Development and validation of bead-based assay quantifying Tetanus, Diphtheria, Pertussis Toxin, Filamentous haemagglutinin and Pertactin specific IgG in human serum

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Abstract

Conventional ELISA platforms have been used for vaccine immunogenicity testing. However, due to limitations in sourcing and accessibility to human serum samples, we report the development and validation of Luminex-based multiplex immunoassay (MIA), using monovalent beads, which would reduce the analysis time, cost, and sample volume while simultaneously measuring the concentration of serum immunoglobulin G (IgG) antibodies specific for tetanus (TT), diphtheria (DT), pertussis toxin (PT), filamentous hemagglutinin (FHA) and pertactin (PRN), using the NIBSC reference standards. Additionally, we also report the development of a multiplex reference standard (MRS) focused on the simultaneous evaluation of antibodies against T, D, PT, PRN, and FHA in healthy human sera samples. As an assay evaluation parameter, the precision, accuracy, dilutional linearity, minimum and maximum detectable limit, robustness, stability, etc were assessed. The assay exhibited a wide dynamic range for all the five antigens and could quantify the IgG concentrations down to minimum concentrations, demonstrating antigen specificity with no cross-talks among the beads. The results obtained with MIA were consistent with commercially available assays. Thus, to conclude, the study provided a pentaplex assay with increased sensitivity, reproducibility and high throughput capabilities which would allow design of large and robust clinical studies for evaluating natural and vaccine-induced immunity.

1. Introduction

Regardless of good immunization coverage, sporadic cases of vaccine-preventable diseases such as whooping cough/pertussis[1], tetanus (T)[2], and diphtheria (D)[3] are reported globally. Vaccines currently used in children and adults for T, D, and acellular pertussis (aP) are offered as combination vaccines (TDaP)[4]. With the increase in novel vaccine development, it is imperative that clinical studies are conducted to assess their immunogenicity and safety as per the regulatory requirements.

Antigen-specific immunoglobulin G (IgG), as quantified by validated enzyme-linked immunosorbent assay (ELISA) methods is a serologic endpoint to evaluate the immunogenicity of aP-based combination vaccines[5]. Assessment of serum IgG responses to the five antigens present in the aP-based combination vaccines has been reported mainly using conventional ELISA tests or commercial kits[6,7]. Such ELISA methods are expensive, time-consuming, and most importantly require a considerable amount of serum samples[8]. Multiplex immunoassays (MIA) such as Luminex x-MAP® and Meso Scale Diagnostics (MSD) offer opportunities for simultaneous quantification of multiple antigens in a single well. Several studies have reported the use of multiplex assays for screening antibodies against the five vaccine antigens: tetanus toxoid (TT), diphtheria toxoid (DT), pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin (PRN)[9,10,11]. It is important that such multiplex assays are developed and validated by reporting the results in units that are traceable to an appropriate international reference standard[12]. There are challenges in such traceability as currently available international standards are more suited to monoplex assays[13,14]. The development of in-house reference standards for MIA has its own challenges as it requires serum samples with high antibody titers for all five antigens[15,16]. Due to limitations in sourcing and accessibility to human serum samples with higher concentrations of antibodies against all five antigens, approaches based on the utilization of serum pools of vaccinated people may not be practical for all laboratories.

Microsphere-based Luminex immunoassays use spectrally distinct fluorescent microspheres as the solid support matrix onto which the target antigens are bound to simultaneously measure antibodies against multiple analytes from a single reaction well, thus reducing the analysis time, cost, and sample volume[17]. The pentaplex microsphere based Luminex immunoassays platform met the standards for specificity, selectivity, reproducibility, and accuracy for the detection of TDaP antibodies in mouse serum[15]. This study describes the development and validation of a 5-plex magnetic bead-based Luminex-based assay to quantify human IgG antibodies to DT, TT, and pertussis antigens using National Institute for Biological Standards and Control (NIBSC) reference standards, the globally accepted agency for the provision of working standards for testing of biologicals[12]. Additionally, the study has evaluated the suitability of currently available international reference standards in MIAs, in order to address the challenges associated with a lack of international reference standards.

2. Results

The development and validation of 5-plex bead-based immunoassay for simultaneous quantification of IgG concentrations against D, T, PT, FHA and PRN antigens in human serum samples are reported. The IgG concentrations are reported in IU/ml traceable to international reference standard.

2.1. Method Development

2.1.1. Optimization of bead coupling procedures

Two methods, EDAC/sulfo NHS using Luminex cookbook and AMG kit from AnteoTech were evaluated. The optimization of antigen coupling procedures, defining the antigen characteristics for coupling, volume of coupling reaction and lot-to-lot variability were compared for all the five antigens for the two assay methods. The assessment was based on the potential of the assay to achieve maximum sensitivity with high level of consistency. Both the coupling methods were found suitable for all the five antigens. However, in terms of yields and recoveries, EDAC coupling method showed good performance for PT, FHA and TT beads, while DT and PRN antigens performed well with AMG coupling. The impact of using different antigens (PT toxin and PT toxoid; FHA and formalin treated FHA; DT toxin, CRM 197 and D toxoid; T toxin and formalin treated tetanus toxin) on the coupling demonstrated a good correlation between titres generated with different classes of antigens. The assay was also optimized with respect to the use of serum dilution buffers. Dilution buffers were optimized to work with minimum sera dilution of 1:100. The use of serum dilution buffer with a composition of1% BSA, 0.2% sodium azide, 0.1% Tween20 and PBS was found optimum for the performance of the assay. A comparative assessment of assay specificity in assay buffer and blank human serum suggested no impact on the performance of the assay. The assay was optimized to cover maximum possible concentration range (0.03-4 mIU/ml for DT, 1.02-131mIU/ml for TT, 3.86–494 mIU/ml for PRN, 2.63–336 mIU/ml for PT and 4.67–598 mIU/ml for FHA.) for all the five antigens using the NIBSC standard **(Supplementary material, Table S1)**.

