buffer and reducing agent (Bolt, Life Technologies) and were heated for 10 min at 70 °C. A protein standard and the samples (0.5 μg) were then loaded onto the gel. For electrophoresis, the assay was performed using the Life Technologies Bolt Bis-Tris system with MES running buffer, pre-made gels (12% bis-tris), and run at a constant voltage of 165 V for approximately 1 h. Western blotting was then performed using a wet-transfer system using the Bolt mini blot module. The transfer was performed using a 0.2 μM nitrocellulose membrane at a constant voltage of 10 V for 1 h. Following transfer, the membrane was blocked overnight at +4 °C (PBST + 5% Marvel), then washed three times with PBST for 15 min each. The diphtheria mAbs (1 μg/ml) were incubated with the membranes for 2 h at room temperature. Following washing as above, the membranes were incubated with an anti-rat (for DT05) or anti-mouse (for all other mAbs) HRP conjugate for 2 h at room temperature. The ECL2 substrate solution was then applied to each membrane and incubated for 5 min at room temperature. The membranes were then developed, and the images captured using the Gel Logic 2200 Pro Imaging System.

2.8. Vero cell toxin neutralisation test (TNT)

This Vero cell method is based on the assay first described by Miyamura and co-workers [20], with modifications to include spectrophotometric determination of assay end points [21]. Complete culture medium was prepared using minimum essential medium (MEM) supplemented with 5% fetal bovine serum, 1x antibiotic-antimycotic solution, 2 mM L-Glutamine, 0.1% D-Glucose and 0.015 M HEPES). Pre-diluted mAb samples (100 μl/well) were added to the first column of a 96-well tissue culture plate (Falcon) and serial two-fold dilutions (50 μl) were prepared across the plate in complete medium. Purified DTxn (NIBSC 02/154) was diluted to 2.5×10^{-5} Lf/ml in complete medium, approximately 4 times the minimum cytopathic dose of toxin for Vero cells. The diluted toxin was added to all wells (50 μl) containing mAb and plates were incubated at +37 °C for 1 h for toxin neutralisation to occur. At the end of the incubation period 50 μl of a Vero cell suspension (in complete culture medium) containing 4×10^5 cells/ml was added to all sample wells. Control wells containing cells only (cell control) or cells in complete medium containing DTxn (toxin control) was included on every plate. A reference serum (NIBSC, 10/262) was also included on each plate to enable neutralising antibody titers to be expressed in IU/ml. Plates were incubated at +37 °C for 6 days. After 6 days, cell viability was assessed using a tetrazolium dye (MTT). Here, 10 μl of MTT (5 mg/ml) was added per well and plates were incubated at 37 °C for a further 4 h. Supernatants were then removed and the MTT-formazan product in viable cells was extracted using 10% w/v sodium dodecyl sulfate in 50% v/v dimethylformamide, pH 4.7 (100 μl/well). Plates were returned to the incubator overnight to allow for complete extraction and solubilisation of the coloured product, and the OD was read at 570 nm. The antibody titre of each mAb sample was determined by comparing the last well of the reference antiserum preparation showing neutralisation of the toxin (defined as OD > 50% of the 'cell only' control wells) with the last well of the test serum preparation demonstrating the same effect.

3. Results

3.1. Binding to DTxn and non-adsorbed DTxd

All of the mAbs recognised DTxn and were able to produce a signal in the direct ELISA. The binding ability, however, was better for some mAbs than for others, with 1–49, 2–18 and Dim9 showing the highest binding to toxin, and 2–25 and Dim27 showing the lowest binding (Fig. 1). The dose response curve for mAb DT05 is shown separately to the other mAbs because a different conjugate antibody (anti-rat) was used for detection in this assay. Direct ELISAs were also performed against non-adsorbed DTxds from HuA and HuB. The mAbs bound well to these DTxds with the exception of Dim27 and Dim33. Dim27 did not produce a dose response curve and Dim33 only showed binding at the highest antibody coating concentrations (Fig. 2). The highest response to both DTxds was seen with mAbs 1–49 and 2–18. A similar high response was seen for Dim9, Dim25 and 2–25 against the DTxd from HuA, however the response for these mAbs against the HuB DTxd was slightly lower.

3.2. Binding to adsorbed DTxd

A direct alhydrogel (DAFIA) method was used to assess binding of mAbs to Ad-DTxd in the presence of adjuvant. In this method, the adsorbed antigen was titrated in the plate and the mAb samples were added at a fixed concentration. All of the mAbs were able to bind Ad-DTxd from HuB – although binding of Dim33 was relatively low compared to other antibodies. Only a subset of the mAbs was tested for binding to Ad-DTxd from HuA, but all antibodies tested were able to bind the adsorbed antigen. We observed only very low signal in control wells containing adjuvant alone and no antigen (Fig. 3).

3.3. Binding to heat altered DTxd

The mAbs were tested to determine whether they could detect antigenic changes in toxoid that had been altered by heat treatment. The DTxd samples were used to coat ELISA plates in a direct ELISA format, alongside a fresh sample that had been diluted immediately prior to the assay. The mAb binding curves to each of the samples at the 8-week time-point is shown in Fig. 4 (HuA DTxd samples) and Fig. 5 (HuB DTxd samples). A quantitative estimate of relative mAb binding, using response to fresh DTxd as the reference, could not be accurately calculated due to differences in the asymptotes of some of the curves (log transformed data). Therefore, only a visual assessment of the differences was made. The trends observed were the same for both toxoids, however the changes were more pronounced in the DTxd from manufacturer HuB. One of the mAbs (2-18) produced a similar signal when binding with fresh toxoid or toxoid stored at elevated temperatures, indicating that it is not able to detect temperature induced changes in the DTxd. The three mAbs (2–25, Dim27 and Dim33) previously shown to be relatively poor binders to DTxn or non-adsorbed DTxd were the most sensitive to temperature induced changes in the toxoid along with Dim9 (Figs. 4 and 5). The recognition of temperature altered toxoid is most pronounced for Dim27 because this mAb binds very poorly to the fresh DTxd. The mAbs Dim9, Dim27 and Dim33 show an increase in binding with elevated temperatures, whereas mAb 2–25 shows a decrease in binding with elevated temperature.

