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Development of a multiplex-based immunoassay for the characterization of diphtheria, tetanus and acellular pertussis antigens in human combined DTaP vaccines

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ABSTRACT

Routine batch quality testing before vaccine release, notably for potency evaluation, still relies on animal use for several animal and human vaccines. In this context, the VAC2VAC project is a public-private consortium of 22 partners funded by EU whose the main objective is to reduce the number of animal used for batch testing by developing immunoassays that could be implemented for routine potency assessment of vaccines. This paper focused on the development of a Luminex-based multiplex assay to monitor the consistency of antigen quantity and quality throughout the production process of DTaP vaccines from two human vaccine manufacturers. Indepth characterized monoclonal antibody pairs were used for development and optimization of the Luminex assay with non-adsorbed and adsorbed antigens and with complete vaccine formulations from both manufacturers. The multiplex assay demonstrated good specificity, reproducibility and absence of cross-reactivity. Analysis of over and underdosed formulations, heat and H2O2-degraded products as well as batch to batch consistency of vaccines from both manufacturers brought the proof of concept for a future application of the multiplex immunoassay as a useful tool in the frame of DTaP vaccine quality control.

1. Introduction

Started in 2016, VAC2VAC is a European consortium regrouping 23 partners from both public and private organisations and funded by the Innovative Medicines Initiative 2 (IMI2).(VAC2VAC, n.d.) The final goal of this project is the development and the (pre-)validation of alternative non-animal techniques for quality control of both human and veterinary vaccines during batch production and release.(De Mattia et al., 2011) Model vaccines were selected among established products at the beginning of the project based on the number of animals currently used and their degree of discomfort during potency testing using existing methods. Generally, the selected products were vaccines manufactured through traditional production methods (inactivation/attenuation) against diseases such as diphtheria, tetanus, pertussis or rabies and for

Abbreviations: aP, Acellular pertussis; BSA, Bovine serum albumin; DT, Diphtheria; DTaP, Diphtheria, tetanus and acellular pertussis combined vaccine; EDC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide; ELISA, Enzyme-linked immunosorbent assays; FHA, Filamentous hemagglutinin; GCV, Geomean coefficient of variation; GMP, Good manufacturing practices; H_2O_2, Hydrogen peroxide; HepB, Hepatitis B; HiB, Haemophilus Influenza type b; HuA, Human DTaP vaccine manufacturer A; HuB, Human DTaP vaccine manufacturer B; IMI2, Innovative medicines initiative; IPV, Inactivated poliovirus; mAb, Monoclonal antibody; MIA, Multiplex immunoassay; MFI, Mean fluorescence intensity; NIBSC, National Institute for Biological Standards and Control; PBS, Phosphate-buffered saline; PRN, Pertactin; PT, Pertussis toxoid; RA, Relative antigenicity; RT, Room temperature; Sulfo-NHS, N-hydroxysulfosuccinimide; TT, Tetanus; VAC2VAC, Vaccine lot to vaccine lot comparison by consistency testing.

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which a control strategy based on use of non-animal methods is not already accepted as it is for many new generation vaccines. Indeed, development, production and control of new generation vaccines are accompanied by an in depth characterization of the product under strict quality system conditions ensuring both production and testing methods consistency. Following this approach allows the production of consistent product batches whose specifications are in line with those of batches already shown to be clinically effective and defined in the marketing authorization. Application of this consistency approach is of paramount importance for moving to an animal-free batch release and is based on in-depth knowledge of the product and on the principle that the quality of new batches is ensured by consistent production compared to previous batches thanks to a GMP quality system.(Bruysters et al., 2017)

Simultaneous immunization against diphtheria (DT), tetanus (TT) and pertussis was among the first attempts of mixing antigens targeting different diseases into a combined vaccine at the end of the 40s. Today, these combined vaccines can confer a protection against up to six different diseases with the development of formulations containing antigens of DT, TT, acellular pertussis (aP), inactivated poliovirus (IPV), *Haemophilus influenza type b* (Hib), and hepatitis B (HepB).(Skibinski et al., 2011) Development of combined DT, TT and aP (DTaP) vaccines allowed the simplification of the immunization schedules of the young children, improved parents' compliance by reducing the number of required injections and, at the same time, improved the coverage compared to multiple monovalent vaccines.(Kalies et al., 2006; Marshall et al., 2007) However, several challenges accompany the development of these more complex formulations due to the panel of analytical techniques required for the characterization of antigens and antigen/adjuvant interactions. In addition, adjuvant(s) and/or excipients can interfere with antigen characterization techniques. Adjuvant dissolution or antigen desorption could solve the problem but generally represent time-consuming procedures and can modify the structure and/or antigenicity of antigens.(Hutcheon et al., 2006; Seeber et al., 1991; Brito et al., 2013) While direct alhydrogel formulation immunoassay(Zhu et al., 2009) and flow cytometry(Ugozzoli et al., 2011) approaches were developed for direct antigen quantification without need of a desorption step, immunoassays are still preferred for their accuracy, sensitivity and specificity.(Dey et al., 2014)

The methodology can vary among different countries but all potency tests for DT, TT and pertussis vaccines are still performed on animals. (Weißer and Hechler, 1997; Suresh et al., 2018; World Health Organization, 2013) Considering the number of produced vaccine batches and the number of animals required for batches quality control, implementation of *in-vitro* techniques monitoring antigenicity consistency as surrogate of potency measurement of these vaccines would have a significant positive impact on the global number of used animals and can considerably shorten the time required to complete batch control testing. To be relevant, the developed *in-vitro* assay should control key quality attributes such as antigen content and functionality through the use of well characterized monoclonal antibodies.(European Phamacopoeia, n.d.) The VAC2VAC consortium already published the characterization of a panel of monoclonal antibodies (mAbs) and their application for the development of enzyme-linked immunosorbent assays (ELISAs) allowing quantification of DT and TT in human and veterinary combined vaccines.(Riches-Duit et al., 2021a; Riches-Duit et al., 2019; Riches-Duit et al., 2021b) A series of mAbs directed against aP antigens was also studied with the aim to identify the best candidates for the development of an *in-vitro* antigenicity immunoassay.(Francotte et al., n.d.) This led to the selection of 4 pairs of mAbs specific for the pertussis toxoid (PT), the filamentous hemagglutinin (FHA), the pertactin (PRN), and the Fimbriae 2/3 antigens on the basis of their binding properties to non-adsorbed antigens, adsorbed antigens, heat-altered antigens as well as their ability to recognize epitopes on both native and detoxified antigens.(Francotte et al., n.d.) Here, we described the development of a multiplex immunoassay (MIA) using well characterized mAbs that could potentially serve as an *in-vitro* relative antigenicity (RA) assay for DTaP vaccines representing an important step forward for the reduction of the number of used animals as well as for the implementation of a batch to batch consistency approach during vaccine quality control.

2. Material and methods

2.1. Antigens/vaccines samples

Non-adsorbed and adsorbed antigens used for vaccine formulation as well as final lot vaccines were obtained from two manufacturers in the VAC2VAC consortium (HuA and HuB). Adsorbed DT and TT from HuA were not tested due to a process particularity. Non-adsorbed DT (NIBSC, 13/212), TT (NIBSC, 16/302), PT (NIBSC, 15/126), FHA (NIBSC, 90/520) and PRN (NIBSC, 18/154) and adsorbed DT (NIBSC, 07/216), TT (NIBSC, 08/218), PT (NIBSC, JNIH-3) and FHA (NIBSC, JNIH-3) were all received from NIBSC. The human vaccines from HuA contained only DT and TT or DT, TT, PT and FHA antigens with additional antigens such as IPV, HepB and/or Hib. Hence, three different formulations from HuA were used in the study and were noted as followed: HuA dt-IPV (booster), HuA DTaP-IPV and HuA DTaP-IPV-HepB-Hib. From the HuB, high dose formulations contained either DTaP antigens only (HuB DTaP) or additional IPV (HuB DTaP-IPV) or IPV and HepB (HuB DTaP-IPV-HepB) antigens. In the same way, booster vaccines from the same manufacturer contained either dtap antigens only (HuB dtap) or additional IPV (HuB dtap-IPV) antigens. Composition and concentration of the vaccines are represented in the supplementary Table 1. Dropout samples provided by HuA and HuB corresponded to DTaP-IPV-HepB-Hib and DTaP formulation respectively.

2.2. Monoclonal antibodies conjugation to Magplex microspheres and biotin

All capture and detection mAbs used during the development of the MIA were obtained from four providers in the VAC2VAC consortium and are shown in Table 1. To develop the 5-plex MIA, 25 µg of capture mAbs were coupled to carboxyl groups of 2.1×10^6 Magplex microspheres corresponding to spectral regions 12, 33, 36, 38, and 72 (#12: MC10012–01; #33: MC10033–01; #36: MC10036–01; #38: MC10038–01; #72: MC10072–01, Luminex Corporation) through activation *via* 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 50 mg/mL, Thermofisher Scientific, 22,980) chemistry following the Luminex xMAP® Cookbook 4nd edition.(Angeloni et al., n.d.) Briefly, microspheres were vortexed (20s) and sonicated (20s) to separate bead aggregates. Each mAb was covalently linked to one specific bead set: #12 (Dim9), #33 (8E1-1H1.2.1), #36 (PS21C2.2.1), #38 (FHADETOX/6), and #72 (69 K/16). For each bead set, approximately 2.1×10^6 beads were activated in 500 µL of activation buffer (0.1 M NaH2PO4, pH 6.2, VWR, 106346 0500) followed by addition of 10 µL of N-hydroxysulfosuccinimide (Sulfo-NHS, 50 mg/mL, Thermofisher Scientific, 24,510) and 10 µL of EDC (50 mg/mL). Activated beads were

Table 1
List of capture and detection mAbs selected during the VAC2VAC consortium and used in the MIA.