2.1.2 Reference standard development for pentaplex assay using NIBSC reference standards

The details of NIBSC reference standards used in the study for development of MRS are provided in (Supplementary material, Table S1). These reference standards, designed for monoplex assays were sourced from healthy subjects who were vaccinated with acellular pertussis-based combination vaccines. To assess their suitability for use in pentaplex assay, these reference standards were initially screened for the presence of IgG antibodies against T, D, PT, FHA and PRN. At screening, it was demonstrated that although the NIBSC provides the unitages against one or three antigens, these standards were found positive for IgG antibodies against all the five antigens.

To develop the MRS, all the three reference standards (06/142, 10/262 and TE-3) were assessed for unitages against all the five antigens. The process followed for unitage assessment is provided in (Fig. 1). The unitages of all the five antigens (IU/ml) that were used to assign unitages to MRS are provided in Supplementary material, Table S2.

2.1.3. Verification of Assigned unitages for MRS

The unitages assigned to the multiplex reference serum using data from six independent runs are provided in **Supplementary material, Table S3**.

The assigned unitages to international reference standard and the MRS for all the five antigens were also verified using commercially available ELISA assays. These commercially available assays report the unitages traceable to international reference standard. **Supplementary material, Table S4** provides the comparative assessment of unitages assigned by bead-based assay and commercially available methods. The unitages by bead-based assay was found to be in good agreement with the commercial ELISA assays wherein the variability of all the unitages were ≤ 20%. Additionally, the evaluation of the NIBSC reference standard *vs.* the MRS at the NIBSC laboratories indicated an excellent agreement (Table 1) between the NIBSC estimates and the multiplex assay estimates wherein all the estimates were within the acceptable range of ≤ 20% of CV.

Table 1
Verification of assigned unitages of MRS using commercial, NIBSC and multiplex assays

Analyte	SIIPL Bead Based Assay	NIBSC ELISA Assay	Commercial ELISA kit	%CV
	(IU/ml)	(IU/ml)	(IU/ml)	
PT	112	122	134	**9**
FHA	199	169	201	**9**
PRN	165	119	174	**19**
DT	1.33	1.30	1.19	**6**
TT	44	43	47	**5**

%CV: percentage coefficient of variation; DT: diphtheria toxoid, ELISA: enzyme-linked immunosorbent assay; FHA: filamentous hemagglutinin; IU/ml: international units per milliliter; NIBSC: National Institute for Biological Standards and Control; PRN: pertactin, PT: pertussis toxin; SIIPL: Serum Institute of India, Pvt, Ltd; TT: tetanus toxoid; Values in bold indicates the %CV of three assays (Bead based, commercial and NIBSC)

2.1.4. Performance of MRS serum in pentaplex assay

MRS serum with assigned IgG antibody concentrations for all the five antigens as indicated in **Supplementary material, Table S3** was used in the 5-plex MIA. **Supplementary material, Figure S1** shows the dilution profiles for each of the five antigens in IU/ml. The back fitted recoveries using 4-PL fits were between 80%-120% for all the calibration levels of all the five antigens (Fig. 2). Replicate %CVs of MFIs for all the calibratable ion levels were found to be less than 10%. Lower (LL) and upper limit (UL) of the assay range is supported by estimates from accuracy, precision and dilutional linearity studies. Samples with values above the UL may be prediluted to bring it within the assay range and thus no formal upper limit of quantification (ULOQ) was defined for the sample concentration (Table 2).

Table 2
Final assay range lower and upper limit of quantification

Antigen	Precision (mIU/ml)		Accuracy (mIU/ml)		Dilutional linearity of sample (mIU/ml)		Dilutional linearity of standard (mIU/ml)		Calibration Curve range (mIU/ml)	
	Lower limit	Upper limit	Lower limit	Upper Limit	Lower limit	Upper limit	Lower limit	Upper limit	Lower Limit	Upper limit
PT	6.9	221	6.9	221	**2.63**	336	0.16	336	2.63	336
FHA	14.3	457	14.3	457	**4.67**	598	0.29	598	4.67	598
PRN	13.3	424	13.3	424	**3.86**	494	0.24	494	3.86	494
DT	0.11	3.4	0.11	3.4	**0.03**	4	0.003	4	0.03	4
TT	3.8	121.7	3.8	121.7	**1.02**	131	0.06	131	1.02	131

DT: diphtheria toxoid, FHA: filamentous hemagglutinin; PRN: pertactin, PT: pertussis toxin; TT: tetanus toxoid; mIU/ml: Milli-international unit per milliliter

Data is representative of estimates in precision, accuracy and dilution linearity validation parameters. Precision, accuracy and dilution linearity estimates to support calibration curve range. Values highlighted in bold were used to estimate the lower limit.

2.1.5. Correlation with sera neutralization assays- diphtheria and pertussis toxin

Toxin neutralization assays using CHO cell and Vero cells for PT toxin and D toxin respectively were used to measure neutralization antibodies. These *invitro* cell-based assays measured the functional antibodies. A total of 10 serum samples were analysed for correlations of results obtained with MIA. Estimates exhibited positive correlations among the assays with correlation coefficients above 0.75 for both the antigens. (Fig. 3).

2.2. Method validation

2.2.1. Specificity

Specificity was demonstrated by comparison of monoplex versus multiplex assay in terms of difference in MFI and inhibition by using the five antigens. Inhibition was assessed with homologous and mix of five antigens. The MFI response (% difference) in monoplex and multiplex format was observed to be < 10% for all the five antigens (Table 3) which showed that there was no cross-reactivity between the five antibodies. Addition of homologous antigens individually and in a mixture resulted in > 85% inhibition of signal for PT, PRN, FHA, DT and TT (Table 3) suggesting the high specificity of the antibodies in binding with their respective antigens.