3.4. Affinity measurements and epitope mapping

First the active concentration was determined to estimate a proper antibody dilution for the kinetic assay (Table 3). Kinetic analysis was done using single cycle analysis, whereby each DTxn concentration was injected in one cycle and the protein G sensorchip was regenerated at the end. In addition, Dim27 was also analysed by multi-cycle analysis. The sensorgrams obtained with each mAb are shown in Fig. 6. Results from the subsequent kinetic analysis are shown in Table 3, arranged in order of decreasing affinity for DTxn (the lower the K_D value, the higher the affinity of the antibody). The mAbs 2–18 and Dim25 show the highest affinity to DTxn, and mAbs Dim33, Dim27 and 2–25 have the lowest affinity to DTxn.

For the epitope mapping, DTxn was captured by a first mAb, and binding of a second mAb was then measured. Additional binding is expected to be observed on the sensorgram if the second mAb binds to a separate epitope. Results are summarised in Table 4. The red cells in the table indicate overlapping epitopes, meaning that Dim25 and 2–25 have overlapping epitopes, and that 1–49 and Dim9 have overlapping epitopes. Interestingly, DT05, Dim25, 2–25 and Dim27 have overlapping epitopes, but not in a reversed order: Dim25, 2–25 and Dim27 do not have an overlapping epitope with DT05 if they bind DTxn first. However, if DT05 binds DTxn first the binding hampers the binding of the three other mAbs. The mAbs 2–18, Dim27 and Dim33 recognise non-overlapping epitopes.

3.5. Diphtheria toxin neutralisation and domain recognition

The neutralising potency of the mAbs was measured using the Vero cell TNT, and the specific activity was calculated from this using the measured IgG concentrations, as shown in Table 5. The ability of the mAbs to neutralise DTxn could be split into 3 groups, those with high neutralising activity (DT05), those with moderate activity (Dim9, Dim25, Dim33, 1–49 and 2–18) and those with low neutralising activity (Dim27 and 2–25). Western blots were performed to determine where the mAbs were binding on the diphtheria toxin. All mAbs were found to be directed against either the catalytic domain of the A fragment, or the receptor binding domain of the B fragment (Table 5).

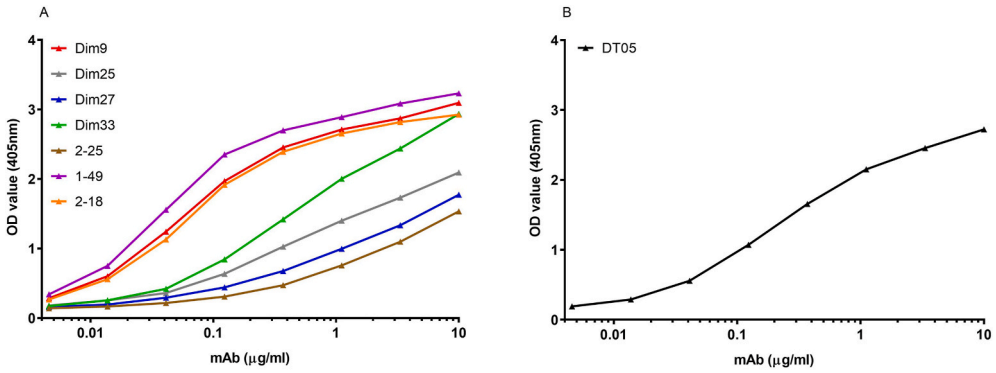

Fig. 1. mAb binding to diphtheria toxin (DTxn). Data shows representative results from one of two independent assays for (A) mouse mAbs and (B) rat mAb binding to DTxn.

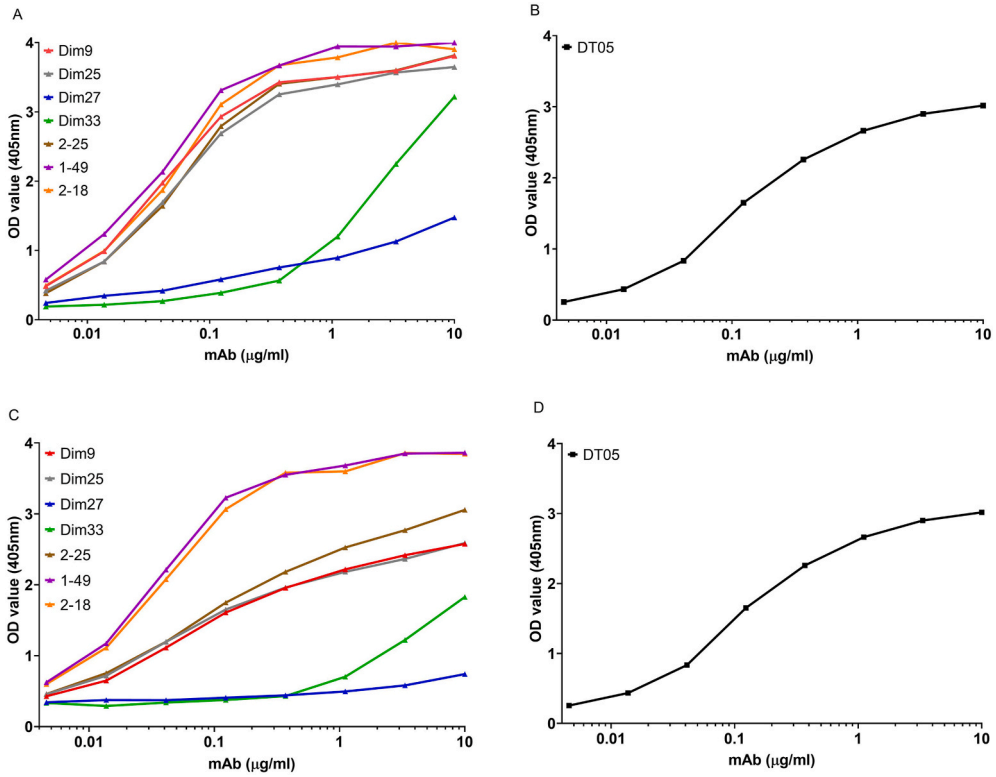

Fig. 2. mAb binding to non-adsorbed detoxified antigen (DTxd) from two vaccine manufacturers. Data shows representative results from one of two independent assays for (A) mouse mAbs binding to non-adsorbed toxoid from HuA; (B) rat mAb binding to non-adsorbed toxoid from HuA; (C) mouse mAbs binding to non-adsorbed toxoid from HuB; (D) rat mAb binding to non-adsorbed toxoid from HuB.