Target	Species	Capture/detection mAb	mAb ID	Provider
DT	Mouse	Capture	Dim9	Intravacc
DT	Rat	Detection	DT05	NIBSC
TT	Mouse	Capture	8E1-1H1.2.1	Sanofi Pasteur
TT	Rat	Detection	TT010	NIBSC
PT	Mouse	Capture	PS21C2.2.1	Sanofi Pasteur
PT	Mouse	Detection	629E1	GSK
FHA	Mouse	Capture	FHADETOX/6	GSK
FHA	Mouse	Detection	32–1	Sanofi Pasteur
PRN	Mouse	Capture	69 K/16	GSK
PRN	Mouse	Detection	3–5	Sanofi Pasteur

washed and resuspended in 100 µL of coupling buffer (2-(N-morpholino) ethanesulfonic acid (50 mM, pH 5.0, Merck Life Science, M2933). Coupling of each mAb was performed by addition of 25 µg of mAb followed by addition of coupling buffer to reach a final volume of 500 µL and an incubation step of 2 h at room temperature (RT) in the dark and under rotating agitation. Coupled beads were then washed and resuspended in 700 µL of blocking/storage buffer (PBS 1% BSA [Bovine Serum Albumin, Merck Life Science, A7030], 0,1% sodium azide [VWR, 1.06688], 0.05% Tween-20 [Merck Life Science, P9416]) to reach a bead concentration of 3×10^6 beads/mL and stored at 2–8 °C until use. Biotinylation of anti-DT (DT05), anti-TT (TT010), anti-PT (629E1), anti-FHA (32−1), and anti-PRN (3–5) detection antibodies was performed using the EZ-Link™ Sulfo-NHS-Biotin (Thermofisher Scientific, 21,217) to reach a final antibody concentration of 500 µg/mL and 50-fold molar excess of biotin.

2.3. Assay procedure

Assay procedure is represented in the supplementary fig. 1. Briefly, antigen/vaccine samples were diluted in working buffer (PBS, 0.1% BSA, 0.01% Tween-20) and added in opaque 96-well plates (Bio-rad,171,025,001) with a volume of 50 µL/well. Then, 50 µL of the mAbs-coupled microbeads diluted at a concentration of 40.000 beads/mL in working buffer were added to each well and the plate was incubated for 1 h30 at 37 °C under agitation (700–800 rpm). A washing step was then performed as followed: 1) the plate was placed on a magnetic separator (Merck Millipore, 40–285) for 30–60s to immobilize the beads at the bottom of each well and avoid their loss during the following step, 2) the content of the plate was emptied 3) all wells were filled with 150 uL of working buffer 4) the plate was washed for 2 min at 37 °C under agitation at 700–800 rpm 5) repetition of steps 1 and 2. Biotinylated detection antibodies diluted in working buffer at a concentration of 1 µg/mL were then added with a volume of 100 µL/well followed by an incubation of 1 h30 at 37 °C under agitation at 700–800 rpm. The plate was washed as described above and 100 µL of streptavidin-phycoerythrin conjugate (Streptavidin, R-Phycoerythrin Conjugate, Life Technologies Europe, S866) diluted in working buffer at a concentration of 1.5 µg/mL/bead set was added to each well and incubated for 45 min at 37 °C under agitation at 700–800 rpm. Following two washing steps, 100 µL of working buffer was added to each well and the plate was analysed on the Magpix or Luminex-200 instrument.

2.4. Data analysis

Raw data from each run were analysed with the CombiStats™ software using a four parameter logistic regression model to determine the RA of "test samples" against a reference. Validity criteria were fixed as follows: slope ratio between 0.8 and 1.25,(United States Pharmacopoeia, 2010) $R^2 > 0.975$ and 95% confidence interval limits not wider than 80% to 120% of the estimated potency. In the case of an invalid criteria, the result was not taken into account.

2.5. Cross-reactivity evaluation

Non-adsorbed antigens from HuA and HuB were used to evaluate the cross-reactivity as recommended in the Luminex xMAP® Cookbook 4nd edition.(Angeloni et al., n.d.) Briefly, three conditions were compared using beads coupled to the five mAbs. In the first one, only one antigen and its specific detection antibody were used to determine antigen-non target beads cross-reactivity. In the second one, only one antigen was used in combination with all detection antibodies to check for cross-reactivity between detection antigens and untargeted beads. The third condition was the full multiplex combination containing all coupled beads, all antigens and all detection antibodies. Supplementary fig. 2 represents these different conditions used to monitor cross-reactivity.

2.6. Specificity evaluation

Specificity of the 5-plex MIA was evaluated using dropout samples of the HuA DTaP-IPV-HepB-Hib and HuB DTaP vaccines obtained from both manufacturers. Each dropout sample corresponded to the same formulation as the complete vaccine but lacking one specific antigen. Using a complete formulation as control, each dropout was used as sample to evaluate the signal obtained for each antigen. Dilution curves obtained for dropout samples and control were compared to determine the RA of each dropout using the CombiStats™ software (7th version, European Directory for the Quality of Medicine and HealthCare).

2.7. Reproducibility

Two batches of HuB DTaP and two batches of HuB dtap vaccines were titrated with the MIA in duplicate on the same plate. One batch was assigned as the reference while the second was assigned as the test. At two different locations, two plates were run per day during three different days ($n = 12$). Variability of the method was evaluated by calculation of the RA of the TEST against its homologous REF of each vaccine using CombiStats™. From this, the geometric coefficient of variation (GCV) was calculated between the 12 RA using the following formula: $GCV = (e^s\text{-}1)x100$ where s is the standard deviation of the ln transformed data.

2.8. Application of the MIA

2.8.1. Detection of heat-induced antigen alteration

Several vials of one batch of HuA DTaP-IPV-HepB-Hib and of one batch of HuB DTaP and HuB DTaP-IPV-HepB were incubated at RT, 37 °C and 45 °C for four weeks then titrated with the developed MIA. Vials of one batch of HuA DTaP-IPV and HuA dt-IPV vaccines were also incubated at 37 °C and 45 °C for four weeks but not at RT due to a technical issue. Vaccines were tested at the end of the incubation period or kept at 4 °C until the analysis The RA of altered samples were determined in CombiStats™ using the batch kept at 4 °C as control.

2.8.2. Detection of oxidative damaged antigens

Vials of one batch of HuA dt-IPV, HuA DTaP-IPV-HepB-Hib, HuB DTaP and HuB DTaP-IPV-HepB were incubated for one week at 4 °C and 37 °C with milliQ water and at 37 °C with 10, 100 and 1000 µg/mL of H_2O_2 (Merck, H1009). The HuA DTaP-IPV could not be incubated with 10 µg/mL of H_2O_2. For the control preparations, an equivalent volume of milliQ water was added to avoid any difference due to a dilution of the samples. At the end of the week of incubation, altered vaccines were directly used for the assay or kept at 4 °C until the analysis. The RA of altered samples were determined in CombiStats™ using the batch kept at 37 °C without H_2O_2 as control.

2.8.3. Detection of underdosed formulations

For the preparation of underdosed formulations, dropout samples donated by the manufacturers were used. Formulations containing 25, 50, or 75% of target antigen were prepared by dilution of the complete formulation in the different dropouts while formulations containing 0% and 100% of antigen corresponded to dropout and complete formulation, respectively. All preparations were titrated using the 5-plex MIA and the relative amount of antigen was calculated in the CombiStats™ software taking the complete formulation as reference. The percentage of recovery was calculated as the ratio between calculated and theoretical amount of antigen.

2.8.4. Detection of overdosed formulations

Formulations containing 100% (corresponding to the antigen concentration in the final vaccine), 150% or 200% of antigen were prepared by addition of adsorbed antigens in the dropout samples. The formulations for vaccines from HuA were prepared using adsorbed PT and FHA

in the dropout without PT and without FHA, respectively while for the vaccines from HuB, adsorbed DT, TT, PT, FHA and PRN were used to prepare the formulations in dropout without DT, TT, PT, FHA and PRN, respectively. All preparations were titrated using the MIA and the amount of antigen were calculated using the CombiStats™ software taking the formulation containing 100% of antigen as reference. The percentage of recovery was calculated as the ratio between calculated and theoretical amount of antigen.