Table 3
Specificity of assay

Serotype	MFI difference monoplex vs. multiplex	Inhibition	
	Difference (%)	Monoplex Inhibition (%)	Multiplex Inhibition (%)
PT	1	88	87
FHA	1	91	90
PRN	8	99	100
DT	8	99	100
TT	-1	99	99

DT: diphtheria toxoid, FHA: filamentous hemagglutinin; MFI: mean fluorescence inhibition; PRN: pertactin, PT: pertussis toxin; TT: tetanus toxoid

2.2.2. Selectivity

No interference of blank human sera was observed based on the back fitted standard curve and controls recovery. Based on the data from three different matrices, the assay was found to be selective, and no interference was observed from any of the matrices (**Supplementary material, Table S5**).

2.2.3. Assay range

The assay range was selected based on the estimates from accuracy, precision and dilutional linearity study sets. LL and UL of assay range were established ranging from 0.03-4 mIU/ml for DT, 1.02-131mIU/ml for TT, 3.86-494mIU/ml for PRN, 2.63–336 mIU/ml for PT and 4.67-598mIU/ml for FHA. The LL of an assay range was the lowest concentration which showed acceptable accuracy, precision and dilution linearity experiments (Table 2).

2.2.4. Precision

Precision analysis suggested that the assay was precise between different analysts, over different days and bead lots. The %CV for the combined precision of the two analysts were below 20% for each of the 5 antigens (Table 4). Based on the data, LL and UL based on precision ranged from 3.8-121.7 mIU/ml for T, 0.11–3.4 mIU/ml for D, 6.9–221 mIU/ml for PT, 14.3–457 mIU/ml for FHA and 13.3–424 mIU/ml and for PRN, respectively (Table 2).

Table 4
Precision and Accuracy of the sera panel

Precision	*Analyst (%RSD)					**Days (%RSD)					***Bead Lot (%RSD)				
	PT	FHA	PRN	DT	TT	PT	FHA	PRN	DT	TT	PT	FHA	PRN	DT	TT
Sample 1	12	10	11	12	10	10	10	13	12	11	9	10	13	16	8
Sample 2	8	8	9	5	7	8	7	11	11	11	5	5	8	11	8
Sample 3	11	10	11	11	8	16	14	17	15	18	14	12	7	13	18
Sample 4	7	9	8	5	13	15	10	12	13	18	9	8	15	13	15
Sample 5	12	11	9	7	3	11	8	16	11	12	6	3	6	14	8
Sample 6	7	10	9	6	1	12	9	17	13	13	7	11	11	12	8
Sample 7	5	8	9	8	8	12	13	11	10	11	10	10	10	9	9
Sample 8	7	9	10	6	14	12	15	12	11	14	7	15	4	5	6
Sample 9	4	5	5	7	13	11	10	10	10	11	8	7	7	6	6
Sample 10	7	6	7	7	10	11	6	8	7	14	9	4	6	7	19
Sample 11	6	10	6	7	6	10	11	8	8	9	9	12	7	7	7
Sample 12	7	8	7	7	8	10	10	9	8	8	8	8	7	6	6
Sample 13	10	10	8	10	8	11	11	10	10	9	10	8	8	8	7
Sample 14	11	13	11	15	13	11	14	12	14	12	8	11	9	15	9
Accuracy	*Analyst (% Recovery)					**Days (% Recovery)					***Bead Lot (% Recovery)				
Sample 1	96	102	106	98	104	91	98	99	96	100	92	96	97	99	102
Sample 2	94	97	111	91	102	91	91	102	90	95	91	88	96	94	95
Sample 3	107	100	101	103	118	105	97	89	98	110	99	89	84	92	100
Sample 4	86	92	91	90	112	89	90	87	96	101	90	90	85	95	95
Sample 5	99	94	106	96	108	96	94	100	104	106	95	93	92	109	107
Sample 6	104	109	106	110	112	100	106	97	106	104	99	103	88	96	99
Sample 7	93	99	101	101	106	92	94	96	99	102	92	93	90	96	103
Sample 8	100	106	104	111	110	90	95	95	102	99	83	90	90	96	95
Sample 9	99	104	92	92	97	90	94	87	86	93	85	92	86	84	92
Sample 10	92	106	93	98	93	101	110	96	95	96	100	111	93	94	93
Sample 11	90	106	93	100	83	96	109	96	96	88	97	108	95	97	87
Sample 12	91	95	95	101	86	98	96	97	98	92	98	99	98	101	90
Sample 13	89	91	94	104	94	96	94	96	98	93	93	93	96	100	88
Sample 14	90	94	94	102	97	95	98	100	98	94	100	100	100	100	100

DT: diphtheria toxoid, FHA: filamentous hemagglutinin; PRN: pertactin, PT: pertussis toxin; RSD: relative standard deviation; TT: tetanus toxoid. Table reports % recovery which is calculated using observed concentration /assigned concentration*100. Precision and Accuracy results are determined with respect to different analysts, days and bead lots. *Combined precision and accuracy of analyst 1 and 2, **Combined precision and accuracy of 6 runs over 3 days, ***Combined precision and accuracy of multiple bead lots.

2.2.5. Accuracy

Acceptable recoveries were observed within the range of 80%-120% for DT, TT, PT, FHA and PRN antigens as mentioned in the Table 4. The UL and LLs based on accuracy ranged from 121.7–3.8 mIU/ml for T, 3.4 – 0.11 mIU/ml for D, 221 – 6.9 mIU/ml for PT, 457 – 14.3 mIU/ml for FHA and 424 – 13.3 mIU/ml and for PRN, respectively (Table 2).