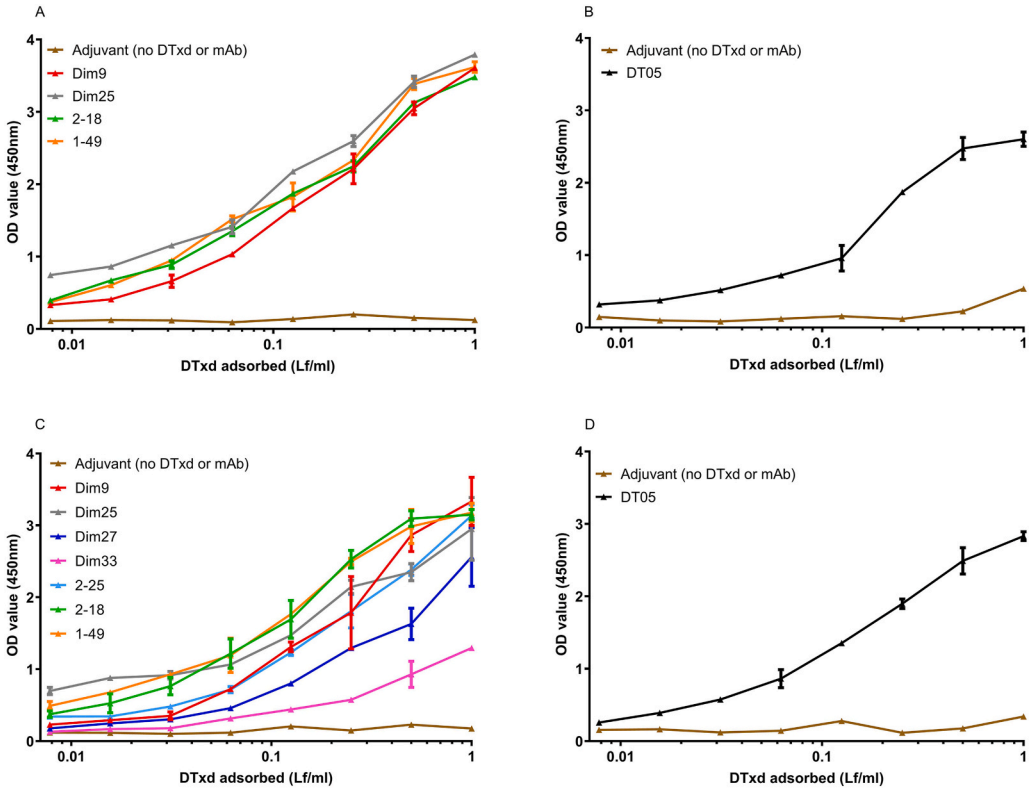

Fig. 3. mAb binding to adsorbed diphtheria toxoid (Ads-DTxd). Data shows representative results from one of two independent DAFIA assays for (A) Mouse mAbs binding to adsorbed toxoid from HuA; (B) Rat mAb binding to adsorbed toxoid from HuA; (C) Mouse mAbs binding to adsorbed toxoid from HuB; (D) Rat mAb binding to adsorbed toxoid from HuB. Adsorbed antigen samples were titrated in a fixed adjuvant concentration and detected using a single concentration of mAb. Data points are the average OD values from duplicate wells (±SEM).