2.8.5. Consistency evaluation

The vaccine batch consistency was evaluated on 11 batches of the HuA DTaP-IPV-HepB-Hib vaccine, 7 batches of the HuB DTaP, 9 batches of the HuB DTaP-IPV, 13 batches of the HuB DTaP-IPV-HepB, 16 batches of the HuB dtap, and 12 batches of the HuB dtap-IPV vaccines. In each group, one homologous batch was randomly selected to be used as a reference.

3. Results

3.1. Development of the 5-plex MIA

The feasibility of the MIA was firstly investigated by titration of non-adsorbed and adsorbed single antigens from the two manufacturers and from NIBSC using the 5-plex immunoassay (Fig. 1). Possible interferences were investigated by comparison of the mean fluorescence intensity (MFI) signal obtained in single and multiplex assay. The Fig. 2 represents the correlation between signals obtained in single and multiplex assays with Pearson coefficients close to 1 for antigens of manufacturer A (DT: 0.999, TT: 0.999, PT: 0.999, FHA: 0.981) and B (DT: 0.991, TT: 0.992, PT: 0.997, FHA: 0.999, PRN: 0.990).

3.2. Specificity analysis

To evaluate the specificity of the MIA, adjuvanted dropout samples

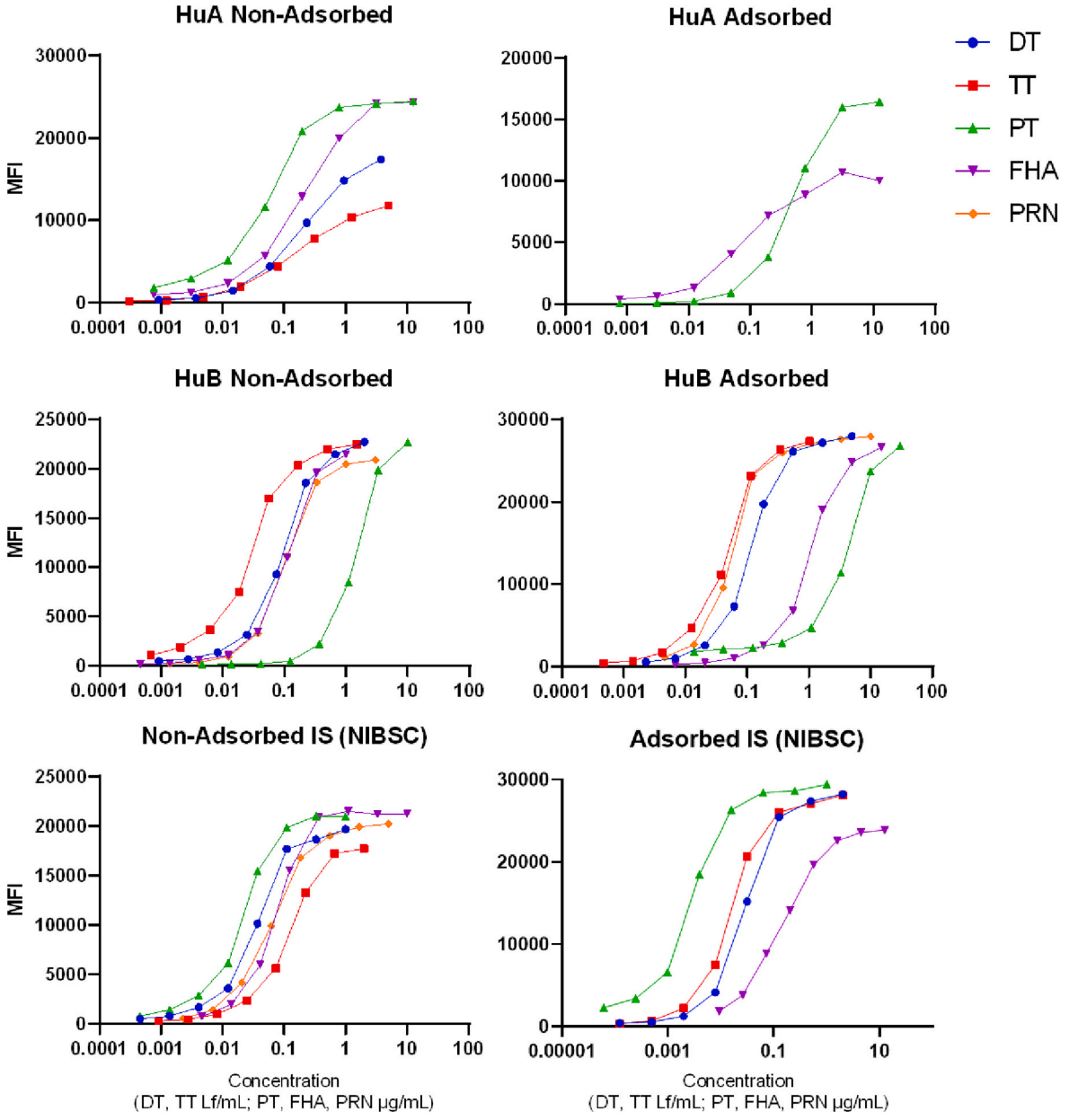


Fig. 1. Titration curves of non-adsorbed and adsorbed antigens from manufacturers A and B and from NIBSC obtained by MIA. HuA DT and HuA TT were not available in the adsorbed form.

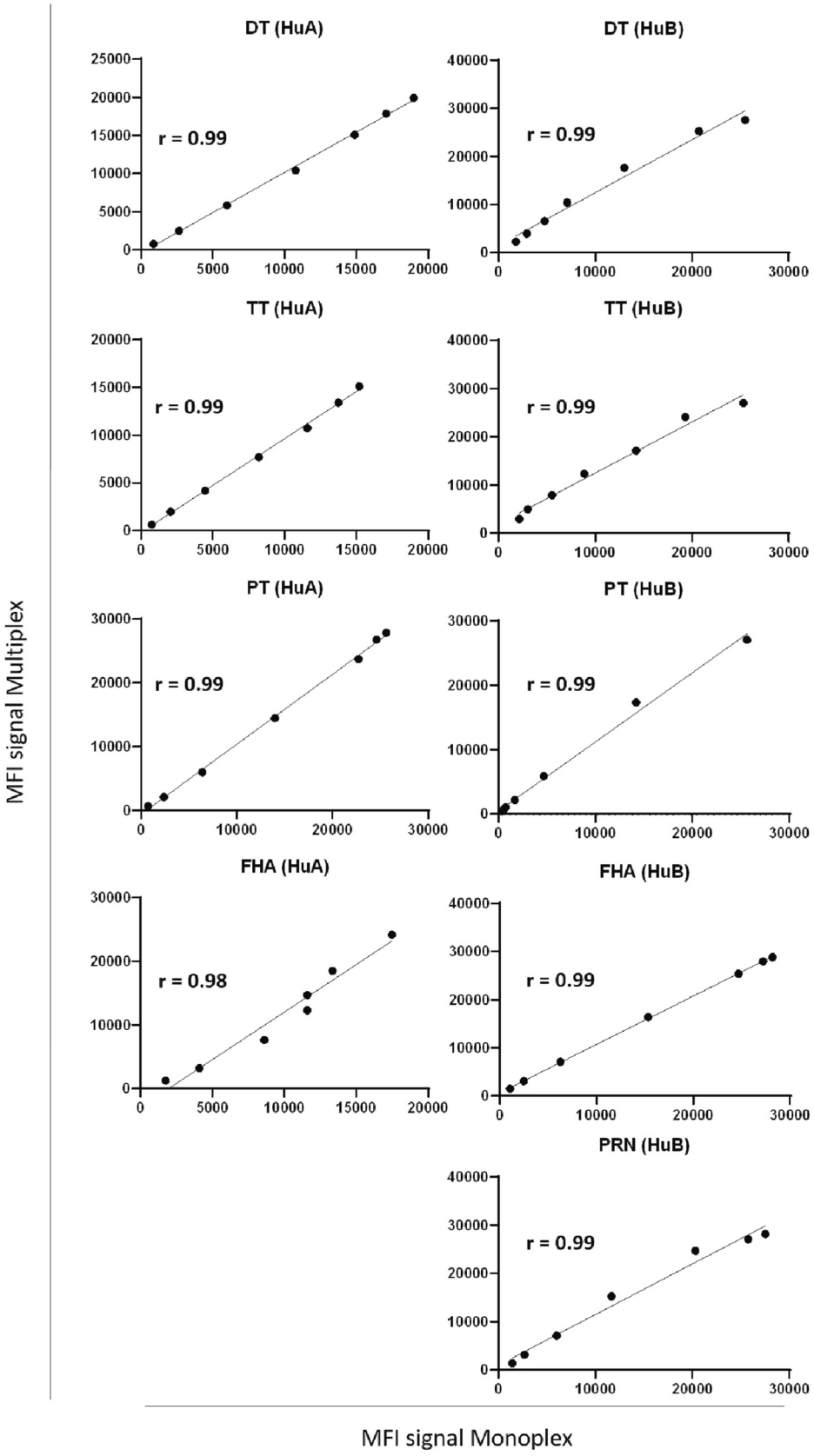


Fig. 2. Correlation of MFI signal obtained in mono- and multi-plex assay formats for antigens from HuA and HuB. r = Pearson correlation coefficient.

from both manufacturers were serially diluted and titrated with the developed MIA. The resulting titration curves are shown in the Fig. 3 and demonstrate that the MFI signal is null when the antigen is lacking excepted for the TT antigen in the dropout without TT of HuA where a low signal was observed.