2.2.6. Dilution linearity

The panel samples were tested in three independent runs across a series of sera samples ranging from dilution of 1:100-6,81,984. No loss in dilution integrity was observed with 2-fold increase in dilution in the dilution range studied for the respective antigens **(Supplementary material, Figure S1)**.

2.2.7. Robustness

Robustness data on IQCs with respect to incubation time, temperature, bead lot and PE lots were assessed. % Relative standard deviation (RSD) of observed *vs* estimated concentrations with respect to deliberate variations in parameter was assessed. Edge effect was calculated using IQC 3 in 96-well filter plate. %RSD of MFIs were noted in 96-wells of the plate. The results demonstrated that sample concentrations of IQCs generated from the assays with deliberate variations were within the acceptable range of less than 20% variability for all the antigens **(Supplementary material, Table S6)**.

2.2.8. Stability study

The reference standard, IQC and sera samples were found stable at room temperature for up to 72 hrs, at 2–8°C until 168 hrs and upto 20 freeze-thaw cycles (FZT)at -20°C.The overall percent difference in sample antibody concentration across the stability conditions (room temperature, 2–8°C and freeze-thaw) were found compliant with the reference standards, the IQCs and the selected sample **(Supplementary material, Figure S2 and Figure S3)**.

2.2.9. Solution stability

The solution stability of an assay was evaluated by analyzing the assay plates at predetermined intervals of 0, 6, 8, 12 and 24 hrs. Results at different intervals suggest that the plate hold time of not more than 12 hrs is recommended for all the antigens as an impact was observed for PRN antigen after 12 hrs of plate hold time **(Supplementary material, Figure S4)**.

2.2.10. Edge effect

Edge effect was evaluated for all the five antigens. No variability was noted in the wells and all the MFIs were within the acceptable variability of 10% RSD **(Supplementary material, Table S4)**.

3. Discussion And Conclusion

This study was to develop a multiplex reference standard for detection of IgG antibodies in the DTaP vaccine and to validate the multiplex bead-based assay against the international NIBSC standards. Additionally, in collaboration with the NIBSC laboratories, we also validated the assigned unitages for multiplex assay against commercially available ELISA assays at the NIBSC laboratories. The bead-based pentaplex assay was found to be robust, specific, accurate and precise and was stable over different temperature ranges and freeze-thaw cycles. The neutralization of D and T were also found to correlate well with this assay.

Immunogenicity testing of aP-based vaccines have been historically carried out using commercially available diagnostic kits. The commercial kits although validated using international standards[18], have had concerns on lot-to-lot variability owing to sourcing and quality of coating antigens. There are studies that support the use of purified antigens in single antigen ELISA or multiplexed immunoassays which have resulted in improved performance[19]. Additionally, single antigen ELISA assays or kits are time and labour intensive and needs large quantities of sera which is often challenging[20–24].

MIAs provides alternatives allowing high sensitivity, reproducibility and specificity. Several studies have reported the usefulness of multiplex platforms for immunogenicity assessment of AP-based combination vaccines[9,10,16,25,26]. A study evaluating a tetraplex microsphere assay for pertussis antigen showed high concordance with an in-house ELISA. The assay demonstrated that pertussis antigens can be measured easily and accurately using the multiplex assay[19]. However, very few studies are available wherein AP antigens are multiplexed with diphtheria and tetanus antigens. We reported previously, a 5-plex Luminex assay covering AP antigens, D and T antigens for evaluating the immunogenicity of combination vaccines in mice models[16]. Here, we report development and validation of a 5-plex assay for evaluating antibody IgG concentrations against T, D, PT, FHA and PRN in human serum samples. The assay reports the IgG concentrations in IU/ml which is traceable to NIBSC reference standard.

Luminex technology is based on use of beads that facilitates the measurement of multiple analytes from a single sample[27]. The beads are color-coded microspheres that contain different proportions of red and infrared fluorophores. These beads when activated at specific light spectrum, aid in quantification of the analyte. Luminex technology allows use of both non-magnetic and magnetic beads. The use of magnetic beads in the assay was shown to have high coupling efficiency and higher reproducibility due to lower inter-assay variation[28]. In our study, we used the magnetic beads for coupling the antigens and observed high coupling yields and minimum interferences from the matrices. The reproducibility of coupling method is an important factor for ensuring the consistency of test results especially from larger clinical trials. One of the prerequisites to the development of bead-based immunoassays is the validation to rule out impact of conjugation method on the antigen epitopes. Van Gageldonk PG et al. described the use of commonly used conjugation protocols for D, T, PT, FHA and PRN antigens[9]. In this study, we evaluated two commercially available conjugation procedures (Luminex cookbook and the commercial kit available from AnteoTech.) for coupling the antigens to beads. Both the methods demonstrated assay specificity and linearity for all the antigens. The specificity experiments involving inhibition assays using homologous antigen confirmed that antigenic epitopes were not affected by coupling process as addition of 2.62 ug/ml of antigen inhibited signalling by more than 85%. The robustness of conjugation process was further demonstrated using three different lots of coupled beads assays which demonstrated good reproducibility. The improved performance and sensitivity of MIAs is attributed to control over the purity of antigens used in the assays and correlation of Luminex technology to single antigen ELISA using purified antigens is previously reported [13]. It was noted that purity of antigens such as PT, FHA and PRN antigens was critical to the assay. The in-house manufactured antigens with purity of > 95% showed excellent results in the MIA. For TT and DT antigens, toxoids as compared to toxins showed higher consistency in the MIA. With tight control on purity of target antigens and use of magnetic beads, both the methods showed good agreement and was found suitable for the assay.