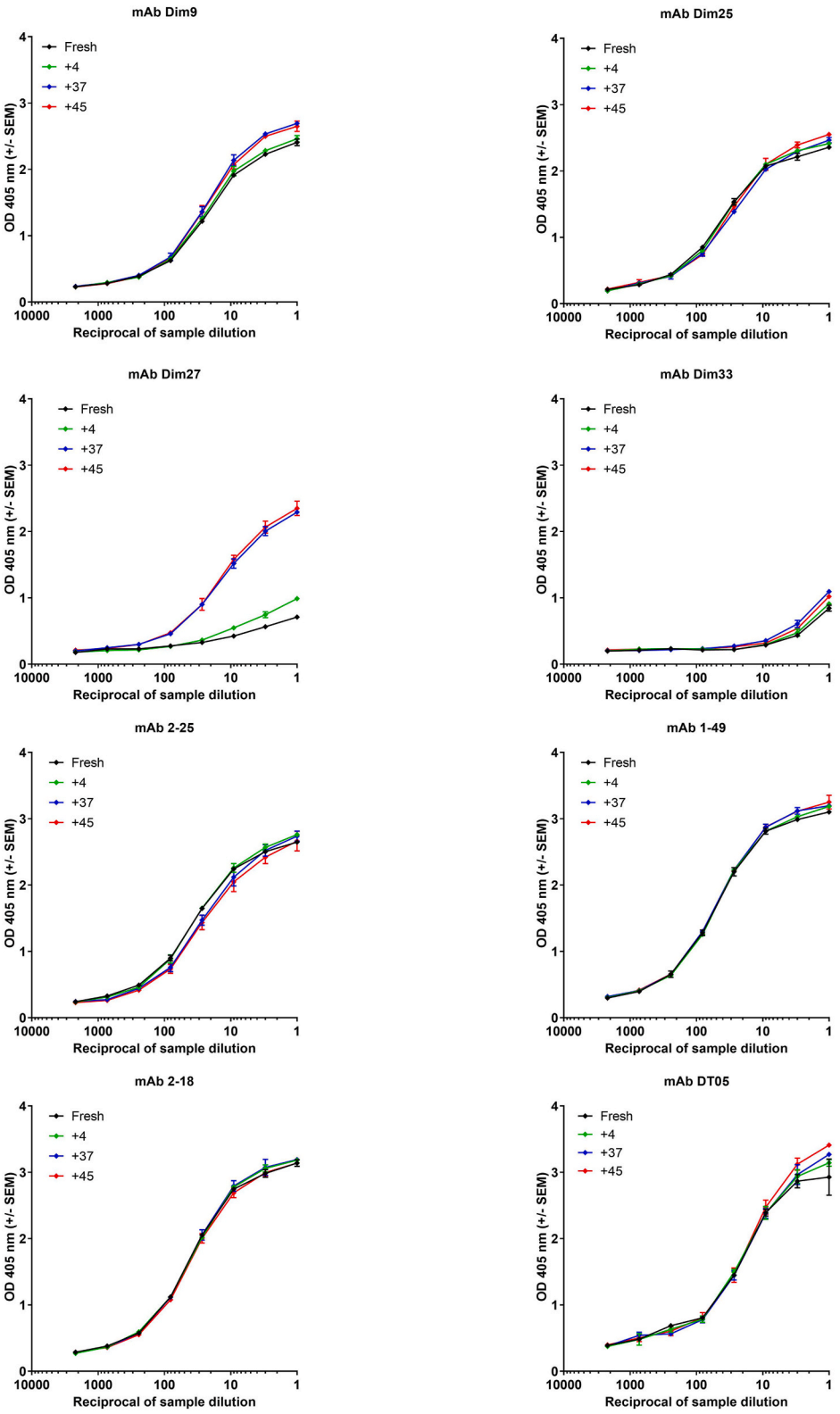

Fig. 4. mAb binding to non-adsorbed diphtheria toxoid (HuA) exposed to elevated temperature for 8 weeks. Freshly diluted toxoid and toxoid samples that had been incubated at +4, +37 or +45 °C for 8 weeks, were titrated in the same assay. Data shown is the average OD value from duplicate wells (±SEM).

4. Discussion

The development of a monoclonal antibody immunoassay that can be implemented into a control strategy for diphtheria vaccines will be dependent on the use of suitable, well characterised antibodies as critical reagents for the assay. Where an assay of this type is intended to serve as a potency test, the antibody used should be of high affinity, should target an epitope that is relevant for protection and is stability indicating [22–24]. We report here the thorough characterisation of a panel of existing diphtheria mAbs with a view to selection of a pair of

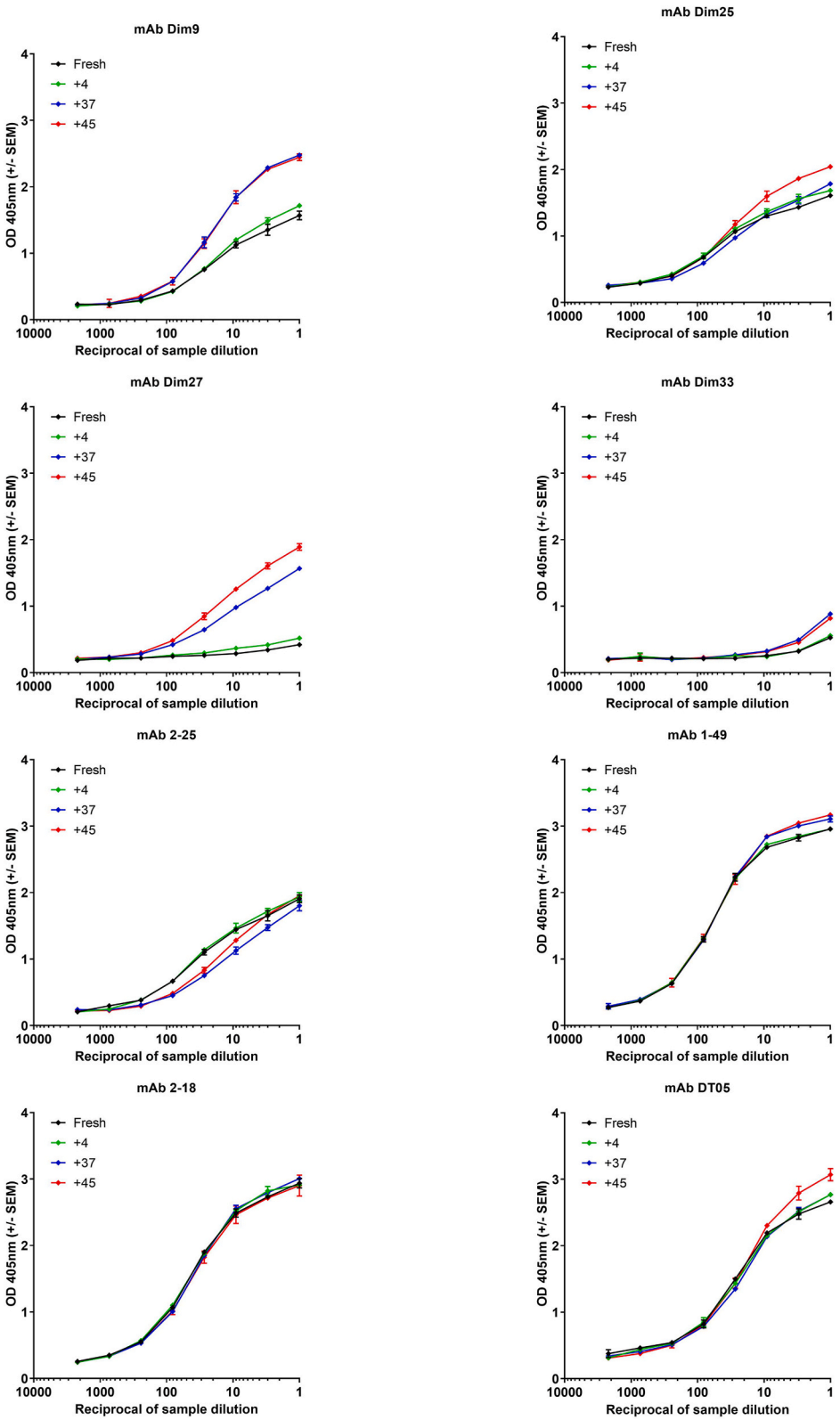

Fig. 5. mAb binding to non-adsorbed diphtheria toxoid (HuB) exposed to elevated temperature for 8 weeks. Freshly diluted toxoid and toxoid samples that had been incubated at +4, +37 or +45 °C for 8 weeks, were titrated in the same assay. Data shown is the average OD value from duplicate wells (±SEM).

antibodies that can be used for development of a replacement *in vitro* diphtheria vaccine potency assay.