3.3. Reproducibility

The evaluation of the reproducibility was performed on 12 independent assays. The geomean and the GCV calculated between the 12 RA obtained for both drug products was below 10% in all cases and are represented in Table 2.

3.4. MIA sensitivity to abnormal or degraded vaccines

3.4.1. Detection of heat-induced alteration of antigens

Graphs of the Figs. 4 and 5 and supplementary Table 2 show that in vaccines from both manufacturers, a decrease of the signal and RA is generally observed when the temperature and incubation time increased for all antigens excepted for PT in both HuB vaccines and to a lesser extent, for DT in HuA DTaP-IPV-HepB-Hib and PRN in HuB DTaP-IPV-HepB vaccines. Indeed, for these latter and especially for the PT, the opposite effect was observed as the signal increased when vaccines were exposed to higher temperatures.

3.4.2. Detection of oxidation-induced alteration of antigens

Compared to the control batch incubated at 37 °C without H_2O_2, the MFI signal and the RA of DT, TT and PRN decreased in all tested vaccine following treatment with H_2O_2. While a decrease was observed in HuA vaccines following hydrogen peroxide treatment, the signal of PT increased in HuB vaccines. Finally, FHA MFI was globally not affected in HuB vaccines and tended to increase in HuA vaccines (Figs. 6 and 7 and supplementary Table 3).

3.4.3. Detection of underdosed formulations

To evaluate the capacity of the MIA to detect an abnormally low amount of one or several antigens in the vaccine, underdosed formulations were prepared with dropout samples to reach final concentrations corresponding to 25%, 50% or 75% of the complete formulation while unmodified dropout and complete formulation were considered as 0% and 100%, respectively. The Fig. 8 shows the shifts of the curves for each dilution of dropout from HuA and HuB. As observed during the specificity analysis, a low signal was observed in the dropout without TT from HuA. The percentage of recovery was in the 80–120% range for the three dilutions of the five antigens except for the 25% dilution of the FHA antigen in vaccines from both manufacturers (Supplementary Table IV).

3.4.4. Detection of overdosed formulations

To determine if the MIA is capable of detecting overdosed formulations, abnormally high antigen formulations of HuA were prepared by mixing adsorbed antigens with dropouts. Results in the Fig. 9 demonstrate that compared to the 100% formulation, the signal obtained for the formulation containing 150% and 200% of antigen was higher. The percentage of recovery were in the 80–120% range for the two dilutions of the five antigens excepted for the 200% dilution of the FHA antigens in vaccines from both manufacturers (Supplementary Table V).

3.4.5. Consistency evaluation

To evaluate the capability of the MIA to monitor the batch to batch consistency in the frame of vaccine quality control, 11, 29, and 28 batches HuA DTaP, HuB DTaP and HuB dtap vaccines were analysed with the MIA, respectively (see supplementary Table VI and Fig. 10). Generally, the geomean of the RA of DT, TT, PT, FHA and PRN antigens compared to the homologous reference batches were within the 0.8–1.2 interval excepted for the PT antigens in HuB DTaP and HuB DTaP-IPV where the geomean RA were 0.24 and 0.35, respectively. In HuB DTaP-IPV, higher values were also observed for DT and FHA antigens with geomean RA of 1.25 and 1.22, respectively. A higher geomean RA of 1.28 was also obtained for FHA in the HuB dtap vaccine. The batch to batch variability of RA was acceptable (< 20%) for all antigens excepted PT in the HuB vaccines where high GCV were observed (from 68 to 184%). To a lesser extent, GCV > 20% were observed for DT (23%) and TT (22%) in HuB DTaP, and for FHA in HuA DTaP-IPV-HepB-Hib (22%) and HuB dtap-IPV (34%).

The ratio of the upper and lower limits of the 95% confidence interval of the geomean with the geomean itself were in the 80%–120% range for all antigens in the HuA DTaP-IPV-HepB-Hib vaccine as well as in most HuB vaccines excepted for the PT antigen for which the variability was higher compared to the variability observed for the other antigens. Upper limits were also slightly out of the interval for DT and TT in HuB DTaP and for FHA in HuB dtap-IPV vaccines.

4. Discussion

Considering the importance of the quality and content of vaccine antigen for its safety and effectiveness, the development of *in-vitro* assays able to measure these parameters represents an attractive approach to replace the animal potency tests currently in place in the frame of vaccine quality control. Actually, a number of immunoassays have already been routinely set up for quality control of several vaccines.(Suresh et al., 2018; Costa et al., 2011; Descamps et al., 2011; Shanmugham et al., 2010; Chabaud-Riou et al., 2017; Wang et al., 2018; Maas et al., 2000; Aly et al., 2018; Sigoillot-Claude et al., 2015) Within the VAC2-VAC consortium, our goal is to develop a monoclonal-based MIA and show proof of concept for an assay that can be used in the quality control of human DTaP vaccines with the ultimate aim to replace the current *in-vivo* potency tests. To be used for potency testing, antibodies should be of high affinity for their epitope and good indicator of antigen stability. (European Phamacopoeia, n.d.) An extensive characterization of mAbs targeting DT, TT and aP antigens was already performed in the frame of the VAC2VAC consortium through evaluation of their binding capacity to non-adsorbed, adsorbed and heat-altered antigens as well as antigen affinity assessment and epitope competition analysis leading to the development of sandwich ELISAs directed against DT and TT antigens. (Riches-Duit et al., 2021a; Riches-Duit et al., 2021b; Francotte et al., n. d.) Here we report the development of a MIA that could potentially be applicable for antigenicity testing of combined human DTaP vaccines.

The possibility of assessing several targets in one analysis offered by the Luminex technology has been exploited in different fields such as clinical proteomics(Yurkovetsky et al., 2010; Liu et al., 2005) and vaccinology for immunological response profiling.(van Gageldonk et al., 2008) The feasibility of a Luminex based-characterization of antigen quality and quantity in adjuvanted vaccines was already demonstrated for aP antigens.(Agnolon et al., 2016) In the present study, using a five-plex MIA we demonstrated the feasibility of evaluating the quality and quantity of DT, TT and aP antigens in human combined vaccines from two different manufacturers. Comparison of the MFI signals obtained for each antigen in the single and the five-plex formats showed very good correlation suggesting absence of major interferences in the assay when multiplexed. Antigen-specific dropout samples represent ideal candidates for specificity evaluation of the assay. Analysis of the dropout samples from both manufacturers revealed a good specificity of the MIA as the signal obtained for each antigen in its dropout was null excepted for the TT antigen in the HuA vaccine which can be explained by the presence of TT as a conjugate for the polyribosyl ribitol phosphate antigen of the *Haemophilus influenzae type b bacteria* in the formulation. Reproducibility is also an important parameter to evaluate for validation of RA assays.(European Medicines Agency, 1995) In this study, reproducibility was demonstrated using two drug products from HuB (one DTaP and one dtap vaccine) with GCV never exceeding 10% for any measure of RA. This level of precision is much higher than that reported for *in-vivo* potency tests where repeated testing on a same batch resulted