Development and validation of an assay against the international reference standard provides opportunities to harmonize and pool clinical results across multiple studies with good confidence and reproducibility[29]. With the advent of MIA technologies and with increasing regulatory expectations for validation of clinical immunogenicity assays, it is imperative that the multiplex assays be validated against a traceable standard to provide uniformity and reproducibility. NIBSC provided three reference standards viz, TE-3, 10/262 and 06/142 which respectively had the unitages for T, D, and AP antigens. MIAs being carried out

in single well will require a reference standard which provides the unitage of all the five antigens, which are being multiplexed. As part of assay development, an equimolar mix of NIBSC reference standards mentioned above was assessed as possible reference standard for multiplex assay. However, the assay reported inaccurate unitages for all the five antigens. It is known that NIBSC reference standards are sourced from subjects vaccinated with aP based combination vaccines and it is likely that reference standards will be positive for other antigens and thereby could contribute to inaccurate unitages for all the antigens. Characterization of the reference standard demonstrated that all the three reference standards have considerable number of antibodies and thereby needs to be accounted in an equimolar mix standard for multiplex assay. It was noted that the MIA reported excellent recoveries using the corrected unitages of multiplex reference standard. The observed unitages of multiplex reference serum and other NIBSC standards were also verified at NIBSC and a very good agreement was observed. These unitages will provide opportunities for use of these reference standards in multiplex assays.

Commercially available diagnostic kits for D, T, PT, FHA and PRN are used widely to assess the antibody responses to AP based combination vaccines. We also compared the results of multiplex assay to commercially available ELISA kits calibrated against international standards that provides unitages in IU/ml[18]. A good concordance was observed among the multiplex and monoplex assay methods. However, the multiplex assay was found to be more sensitive (2000 times for PT, 1000 times for FHA, 250 times for PRN, 330 times for DT and 100 times for TT) for all the antigens compared to the monoplex ELISA.

Immunogenicity testing of aP-based combination vaccines is mainly based on detecting IgG antibody concentrations. Cell-based *in-vitro* methods for determination of toxin neutralization antibodies for diphtheria and pertussis toxins were reported earlier[30–32]. These neutralization assays are based on determining the number of antibodies to PT and Diphtheria antigens which inhibit the toxin induced clustering of Chinese hamster ovary (CHO) cells and vero cells respectively[33]. The CHO cell assay for pertussis toxin and vero cell assay for diphtheria toxin which belong to this category are laborious, semi-quantitative and less sensitive in comparison to ELISA based readouts. Various studies have reported a positive correlation between concentration of IgG antibodies and neutralization antibody titres[33,34,30–32]. We also studied the agreement between the IgG concentrations estimated by bead-based assay and toxin neutralization antibodies for diphtheria and pertussis toxin antigens. The assay showed a positive correlation of > 0.75 with both DT and PT neutralization assays.

Immunogenicity testing of vaccines in clinics requires robust method development and validation. Existing regulatory guidance on bioanalytical method validation addresses vaccine immunogenicity assays in limited manner. The method was validated following the ICH Guidance (Q2 R1), US FDA, EMA and ICH M10[35–37]. The pentaplex magnetic bead-based assay exhibited a wide dynamic range and high sensitivity as compared to commercially available assays. The accuracy, precision and linearity of the assay was demonstrated using international reference standards. The assay showed excellent dilutional accuracy for all the antigens which is important to understand the full range of antibody responses to all the five antigens in both pre-vaccinated and post-vaccinated samples. The validation also established the LOQs for all the antigens using the international reference standards. The sample stability, robustness and bead-to-bead lot consistency were also established during the validation. Among all the antigens, PRN antigen was found to be most sensitive to assay conditions of plate hold time. The significance of plate hold time is minimal as PRN was found stable for up to 12 hrs, which is considered suitable to address any instrumental breakdowns during routine use of assay. The assay was found to be robust over different incubation temperatures and different PE lots. This ensures that the assay is unaffected by minor variations thereby ensuring that the performance of the assay is maintained on repeated use.

In conclusion, the study provides a pentaplex assay which aids in the detection of IgG antibodies against T, D, PT, FHA and PRN antigens in IU/ml using NIBSC reference standards. The study also provides characterization of NIBSC reference standards with respect to determination of antibodies against all the five antigens, which will allow their efficient use in multiplex assays. The increased sensitivity, reproducibility and high throughput will allow design of large and robust clinical studies for evaluating natural and vaccine-induced immunity. The assay being developed on Luminex technology further provides opportunities for further expansion to include new antigens.

4. Material And Methods

4.1. Antigens

PT (purified toxin), FHA (purified antigen), PRN (purified antigen), DT (toxoid), and TT (toxoid) were sourced from Serum Institute of India Pvt. Ltd. (SIIPL). All the antigens were tested for their content and purity. The protein content of the antigens was estimated using a validated bicinchoninic acid (BCA) assay[38]. Antigens were stored in aliquots at -20°C or at a lower temperature, according to the manufacturer's recommendation.

4.2. Reagents

R-phycoerythrin (R-PE) - conjugated to anti-human antibody was obtained from Southern Biotech. Beads (carboxylated microspheres) were procured from Luminex corporation, US, and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) was obtained from Bio-Rad Laboratories, India. N-hydroxysulfosuccinimide (Sulfo-NHS) was procured from ThermoFisher Scientific, USA. Bovine Serum Albumin (BSA) was obtained from Sigma Aldrich, India. Tween-20 was purchased from SDFine Chem Limited, India.

4.3. World Health Organisation (WHO) biological reference materials International reference standards- Human reference sera

WHO biological reference materials (WHO International Standards and Reference Reagents) were purchased from the National Institute for Biological Standards and Control (NIBSC, UK). The 1st WHO Reference Reagent for Pertussis Antiserum Human (06/142) is a freeze-dried preparation of pooled re-calcified human serum and has an assigned anti-PT IgG content of 106 IU/ampoule, an anti-FHA IgG content of 122 IU/ampoule and an anti-69K IgG content of 39 IU/ampoule. The 1st WHO International Standard for Diphtheria Antitoxin Human (10/262) is a freeze-dried preparation of normal human IgG with a diphtheria antitoxin potency of 2 IU/ampoule. The 1st WHO International Standard for Anti-Tetanus Immunoglobulin Human (TE-3) is a freeze-dried

preparation of human tetanus immunoglobulin and has an assigned unitage of 120 IU/ampoule. The ampoules of 06/142 and 10/262 were reconstituted in 1 ml sterile water and TE-3 in 1X PBS prior to use. The unitages in IU/ml for these standards is summarised in **(Supplementary material, Table S1)**.