The antibodies in this study were from different sources, produced and purified using different approaches. As a result, the IgG concentration was measured for all mAbs to help standardise antibody concentrations in binding assays. For most binding assays we used a representative batch of non-adsorbed or adsorbed diphtheria toxoid from two different vaccine manufacturers – and in most cases, results

Table 3
Affinity of the mAbs for diphtheria toxin. Calibration Free Concentration Analysis (CFCA) was determined by biosensor analysis. mAbs are ranked from the highest (1) to the lowest affinity (8) for DTxn. [1]Average ± sd (n = 3); [2]K_d generated by the single cycle analysis; [3]K_d generated by the multicycle analysis.

Ranking	mAb	CFCA	Association rate x 10^5 (K_a, 1/Ms)	Dissociation rate x 10^{-3} (K_d, 1/s)	Equilibrium dissociation constant (K_D, M)
1	2–18	1.30 ± 0.10	4.05	0.672	1.66E-09
2	Dim 25	0.10 ± 0.01	21.9	4.24	1.94E-09
3	1–49	1.67 ± 0.06	8.95	3.21	3.59E-09
4	DT05	0.84 ± 0.07	3.61	1.55	4.29E-09
5	Dim 9	0.86 ± 0.12[1]	4.68	2.67	5.71E-09
6	Dim 33	0.24 ± 0.01	3.67	2.23	6.09E-09
7	2–25	0.41 ± 0.08	4.61	3.11	6.76E-09
8	Dim 27[2]	0.48 ± 0.04	4.09	9.44	23.1E-09
8	Dim 27[3]		3.42	8.46	24.8E-09

obtained were not influenced by the source of the antigen used for binding assay. All eight mAbs were able to bind to native antigen (DTxn) and detoxified antigen (DTxd). Two antibodies (Dim33 and Dim27) were notable for reduced binding to detoxified antigen compared to other mAbs and this was true for the antigen from both manufacturers. For one of the antigen samples (HuA), there were 2 populations of antibody in terms of binding profile, with Dim27 and Dim33 notable for reduced binding to DTxd compared to the other mAbs. For the other antigen sample (HuB), 3 populations of antibody were observed and, again, Dim27 and Dim33 were notable for reduced binding.

For development of an *in vitro* potency assay, it is necessary to perform the test on the drug product, which contains aluminium adjuvant, the presence of which may interfere with binding of antibody to the target antigen [7,17,25–27]. To assess binding of mAbs in the presence of adjuvant we used a modified version of the Direct Alhydrogel Formulation Immunoassay (DAFIA) [17,18] where adsorbed antigen samples are titrated in a fixed concentration of adjuvant. The principle of the assay is similar to an ELISA except that DTxd adsorbed to aluminium adjuvant is pelleted by centrifugation in wells of an ELISA plate (rather than being directly bound to the surface of the plate as in a traditional ELISA). The data was quite variable, mainly due to the difficulties in removing all of the reagent/wash buffer consistently from the adjuvant pellet during the wash steps, but allowed us to identify low or high binders to the adsorbed antigen. All of the mAbs were tested against adsorbed antigen from HuB, however due to the time consuming nature of the assay, only a selection of mAbs were tested against adsorbed antigen from HuA; those mAbs which did not bind well to non-adsorbed DTxd and/or DTxn were excluded (Dim 27, Dim 33 and 2–25). All mAbs were able to bind to the HuB adsorbed diphtheria toxoid, but again Dim33 showed reduced binding compared to the other mAbs. Interestingly, Dim27 showed improved binding to adsorbed antigen over non-adsorbed antigen. This may be due to the presentation of the antigen in the immunoassay (for the DAFIA both antibody and antigen are in solution, whereas for ELISA the antigen is coated on a solid surface), or the adsorption of adjuvant may cause a conformation change and increased availability of the epitope. We did not observe binding to adjuvant alone (either aluminium hydroxide or aluminium phosphate). Although specificity testing will be performed as part of future immunoassay development, the results obtained here suggest that it will be feasible to use one or more of these mAbs for testing aluminium containing vaccines in immunoassay.

To provide an indication of whether one or more of these antibodies is sensitive to changes in the antigen that are relevant for quality assessment, we deliberately altered non-adsorbed toxoid samples by exposure to heat. The mAb Dim27 was the most sensitive to a heat-induced change in the toxoid which may be due to the fact that this antibody shows relatively poor binding to fresh toxoid. On exposure to heat, some unfolding of the protein is likely [28–30] and the epitope targeted by Dim27 may become more accessible (from being largely inaccessible to this mAb under normal toxoid storage conditions), resulting in increased binding (relative to the fresh toxoid). A similar pattern was also observed for Dim33 which, together with Dim27, showed relatively poor binding to detoxified antigen under normal conditions. We also observed an increase in mAb binding to heated toxoid for Dim9 and this was more pronounced for one antigen sample than the other. One mAb, 2–25, showed reduced binding to heated toxoid which again was more pronounced for one antigen sample than the other. The mAb 2–18 appeares to be insensitive to heat-induced changes in the toxoid samples.

With the exception of Dim27, all of the mAbs included in this study showed high affinity for the diphtheria toxin antigen with dissociation constants in the low nanomolar range. The on/off rates of the Dim mAbs (Dim25, 33 and 27) are in good agreement with results published by Metz et al. [31]. The lower affinity of Dim27 was reflected in relatively poor binding to both non-adsorbed toxoid antigens and in the results obtained in the cell-based toxin neutralisation test (TNT), where no neutralisation was observed using this mAb. All of the other mAbs were able to neutralise diphtheria toxin in the TNT, but neutralising activity of mAb 2–25 was low. This antibody also showed relatively poor binding to native antigen and had the lowest affinity for DTxn of all mAbs except Dim27. Interestingly, mAb 2–25 showed good binding to detoxified antigen (both non-adsorbed and adsorbed) suggesting that the epitope for this mAb is dependent on the conformation of cross-linked toxoid protein. Western blotting using purified diphtheria toxin domains revealed that the mAbs were directed against either the receptor binding domain (B fragment) or catalytic domain (A fragment) of diphtheria toxin [32]. This is consistent with previous studies that have shown that neutralising monoclonal antibodies may be directed against either fragment of DTxn [33,34]. However, the mAbs with the highest neutralising activity were not all directed towards an epitope on the same toxin domain, and observed differences in neutralising potency may be related to differences in affinity maturation.