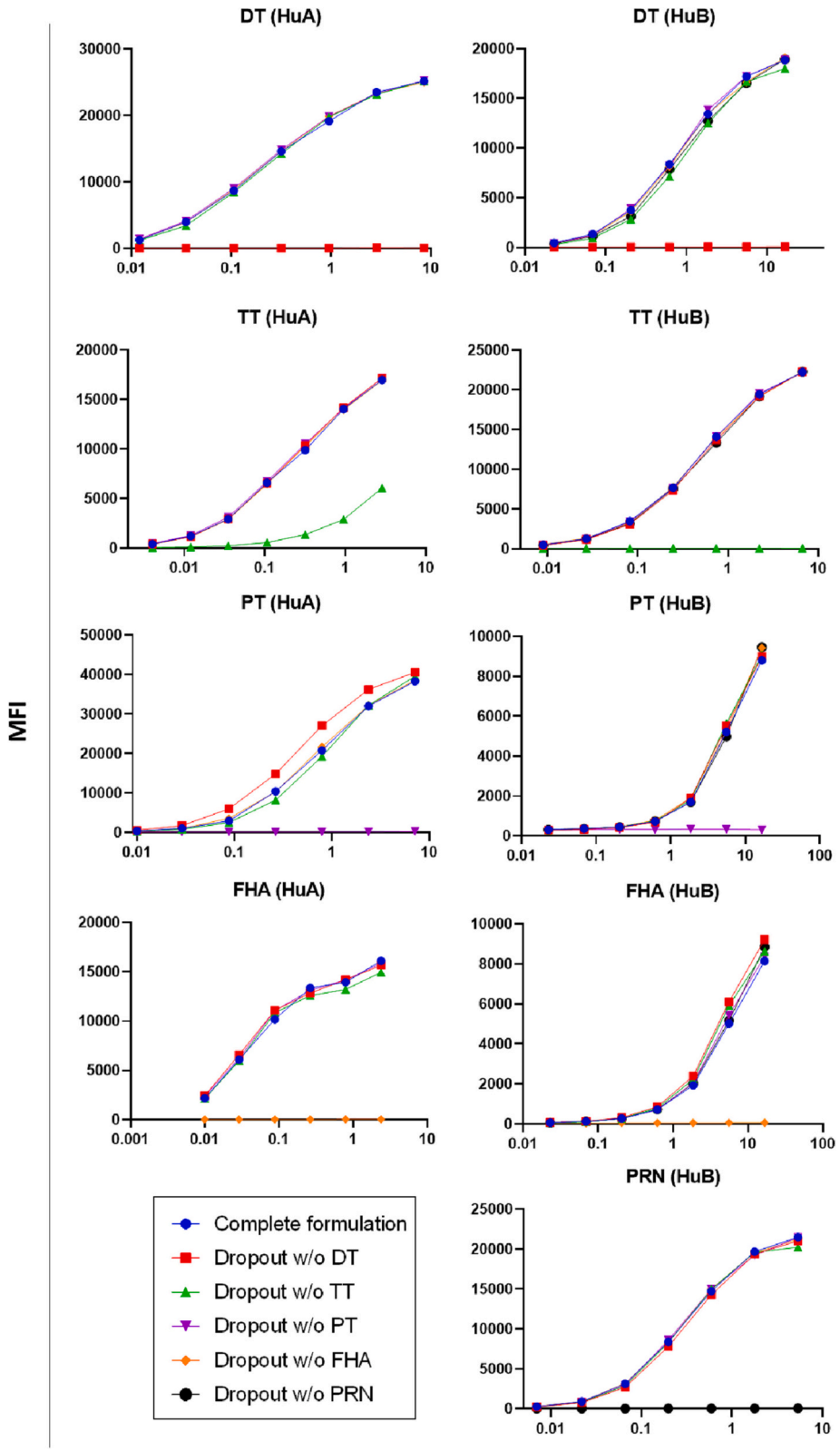


Fig. 3. Representation of the titration curves obtained by MIA for DTaP antigens in complete formulations and in dropouts from HuA and HuB.

Table 2
Geomean and GCV calculated using the RA obtained from 12 assays on DTaP and dtap drug products from HuB.

Antigen	Drug product	RA	GCV
DT	DTaP	0.88	5%
	dtap	1.14	4%
TT	DTaP	0.85	5%
	dtap	0.99	5%
PT	DTaP	0.90	5%
	dtap	1.27	4%
FHA	DTaP	1.15	8%
	dtap	0.94	8%
PRN	DTaP	0.90	5%
	dtap	0.89	5%

in high coefficient of variances ranging from 15 to 101%.(Stalpers et al., 2022; Stalpers et al., 2021)

To replace the *in-vivo* potency test, an *in-vitro* assay should demonstrate its ability to detect potential changes in vaccine key quality attributes.(European Phamacopoeia, n.d.) Antigen quality/functionality is clearly one of those key quality attributes to control before releasing the product onto the market. The capability of the selected pairs of monoclonal antibodies to detect heat-induced alterations of non-adsorbed DT, TT and pertussis antigens was already demonstrated using indirect ELISA.(Riches-Duit et al., 2021a; Riches-Duit et al., 2021b; Francotte et al., n.d.) Using the same pairs of antibodies, the developed MIA could detect heat-induced alterations of adsorbed antigen in several final products from both manufacturers. However, some discrepancies were observed depending on the drug product. While heat exposure systematically led to a reduction of the RA of TT and FHA in final drug products from HuA (dt-IPV, DTaP-IPV and DTaP-IPV-HepB-Hib vaccines) and HuB (DTaP and DTaP-IPV-HepB vaccines), the same pattern was observed for DT and PRN except in the more complex vaccines from HuA (DTaP-IPV-HepB-Hib) and HuB (DTaP-IPV-HepB), respectively, where the RA slightly increased following heat exposure. The folding state of proteins is a complex process that can be notably disturbed by chemical, pH and thermal changes.(Kishore et al., 2012) The increased signal seen for DT and PRN antigens could be explained by

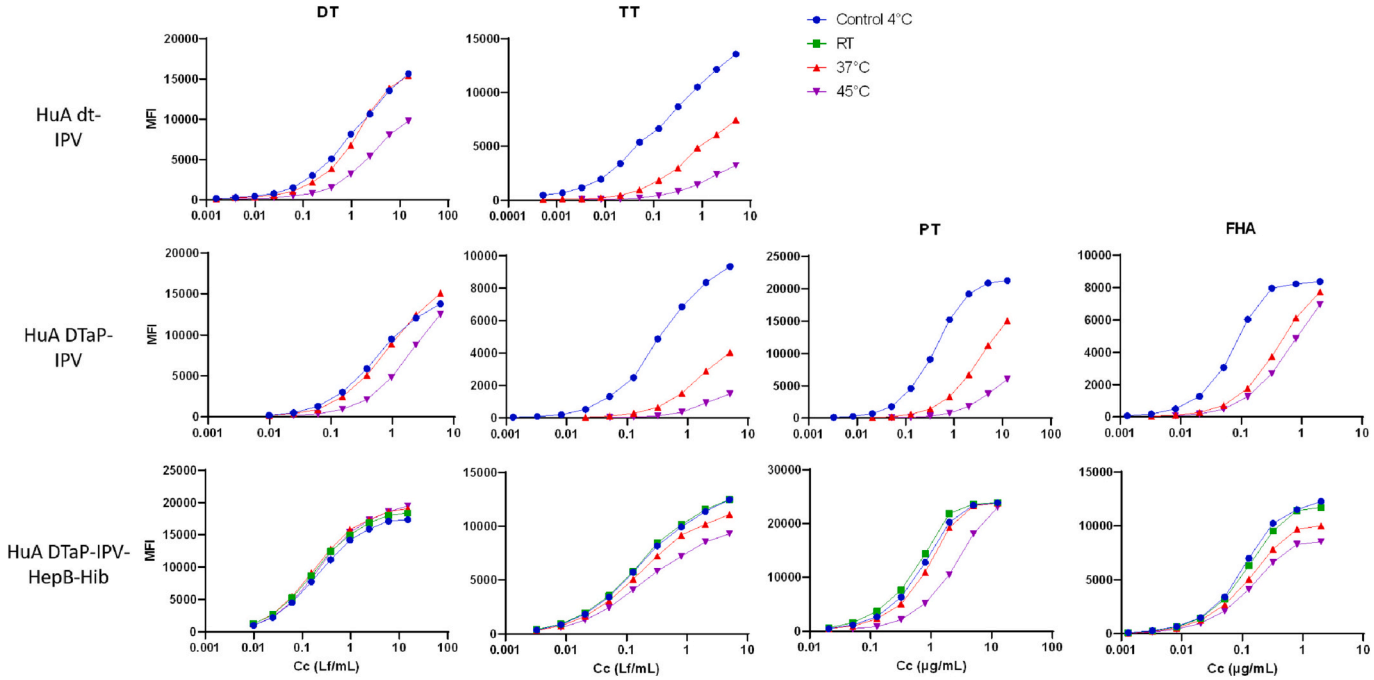


Fig. 4. Titration by MIA of heat-altered samples from HuA. HuA dt-IPV and HuA DTaP-IPV were not incubated at RT due to a technical issue.

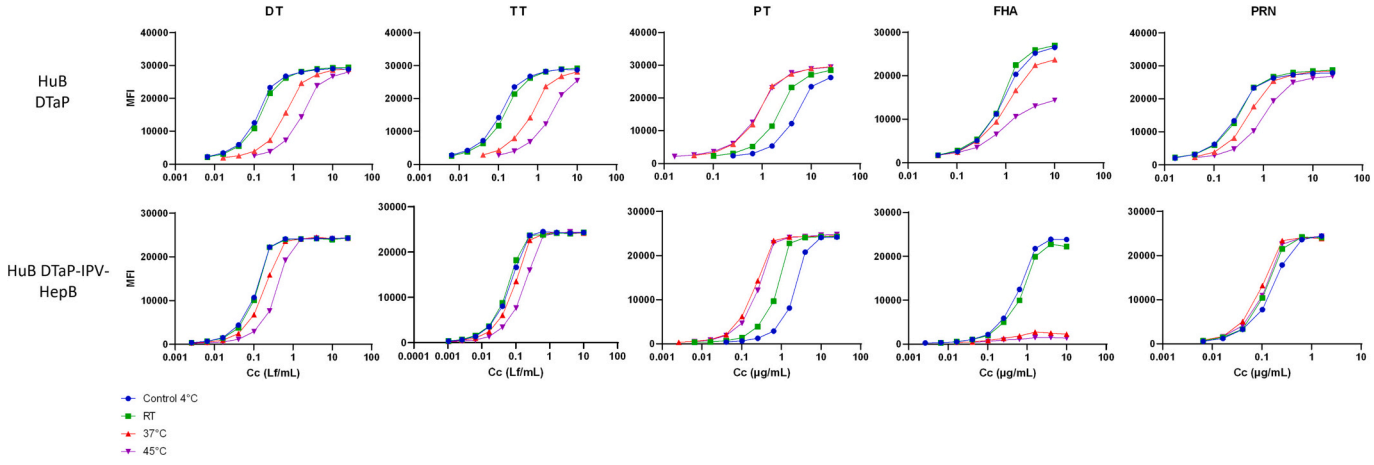


Fig. 5. Titration by MIA of heat-altered samples from HuB.