Multiplex Reference Standard (MRS): WHO international reference reagents for aP (06/142), D (10/262) and T (TE-3) obtained from NIBSC were used to prepare MRS by mixing in equal proportion (1:1:1) and established the unitages of all five antigens.

4.4. Internal quality controls

WHO international reference standards for T and D obtained from NIBSC were used to prepare internal quality controls (IQCs) by mixing equimolar proportion of diphtheria (10/262) and tetanus (TE-3) reference standard and the unitage for all the five antigens were established. Five levels of IQCs (IQC-1 to IQC-5) were prepared. IQC panels were prepared using Diphtheria antitoxin human serum (10/262) and tetanus immunoglobulin human (TE-3) by mixing equal volumes (1:1) to yield IQC stock standard. These were calibrated against the reference standard panel and assigned the following unitages, PT (110.43 IU/ml), FHA (228.70 IU/ml), PRN (212.06 IU/ml), DT (1.737 IU/ml) and TT (61.08 IU/ml). For assigning the limit of the specification to IQC's, they were diluted from 1:500 to 1:16,000 in assay buffer. Assay variability and acceptance limits were established by repeated testing of the IQCs. Acceptable ranges for the estimates were set as average values ± 2 standard deviations (SDs) of the IgG concentrations to each antigen.

4.5. Sera for performance characterization

Sera from healthy adults aged > 18 years working at Serum Institute of India Private Limited were collected after obtaining appropriate ethics committee approval (IEC No. IRECP/015/2020,Independent Research Ethics Committee, Pune). Sera from healthy volunteers with no prior history of the booster with Tdap were selected to develop a panel of serum samples representing high (1:400), medium (1:6400), and low (1:12800) concentrations of antibodies against T, D, PT, FHA, and PRN. These human sera were screened for antibodies to each of the T, D, PT, FHA, and PRN antigens using bead-based multiplex assay. Sera samples outlined in Table 5 represent the panel used for assay validation.

Table 5
Sera panel used in method validation

Sample no.	Panel 1	Panel 2	Panel 3	Panel 4	Panel 5	Panel 6	Panel 7	Pa 8
1.	DU0001/B/2020/IA	AM0015/NB/2020/IA	Haemolytic sera	Blank Human Sera	DU0001/B/2020/IA	DU0001/B/2020/IA	S1	S1
2.	RS0002/B/2020/IA	DG0016/NB/2020/IA	Lipemic sera		RS0002/B/2020/IA	RS0002/B/2020/IA	S2	S2
3.	AS0004/B/2020/IA	ST0017/NB/2020/IA			AS0004/B/2020/IA	SS0007/B/2020/IA	S3	S3
4.	AN0005/B/2020/IA	SS0018/NB/2020/IA			AN0005/B/2020/IA	AS0009/B/2020/IA	S4	S4
5.	SP0006/B/2020/IA	PO0019/NB/2020/IA			MRS	RK0010/B/2020/IA	S5	S5
6.	SS0007/B/2020/IA	SH0020/NB/2020/IA				IQC 1	S6	S6
7.	AZ0008/B/2020/IA					IQC 2	S7	S7
8.	AS0009/B/2020/IA					IQC 3	S8	S8
9.	RK0010/B/2020/IA						DU0001/B/2020/IA	IQ
10.	IQC 1						RS0002/B/2020/IA	IQ
11.	IQC 2						SS0007/B/2020/IA	IQ
12.	IQC 3							IQ
13.	IQC 4							IQ
14.	IQC 5							IQ
15.								IQ

MRS: Multiplex Reference Standard; IQC: internal quality controls; Panel 1: panel for precision and accuracy, Panel 2–4: panel for selectivity parameters; Pane 5: panel for dilution linearity; Panel 6: panel for stability at 2–8°C and RT; Panel 7: panel for freeze-thaw stability (S1-S8: Reference standard); Panel 8: panel solution stability

4.6. Assay procedures

4.6.1. Coupling of antigens to microspheres

Antigens (T, D, PT, FHA, and PRN) were coupled to the spectrally unique magnetic carboxylated microspheres using established and commercially available coupling procedures Two commercially available procedures were evaluated; the first one was based on the Luminex cookbook published previously by Kadam L, et al.[16,39] and second one based on the commercial kit available from AnteoTech (Australia)[40]. Briefly, for Luminex, microspheres were activated with a carbodiimide derivative, EDAC hydrochloride containing buffered solution. The intermediate carboxyl groups formed on the beads by reaction with EDAC were stabilized using sNHS solution. This was followed by 3x washing steps using a magnetic separator. Respective antigens were added to the activated beads and kept in the dark for 2 hrs under constant mixing (15–30 rpm). The resulting mixture was washed, and the supernatant was discarded

during every washing step. After three steps of pelleting and washing, coupled beads were blocked using 1% BSA buffer for 30 minutes and were kept in a storage buffer (0.1% w/v BSA in phosphate buffered saline [PBS] containing 0.05% sodium azide and 0.02% Tween 20).

The coupling, activation and storage buffers used were procured from Anteo tech kit. Briefly, the beads were activated using the activation buffer for 60 minutes. The antigens to be coupled were prepared using conjugation buffer. The antigens were then mixed with the activated beads and kept for incubation at room temperature for 60 minutes. The unbound antigens were removed by washing it three times. The blocking buffer was prepared by adding 0.1% BSA in conjugation buffer and added to the coupled beads. Beads were kept in blocking buffer for 60 minutes and stored in storage buffer.