Epitope competition studies showed that inhibition of mAb binding only occurred with pairs of mAbs directed towards the same toxin domain. Two mAb pairs appear to be targeting overlapping epitopes and inhibition of binding was observed regardless of order of binding to antigen: Dim9 and 1–49 for the C-domain, and Dim25 and 2–25 for the R-domain. We observed asymmetric inhibition between DT05 and 3 other mAbs targeting the same R-domain of the toxin molecule. A possible explanation for this phenomenon is that binding of DT05 to DTxn induces a conformational change in the toxin such that Dim25, 2–25, and Dim27 can no longer bind. Although unidirectional displacement between antibodies targeting minimally overlapping or closely adjacent epitopes has also been demonstrated by others [35] and cannot be ruled out. Overall, we identified a number of potential mAb combinations that were non-inhibitory and therefore suitable for consideration in the development of a sandwich immunoassay.

Not all assays provided strong evidence of superiority for one antibody over another, and the final selection of antibodies to take forward to development of a capture sandwich ELISA was based on the overall

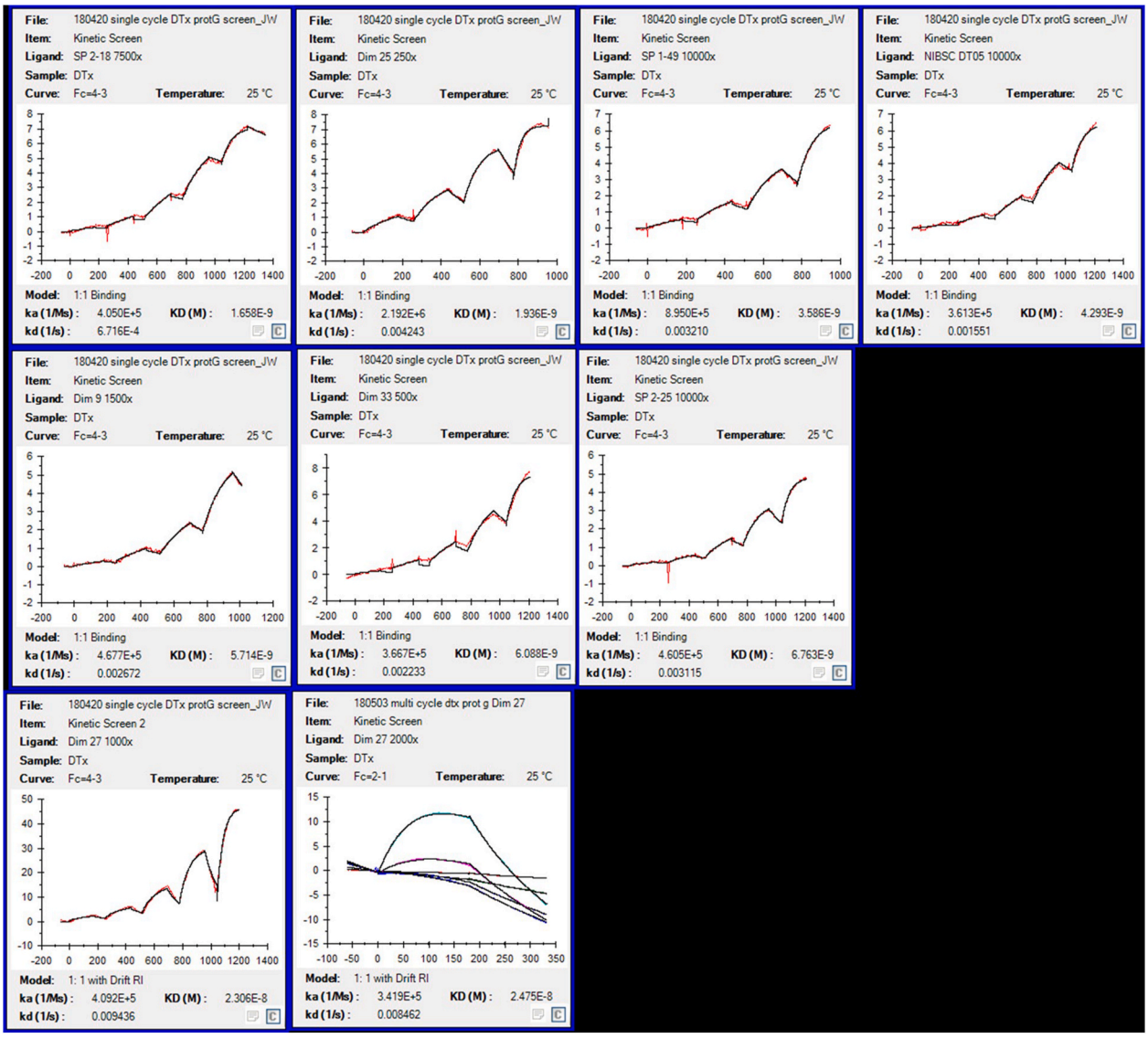

Fig. 6. Representative binding sensorgrams for mAb-antigen interactions using the single cycle kinetic assay. Anti-diphtheria mAb was first captured using a protein G sensorchip followed by the sequential injection of increasing concentrations (0.03, 0.08, 0.23, 0.7 and 2.1 μg/ml) of diphtheria toxin (black curves). Red curves: best fit of the sensorgram with the 1:1 binding model (Bia evaluation software). The last image at the bottom right of the panel is shown as an example of the multicycle kinetic analysis for mAb Dim27 in which each diphtheria toxin concentration was injected in a separate cycle and the analyte was allowed to dissociate fully. Black lines in this image represent the best fit of each sensorgram with the 1:1 binding model. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