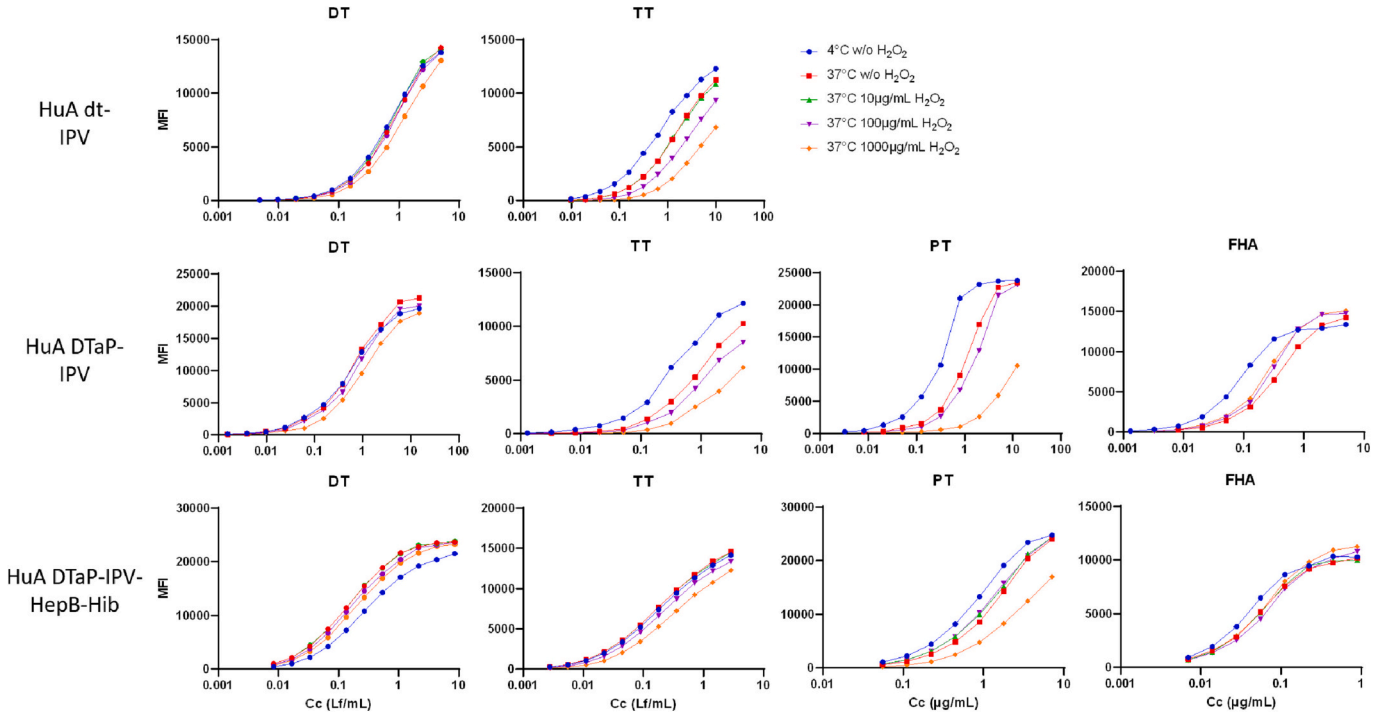


Fig. 6. Titration by MIA of H_2O_2-treated vaccines from HuA. HuA DTaP-IPV data could not be obtained for the 10 µg/mL H_2O_2 concentration due to a technical issue.

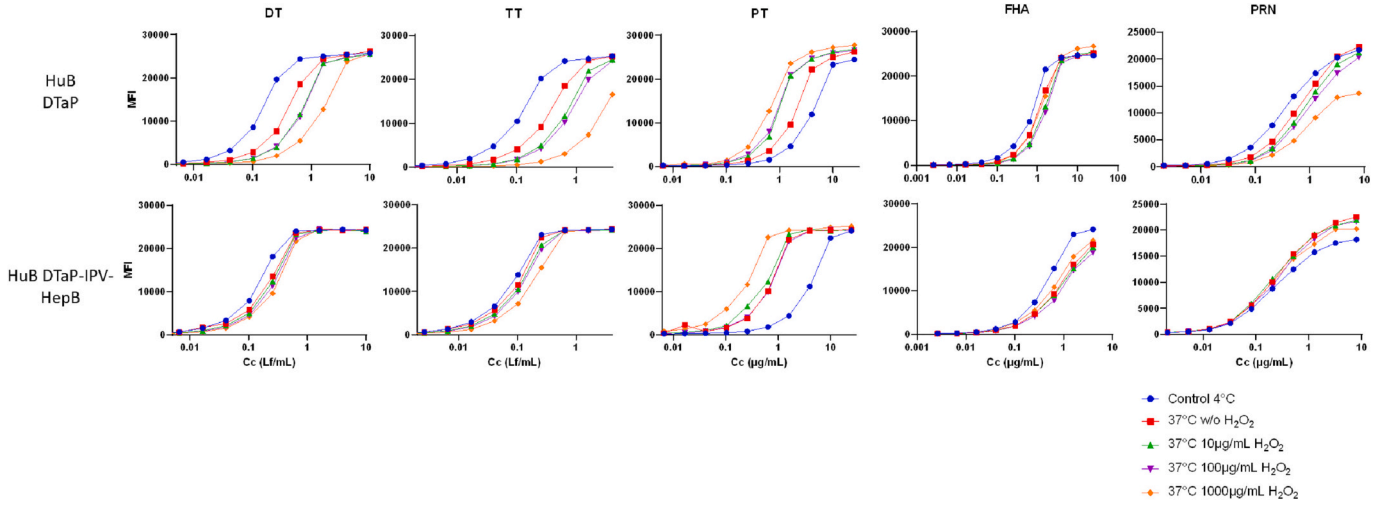


Fig. 7. Titration by MIA of H_2O_2-treated vaccines from HuB.

the unfolding of antigens resulting in the appearance of new antigenic sites as already described in other protein degradation studies.(Davis and Williams, 1998) Additionally, antigenicity of heated proteins can vary in the case of the presence of other proteins which could explain the increased signal for DT and PRN in more complex vaccines due to difference of formulation or presence of additional antigens.(Bogahawaththa et al., 2019) While the signal for PT decreased in HuA vaccines, the opposite effect was observed for the PT of HuB. Process used to detoxify PT are usually based on formaldehyde (and/or glutaraldehyde) treatment. Intra- and inter- molecular bound on PT toxoid created during detoxification can be broken with heat treatment with epitope release or destruction. Aggregation state can also be modified by heat treatment. As PT detoxification process can vary among manufacturer, heat treatment could promote the release of epitope targeted by MIA mAb in one case but reduce the presence of epitope in another case.

During the manufacturing process of a vaccine, some steps of sterilization of filling equipment prior to formulation can necessitate the use of vaporized hydrogen peroxide and although this is a strictly controlled process, residual adsorption of H_2O_2 onto material cannot be ruled out.(Kushwah et al., 2020; Hubbard et al., 2018) To evaluate the ability of the MIA to detect a potential H_2O_2-induced antigen degradation in final products, dt-IPV, DTaP-IPV and DTaP-IPV-HepB-Hib vaccines from HuA and DTaP and DTaP-IPV-HepB vaccines from HuB were exposed to H_2O_2 for one week. Results showed a decrease of the RA of DT and TT in vaccines from both manufacturers. As following heat-alteration, the RA of PT decreased in vaccine from HuA but increased in vaccines from HuB. Heat or H_2O_2 treatment can modify differently epitopes inducing variable effect on different mAb recognition. It could also make epitope more or less available by changing antigen structures. The assay design itself (homogenous like MIA or surface based like ELISA) could also be more or less sensitive to highlight these change. Following alterations of epitopes of the pertussis toxin with H_2O_2, *Ibsen*