4.6.2. Monoplex assay for unitage assessment

Monoplex assays were carried out for unitage assessment of 06/142, 10/262 and TE-3 as detailed in Fig. 3.

The assay used monovalent beads of ~ 4000 beads/well. Briefly, respective human reference standard (NIBSC 06/142, 10/262 and TE-3) diluted serially 2-fold for 8 dilutions from 1:1000 to 1:1, 28,000 were added to the monovalent beads. Test serum samples (NIBSC standards) were also assessed at multiple serial dilutions starting from 1:1000. Assay blanks were included in the plate as a control. All the incubation conditions, number of washes, buffers and instrument settings were followed as for the pentaplex assay (Section 2.6.5).

4.6.3. Commercially available ELISA Methods

Commercially available CE certified ELISA assay kits (IBL, USA and Euroimmun, Germany) were used to confirm the unitages assigned to all the antigens in MRS. ELISA assays were performed for the PT, FHA, PRN, DT and TT antigens. The IBL ELISA kits (PRN, DT and TT) provided the calibrators, positive and negative controls. The NIBSC standards 06/142, 10/262, TE-3 were used as test serum samples. The assays were performed as per the manufacturer's instructions. The samples were diluted from 1:100 and were added on to the pre-coated plate and incubated for an hour at 37°C. Following this, 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgG) was added into each of the microplate wells. This mixture was incubated for 30 minutes at room temperature and washed after the incubation. Into each of the microplate wells, 100 µl of chromogen/substrate solution was added. This was incubated for 15 minutes at room temperature and 100 µl of stop solution was added. Optical density (OD) was read at 450 nm using the Biotek ELISA reader (USA). OD values within the linear part of the curve were converted to IU/ml by interpolation from a 4-parameter logistic (PL) standard curve of the reference serum and averaged.

The Euroimmun (PT, FHA) ELISA test kit was used for *in vitro* quantification of human antibodies of the IgG class in serum or plasma. In the first reaction step, diluted samples were incubated in the wells. Positive samples contained specific IgG antibodies bound to the antigens. To detect the bound antibodies, a second incubation was carried out using an enzyme labelled anti-human IgG (enzyme conjugate) catalysing a color reaction. Photometric measurement of the color intensity was made at a wavelength of 450 nm and a reference wavelength between 620 nm and 650 nm and read within 30 minutes of adding the stop solution. The results of this assay were compared with the bead-based assay.

4.6.4. Unitage confirmation at NIBSC laboratory

For characterization, MRS was also tested at NIBSC using conventional assays for PT, FHA, PRN, DT and TT.

Towards characterization, MRS was also tested at NIBSC using conventional ELISA assays for PT, FHA, PRN, DT and TT. Briefly, for PT, FHA and PRN (after each step, plates were washed with phosphate buffered saline (pH 7.4) containing 0.05% v/v Tween 20 (PBST) and all incubations, unless specified, were at room temperature), ELISA plates, 96 well, (Nunc maxisorp) were coated with 100µl per well of a 2µg/ml solution of either PT (NIBSC in-house), FHA (NIBSC JNIH-4) or PRN (NIBSC 18/154) in carbonate buffer (pH 9.5 containing 0.035 M sodium hydrogen carbonate, 0.015 M sodium bicarbonate, and 7.4 mM sodium azide) overnight. Plates were blocked with 100µl of PBST containing 10% foetal bovine serum (FBS) for 1 hour followed by incubation with samples and reference (WHO reference reagent 06/142) at a starting dilution of 1/100 in blocking buffer for 1.5 hrs. Two-fold serial dilutions were performed using blocking buffer as the diluent. Following this, antigen-specific IgG antibodies were detected with 100µl of rabbit anti-human IgG labelled with horseradish peroxidase (Sigma, A-8792), 1/2000 in blocking buffer, for 1.5 hrs. Finally, 100 µl of 1% 3, 3', 5, 5' - tetramethyl-benzidine (TMB) substrate (Sigma, T-2885) in dimethyl sulphoxide (DMSO) and 0.03% hydrogen peroxide (Sigma, H1009) in acetate buffer (pH 6.0) was added and color developed for 15 minutes, followed by the addition of 50 µl 1M sulphuric acid. The optical density was measured at 450nm using a Multiskan ELISA plate reader (Molecular Devices, United Kingdom). Antibody responses for the MRS were calculated relative to the WHO reference material by parallel line analysis (log optical density vs. log dose), using a minimum of 3 sequential points from the linear section of the dose response curves and expressed in IU/ml.

For DT and TT, ELISA plates were coated overnight at + 4°C with either 100 µl per well of D Toxoid (NIBSC 13/212, 3.7 flocculation units/ml) or 100 µl per well of T Toxoid (NIBSC 02/126, 0.5 flocculation units/ml) diluted in carbonate buffer (0.05 M, pH 9.6). The ELISA plates were washed (3x) in PBST and blocked with 150 µl of PBST containing 5% (wt/vol) dried skimmed milk powder (PBSTM-5%) for 1 h at 37°C. Following a second wash in PBST, serial two-fold dilutions of the WHO reference material (10/262 for D ELISA and TE-3 for T ELISA) and MRS in PBSTM-1% were prepared in the plate (final volume 100 µl), and the plates were incubated at 37°C for 2 h. Plates were washed as described previously, and antigen-specific IgG antibodies were detected using a horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG antibody diluted 1/2000 in PBSTM-1%. After a further 1 h incubation at 37°C and a final wash, 100 µl per well of substrate solution containing 0.5 mg/ml 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma A9941) and 0.008% hydrogen peroxide (Merck, 107209) in 0.05 M citric acid buffer (pH 4.0) was added and the reaction was allowed to develop for up to 30 min. The optical density was measured at 405 nm using a Multiscan ELISA plate reader. Antibody responses for the MRS were calculated relative to the WHO reference material as before. Analysis of variance was used to test for any significant deviation from linearity or parallelism of the dose response relationship ($p < 0.01$).