assessment of binding profile to normal and stressed antigen, antibody function and competition. Some antibodies were clearly notable for low neutralising activity and relatively poor binding to antigen. These antibodies (Dim27, 2–25 and Dim33), which also had the lowest affinity for native antigen, were therefore not considered for use in development of a sandwich ELISA. Despite its high affinity, mAb 2–18 was insensitive to potential antigenic changes caused by prolonged storage of the toxoid antigen at elevated temperature and is therefore less suitable for assay development where detection of changes indicative of quality or stability is essential. Three of the antibodies in the panel studied here (Dim9, Dim25 and DT05) all showed good binding to antigen (toxin and non-adsorbed or adsorbed toxoid), had affinities in the nano molar range and neutralised diphtheria toxin in a cell-based TNT. Of these, Dim9 was most sensitive to detect heat altered antigen and is the preferred antibody to take forward to assay development. Competition analysis revealed that only certain pairs of antibody could be deployed in a sandwich ELISA format and based on the overall profile we propose that DT05 is selected as the second antibody for use in a sandwich format because it has the highest neutralising activity of all the antibodies and is a different species to Dim9 (and Dim25) avoiding the need for biotinylating one of the antibodies in a sandwich ELISA. The development of the ELISA, using these two antibodies, is now underway in our laboratory and will assess the ability of the assay to detect antigenic changes that are indicative of vaccine quality/stability covering a wider range of diphtheria vaccine types.

The extensive characterisation of the monoclonal antibodies performed in this study, including affinity, functional activity and recognition of heat-altered antigen, provides a platform of evidence to support their use in development of quantitative immunoassays that, subject to appropriate validation and regulatory approval, can be implemented as part of a control strategy for diphtheria vaccines, potentially as a substitute for *in vivo* potency. The possibility to produce recombinant versions of the selected antibodies can be explored to ensure sustainability of critical reagents.

Table 4
Epitope competition for diphtheria monoclonal antibodies. Epitope competition was determined by biosensor analysis. "-" indicates no binding of the secondary antibody (response second antibody/primary antibody < 20%) with shaded cells indicating that binding was reduced but only in one direction; "+" indicates substantial binding of the secondary antibody (response secondary antibody > 5x response background).

		Second mAb							
		2-18	Dim 25	1-49	DT05	Dim 9	Dim 33	2-25	Dim 27
First mAb	2-18	-	+	+	+	+	+	+	+
	Dim 25	+	-	+	+	+	+	-	+
	1-49	+	+	-	+	-	+	+	+
	DT05	+	-	+	-	+	+	-	-
	Dim 9	+	+	-	+	-	+	+	+
	Dim 33	+	+	+	+	+	-	+	+
	2-25	+	-	+	+	+	+	-	+
	Dim 27	+	+	+	+	+	+	+	-

Table 5
Diphtheria toxin neutralisation and domain recognition for the monoclonal antibodies.

mAb ID	Toxin binding domain	Neutralising activity (IU/mg)
Dim9	A fragment, C domain	1.03
Dim25	B fragment, R domain	0.87
Dim27	B fragment, R domain	≤0.002
Dim33	A fragment, C domain	0.70
DT05	B fragment, R domain	46.55
2–25	B fragment, R domain	0.06
1–49	A fragment, C domain	4.52
2–18	A fragment, C domain	3.96

Author contributions

RRD and LH participated in the design of the study, designed and performed experiments, analysed data and drafted the manuscript. AK and JW participated in the design of the study, designed and performed experiments and analysed data. AD and AF participated in the design of the study. PS conceived the study, participated in the design of the study and drafted the manuscript. All authors reviewed and contributed to final editing and refinement of the manuscript.

Funding

This work has been funded by the EU/EFPIA/Innovative Medicines Initiative 2 Joint Undertaking (VAC2VAC grant No. 115924).

Declaration of competing interest

None.

Acknowledgements

The authors are grateful to the industry partners in VAC2VAC who provided antigen and vaccine samples for use in this study.