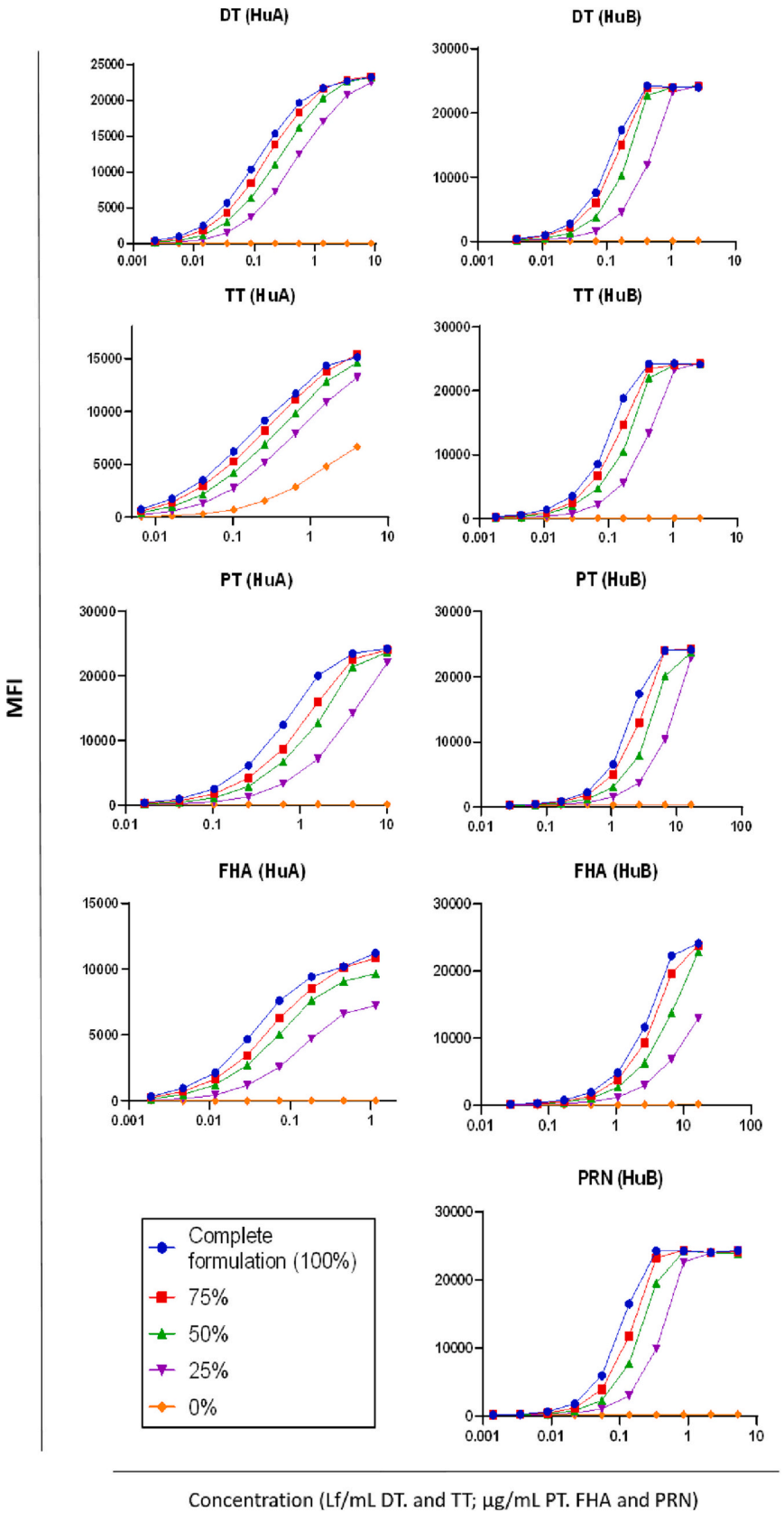


Fig. 8. Titration by MIA of underdosed formulations from HuA and HuB.

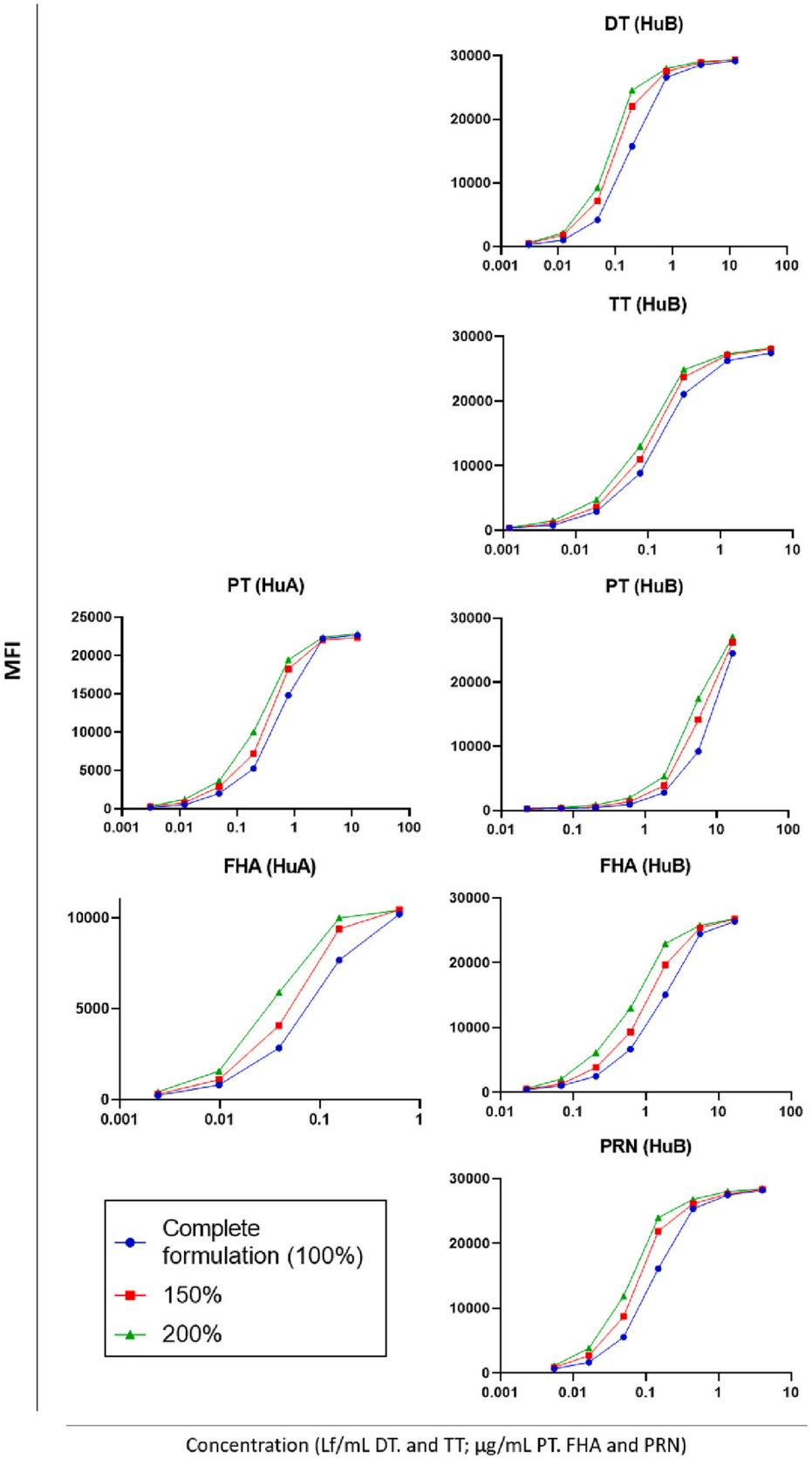
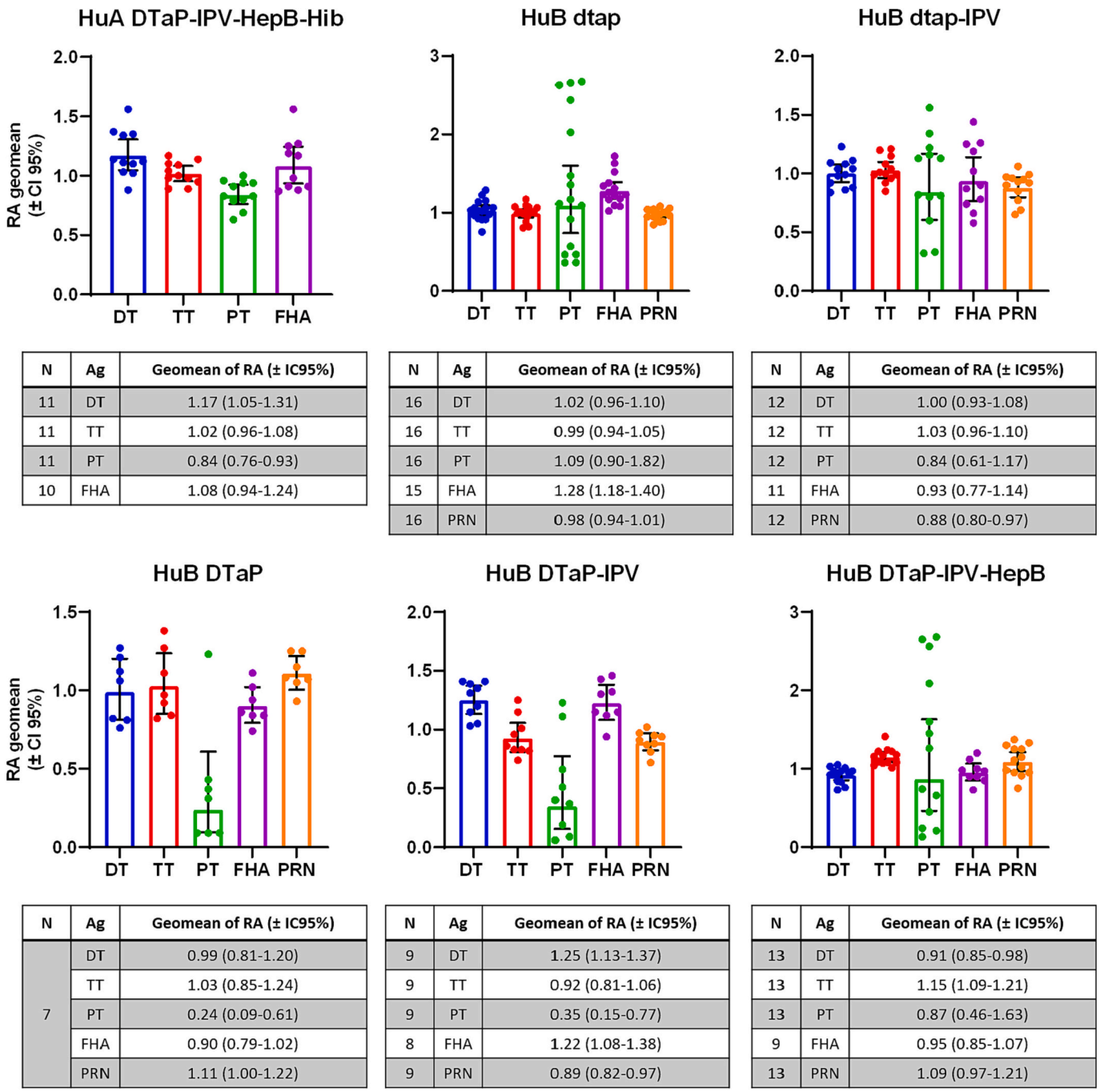