References

[1] De Mattia F, Chapsal JM, Descamps J, Halder M, Jarrett N, Kross I. The consistency approach for quality control of vaccines – a strategy to improve quality control and implement 3Rs. Biologicals 2011;39:59–65.
[2] VAC2VAC website. http://www.vac2vac.eu/. accessed 04-Aug-2020.
[3] Suresh K, Mahendra PS, Vijay KB, Pandey RM. Quality control of vaccines-A journey from classical approach to 3Rs. Microbiol Curr Res 2018;2:45–61.
[4] Weisser K, Hechler U. Animal welfare aspects in the quality control of immunobiologicals. A critical evaluation of the animal tests in pharmacopoeial monographs. Nottingham: FRAME; 1997. p. 276–84.
[5] Coombes L, Stickings P, Tierney R, Rigsby P, Sesardic D. Development and use of a novel *in vitro* assay for testing of diphtheria toxoid in combination vaccines. J Immunol Methods 2009;350:142–9.
[6] Coombes L, Tierney R, Rigsby P, Sesardic D, Stickings P. *In vitro* antigen ELISA for quality control of tetanus vaccines. Biologicals 2012;40:466–72.
[7] Riches-Duit R, Hassall L, Rigsby P, Stickings P. Evaluation of a capture antigen ELISA for the characterisation of tetanus vaccines for veterinary use. Biologicals 2019;61:8–14.
[8] Costa CI, Delgado IF, da Costa JA, de Carvalho RF, Mouta Sda Jr S, Vianna CO, de Moraes MT. Establishment and validation of an ELISA for the quantification of HBsAg in recombinant hepatitis B vaccines. J Virol Methods 2011;172:32–7.
[9] Descamps J, Giffroy D, Remy E, Mortiaux F, Mareschal JC, Ponsar C, Duchene M. A case study of development, validation, and acceptance of a non-animal method for assessing human vaccine potency. Proc Vaccinol 2011;5:184–91.
[10] Shanmugham R, Thirumeni N, Rao VS, Pitta V, Kasthuri S, Singanallur NB, Lingala R, Mangamoori LN, Villuppanoor SA. Immunocapture enzyme-linked immunosorbent assay for assessment of *in vitro* potency of recombinant hepatitis B vaccines. Clin Vaccine Immunol 2010;17:1252–60.
[11] Chabaud-Riou M, Moreno N, Guinchard F, Nicolai MC, Niogret-Siohan E, Seve N, Manin C, Guinet-Morlot, Riou P. G-protein based ELISA as a potency test for rabies vaccines. Biologicals 2017;46:124–9.
[12] Wang Z, Sun Y, Wu X, Carroll DS, Lv W, You L, Ji Y, Shi J, Yan J, Xu G, Meng S. Development of a relative potency test using ELISA for human rabies vaccines. Biologicals 2018;55:59–62.
[13] Maas RA, de Winter MPM, Venema S, Oei HI, Claassen IJTM. Antigen quantification as *in vitro* alternative for potency testing of inactivated viral poultry vaccines. Vet Q 2000;22:223–7.
[14] Aly SE, Hussein HA, Aly AHM, Abdel-Baky MH, El-Sanousi AA. Assessment of *in vitro* potency of inactivated Newcastle disease oil-adjuvanted vaccines using hemagglutination test and blocking ELISA. Vet World 2018;11:1222–8.
[15] Sigoillot-Claude C, Battaglio M, Fiorucci M, Gillet D, Vimort AS, Giraud Y, Laurent S, Vaganay A, Poulet H. A versatile *in vitro* ELISA test for quantification and quality testing of infectious, inactivated and formulated rabies virus used in veterinary monovalent or combination vaccine. Vaccine 2015;33:3843–9.
[16] The immunological basis for immunization series. Module 2: diphtheria update 2009. Geneva: World Health Organization; 2009.
[17] Zhu D, Huang S, Gebregeorgis E, McClellan H, Dai W, Miller L, Saul A. Development of A Direct alhydrogel formulation immunoassay (DAFIA). J Immunol Methods 2009;344(1):73–8.
[18] Westdijk J, Metz B, Spruit N, Tilstra W, van der Gun J, Hendriksen C, Kersten G. Antigenic fingerprinting of diphtheria toxoid adsorbed to aluminium phosphate. Biologicals 2017;47:69–75.
[19] Wenzel EV, Bosnak M, Tierney R, et al. Human antibodies neutralizing diphtheria toxin in vitro and in vivo. Sci Rep 2020;10(1):571. https://doi.org/10.1038/s41598-019-57103-5. Published 2020 Jan 17.
[20] Miyamura K, Nishio S, Ito A, Murata R, Kono R. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using VERO cells. I. Studies on factors affecting the toxin and antitoxin titration. J Biol Stand 1974;2(3):189–201.
[21] Aggerbeck H, Heron I. Improvement of a Vero cell assay to determine diphtheria antitoxin content in sera. Biologicals 1991;19(2):71–6.
[22] Council of Europe. 5.2.14 Substitution of in vivo method(s) by in vitro method(s) for the quality control of vaccines. In: European pharmacopoeia. EDQM; 2016.

[23] Metz B, Brunel F, Chamberlin C, Van der Gun J, Halder M, Jiskoot W, Kersten G, Van Opstal O, Petersen JW, Ravetkar SD, Redhead K, Sloth Wilhelmsen E, Vann WF, Hendriksen C. The potential of physicochemical and immunochemical assays to replace animal tests in the quality control of toxoid vaccines - the report and recommendations of ECVAM workshop 61a. Atla-Altern Laboratory Anim 2007;35:323–31.

[24] Hendriksen C, Arciniega JL, Bruckner L, Chevalier M, Coppens E, Descamps J, Duchene M, Dusek DM, Halder M, Kreeftenberg H, Maes A, Redhead K, Ravetkar SD, Spieser JM, Swam H. The consistency approach for the quality control of vaccines. Biologicals 2008;36:73–7.

[25] Ugozzoli M, Laera D, Nuti S, Skibinski DAG, Bufali S, Sammicheli C, Tavarini S, Singh M, O'Hagen DT. Flow cytometry: an alternative method for direct quantification of antigens adsorbed to aluminum hydroxide adjuvant. Anal Biochem 2011;418:224–30.

[26] Katz J. Desorption of porcine parvovirus from aluminium hydroxide adjuvant with subsequent viral immunoassay or hemagglutination assay. Vet Res Commun 1987; 11:83–92.

[27] Katz J, Hanson SK, Patterson PA, Stoll IR. *In vitro* assessment of viral antigen content in inactivated aluminum hydroxide adjuvanted vaccines. J Virol Methods 1989;25:101–8.

[28] Ho MM, Mawas F, Bolgiano B, Lemercinier X, Crane DT, Huskisson R, Corbel MJ. Physico-chemical and immunological examination of the thermal stability of tetanus toxoid conjugate vaccines. Vaccine 2002;20:3509–22.

[29] Salnikova MS, Joshi SB, Rytting JH, Warny M, Middaugh CR. Physical characterization of Clostridium difficile toxins and toxoids: effect of the formaldehyde crosslinking on thermal stability. J Pharmacol Sci 2008;97:3735–52.

[30] Gribenko A, Severina E, Sidhu MK, Jansen KU, Green BA, Matsuke YV. Development of a subunit vaccine for prevention of Clostridium difficile associated diseases: biophysical characterization of toxoids A and B. Biochem Biophy Report 2017;9:193–202.

[31] Metz B, Jiskoot W, Hennink WE, Crommelin DJA, Kersten GFA. Physicochemical and immunochemical techniques predict the quality of diphtheria toxoid vaccines. Vaccine 2003;22:156–67.

[32] Collier RJ. Understanding the mode of action of diphtheria toxin: a perspective on progress during the 20th century. Toxicon 2001;39:1793–803.

[33] Kakita M, Takahashi T, Komiya T, Iba Y, Tsuji T, Kurosawa Y, Takahashi M. Isolation of a human monoclonal antibody with strong neutralising activity against diphtheria toxin. Infect Immun 2006;74:3682–3.

[34] Usuwanthim K, Pootong A, Chaisri U, Tongtawe P, Tapchaisri P, Chongsa-Nguan M, Chaicumpa W. Murine monoclonal antibodies neutralising the cytotoxic activity of diphtheria toxin. Asian Pac J Allergy Immunol 2008;26:47–55.

[35] Abdiche YN, Yeung AY, Ni I, Stone D, Miles A, Morishage W, Rossi A, Strop P. Antibodies targeting closely adjacent or minimally overlapping epitopes can displace one another. PloS One 2017;12(1):e0169535. https://doi.org/10.1371/journal.pone.0169535.