Fig. 9. Titration by MIA of overdosed formulation prepared with adsorbed antigens from HuA and HuB.



N	Ag	Geomean of RA (± IC95%)
11	DT	1.17 (1.05-1.31)
11	TT	1.02 (0.96-1.08)
11	PT	0.84 (0.76-0.93)
10	FHA	1.08 (0.94-1.24)

N	Ag	Geomean of RA (± IC95%)
16	DT	1.02 (0.96-1.10)
16	TT	0.99 (0.94-1.05)
16	PT	1.09 (0.90-1.82)
15	FHA	1.28 (1.18-1.40)
16	PRN	0.98 (0.94-1.01)

N	Ag	Geomean of RA (± IC95%)
12	DT	1.00 (0.93-1.08)
12	TT	1.03 (0.96-1.10)
12	PT	0.84 (0.61-1.17)
11	FHA	0.93 (0.77-1.14)
12	PRN	0.88 (0.80-0.97)

N	Ag	Geomean of RA (± IC95%)
7	DT	0.99 (0.81-1.20)
	TT	1.03 (0.85-1.24)
	PT	0.24 (0.09-0.61)
	FHA	0.90 (0.79-1.02)
	PRN	1.11 (1.00-1.22)

N	Ag	Geomean of RA (± IC95%)
9	DT	1.25 (1.13-1.37)
9	TT	0.92 (0.81-1.06)
9	PT	0.35 (0.15-0.77)
8	FHA	1.22 (1.08-1.38)
9	PRN	0.89 (0.82-0.97)

N	Ag	Geomean of RA (± IC95%)
13	DT	0.91 (0.85-0.98)
13	TT	1.15 (1.09-1.21)
13	PT	0.87 (0.46-1.63)
9	FHA	0.95 (0.85-1.07)
13	PRN	1.09 (0.97-1.21)

Fig. 10. Consistency analysis of a panel of batches of different drug products from HuA and HuB obtained by MIA. Tables bellow each graph represent the geomean of the relative antigenicity calculated for each antigen with the lower and upper limits of the 95% confidence interval.

already demonstrated, for example, that the level of detection measured by ELISA compared to non-altered toxin could increase or decrease depending of the mouse antibody used for the assay.(Ibsen, 1996) In vaccines from both manufacturers, the RA calculated for FHA was not really affected by H_2O_2 suggesting that the treatment did not affect the epitope(s) targeted by the pair of antibodies used in the assay. Regarding PRN in HuB vaccines, the decrease of RA was higher in DTaP compared to DTaP-IPV-HepB vaccine. Although no sign of cross reactivity with additional antigens (IPV, HepB and Hib) was observed in HuA dropouts, further investigations could be performed to exclude cross reactivity of the assay with additional antigens (HepB and IPV) in HuB dropouts.

Concentration of antigen composing the vaccine represent another important quality attribute that has to be ensured before vaccine batch release. Here, the ability of the MIA to detect a change in antigen content was demonstrated on under- and overdosed formulations prepared for each antigen composing the vaccines from the two manufacturers. Indeed, compared to the antigen concentration in the final product, the MIA allowed to detect abnormally low (25%, 50% and 75%) and high (150% and 200%) antigen content with acceptable linearity for all DTaP antigens excepted for more extreme FHA concentrations of 25% and 200%. While the poorer linearity observed at high FHA concentration could be due to a saturation of antibody, the loss of linearity at low FHA concentration could be explained by the lower MFI signal at this concentration. Although not investigated here, increasing the capture and/or detection antibodies concentration could improve the linearity range of detection of FHA. A low signal was also observed for TT in the dropout

without TT from HuA and can be explained, as for the specificity analysis, by the presence of TT as a conjugate for the polyribosyl ribitol phosphate antigen of the *Haemophilus influenzae type b bacteria* in the formulation.

Batch to batch consistency is a key parameter to ensure clinical safety and efficacy of vaccines established at the time of licensing.(De Mattia et al., 2011; Akkermans et al., 2020) To mimic consistency monitoring of DTaP vaccine using the MIA, the RA of DTaP antigens were determined for several batches of HuA DTaP-IPV-HepB-Hib, HuB DTaP, HuB DTaP-IPV, HuB DTaP-IPV-HepB, HuB dtap and HuB dtap-IPV vaccines compared to one homologous batch fixed as reference. Results revealed that the lower and upper limits of the 95% CI expressed as a percentage of the geomean were generally inside or close to the 80%–120% interval suggesting an acceptable batch to batch variability except for the PT antigen in HuB vaccine. For the later, a high variability was observed between batches in all HuB products. This variability can be explained by the strong link measured between the antigenicity of PT and its adsorption date. Indeed, we observed that in the older batches, the RA of PT was systematically higher and that the correlation between final container production and the RA of PT displayed a $R^2 > 0.9$ in all HuB drug products (data not shown). This increase of antigenicity during antigen ageing is in line with degradation studies where an increase of antigenicity was observed for PT following heat and H_2O_2 treatments. Further studies with another antibody pair format using VAC2VAC-characterized anti-PT mAbs is currently under investigation.

The extensive work performed in this study demonstrated good specificity, reproducibility and absence of cross reactivity in the developed MIA. In addition, graded-doses, forced degradation and consistency analysis performed on final products brought the proof of concept for a future application of the MIA as a useful tool for rapid quality control of DTaP vaccine compared to *in-vivo* testing. Considering the differences of formulation among all DTaP products on the market, the MIA could require some drug product-specific adaptations. In this study, MIA was developed without desorption step which would be an additional important source of variability making a validation process complicated. In the consistency approach applied to immunoassays and vaccine antigenicity, an assay should show an appropriate sensitivity and precision but should mainly be able to detect sensitively any relevant modification or degradation. The antigen recovery after desorption would therefore not be strictly necessary if previous parameters are demonstrated. If required, a completeness of adsorption test could also be developed in addition to the MIA.

In the future, it will be important to continue the promotion of the MIA to other companies and OMCLs. The ideal future scenario would be the qualification of an in-house standard and generation of consistency data on a sufficient number of batches (using ICH validation guidelines (European Medicines Agency, 1995)) by the companies allowing them to submit a variation dossier to EMA. Generation of these data will also be useful for the calculation of upper and lower limits as acceptance criteria. The launch of an extensive collaborative study (under the aegis of the EDQM) to evaluate the inter-lab transferability of the method would also be valuable and encouraged by the successful transfer performed in this study with HuB but is a process that can take some time due to the number of labs to recruit.

Considering the relevance of the immunoassays developed during the VAC2VAC project with the European directive 2010/63/EU,(European Parliament AND Council of the European Union, 2010) efforts should be made to further evaluate this approach for future inclusion in regulatory guidelines. As described in a recent letter, the VAC2VAC project induced mindest adaptation and a consensus between experts from industries and regulatory bodies towards a progressive abolishment of *in-vivo* testing for routine vaccine release testing.(Dierick et al., 2022) To facilitate this long term objective, the VAC2VAC consortium have worked to ensure availability of the selected monoclonal antibodies through NIBSC (https://www.nibsc.org/) for laboratories wishing to implement and further validate this method.

Author contributions

AF contributed to the first steps of development of the MIA and to the design of the study. IFE performed experiments. MVE designed the study, performed experiments, analysed data and wrote the manuscript. AD, PS, LH, RP, THL and CH participated in the design of the study and revised the manuscript. LT and WVM revised the manuscript. All authors have approved the final article.

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Declaration of Competing Interest

NA.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2023.113483.

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