The unitages were compared against the acceptance criteria of assigned unitages of MRS within 30% variability margin attributed to the use of different assay platforms, different antigens, and inter-laboratory variations.

4.6.5. Pentaplex Immunoassay

Multiplex reference standard (Mix of 06/142, 10/262, TE-3) prepared as detailed in Section 2.3 was used as a calibrator for the assay.

The multiplex reference standard was serially diluted 2-fold from 1:333 to 1:42,624. The test sera samples were diluted serially 2-fold from 1:100 to 1:12800 using assay buffer and tested at multiple dilutions. The filter plate was used for the assay. The multivalent beads were added in each well at 50 µl/well (~ 4000 beads/well) and aspirated. From the dilution plate, 50 µl of reference standard and samples were transferred in duplicate on to the filter plate and incubated in the dark for 60 minutes at 37°C and centrifuged at 150 rpm. The plate was aspirated and was washed 3 times with 100 µl assay buffer. To each well, 50 µl of a 1:100 diluted R-PE goat anti-human antibody was added and incubated in dark for 30 minutes at 37°Cand centrifuged at 150 rpm with agitation. The plate was aspirated, washed 3 times with 100 µl assay buffer and the microspheres were resuspended in 100 µl assay buffer. The plate was read in Protein Suspension Array System (Bioplex-200). The assay blank was run in each plate. Reference standard's backfit of 70–130 and percentage coefficient of variation (%CV) of ≤ 20% was monitored as a system suitability criterion.

4.6.6. Toxin neutralization assays

Toxin neutralization assay was performed to verify the ability of antibodies in the serum samples to neutralize active P toxin and D toxin. The Chinese Hamster Ovary (CHO) cell-clustering assay (CHO assay) based on the induction of clusters in non-confluent CHO cell cultures by aP toxin was performed to assess the toxin neutralization. Briefly, serially diluted sera samples were incubated with known concentration of toxins at 37ºC for NLT 60 min. After incubation, CHO cells with 3 X 10^4 cells/ml concentration were added in all the wells of antigen-antibody mixture and the plates were incubated at 37 ± 1°C for about 48 hrs. Following this, CHO cells were observed for clustering under an inverted microscope. The highest dilution of sera, which showed cluster neutralization, was recorded as the sample titer and a positive score is assigned when 10 or more CHO cell clusters are evident within a single well[33].

The Vero cell assay has been used to determine the protective level of D antitoxin in human sera[34]. The metabolic activity and survival of Vero cells in cultures are inhibited by diphtheria toxin and this effect of the toxin may be neutralized by D antitoxins in serum samples. Titration of serum samples on Vero cells in the presence of fixed amounts of D toxin is carried out and the reading of the Vero assay is based on a microscopic examination of cells under microscope for determination of color change in wells of microtiter plates from red to yellow due to metabolic formation of acid. For the preparation of diphtheria toxin, serum dilutions were prepared in a micro titer plate in MEM supplemented with 10% FBS. Diphtheria toxin at dose of Ltc/100 were added and incubated for 45 minutes to 1 hour at room temperature (20–25°C) for toxin neutralization. Prepare vero cell suspension containing 3.5–4.5 x 10^5 cells/ml and were added 100 µl to the 96-well microtiter plates and were incubated for 5 days at 36 ± 1°C under 5% CO_2 atmosphere and cells were observed for metabolic inhibition caused by un-neutralized toxin. Vero cell assay was performed to determine the neutralization to D toxin. Fixed sera dilutions of D toxin were prepared, and the titer of serum sample was calculated by comparing the test results with standard diphtheria antiserum. Factor of the highest dilution, showing metabolic inhibition was multiplied with 0.2 (limit of detection of this method) to report the results in IU/ml.

4.7. Method validation

The methods for assay validations were carried out based on the International Council of Harmonization (ICH M10), European medicines agency (EMA) and Food and Drug Administration (FDA) guidelines for bioanalytical methods. The validation study was approved by the respective ethics committees.

4.7.1. Specificity

The specificity of the method was evaluated by comparing the median fluorescent intensity (MFI) of a multiplex assay which assessed the 5 antigens simultaneously, with a monoplex assay which assessed each individual antigen separately. Further, IgG specificity was evaluated by performing inhibition experiments with both the multiplex and monoplex assays. For inhibition experiments, MRS was incubated separately with each of the purified antigens (DT, TT, PT, FHA and PRN) and with a mixture of antigen (DT + TT + PT + FHA + PRN) for 1 hour before analysis. Percentage reduction in MFIs due to presence of purified antigen (specific antigen) or mixture of antigens was compared to demonstrate the specificity.

4.7.2. Selectivity

The selectivity of the method was evaluated using three human serum matrices) Matrix 1 (Panel 2 Sample 1–6), ii) Matrix 2 (Panel 3 Sample 1–2), iii) Matrix 3(Panel 4 Sample 1) as mentioned in Table 5. Matrix 1 and Matrix 2 were spiked with different concentrations of reference standard and tested at 1:400 (high), 1:6400 (medium), and 1:12800 (low). Matrix 3 was spiked with the MRS and IQC. Recovery of spiked samples from the different matrices was calculated with the acceptance criteria within the range of 70%-130% of expected concentrations.

4.7.3. Assay range

The assay range was established by using MRS. The reference standard for the determination of the assay range was prepared by serially diluting the MRS2-fold from 1:333 to 1:42624. The assay range was also supported by estimates from accuracy, precision, and dilution linearity studies.

4.7.4. Precision

Assay precision was evaluated with respect to variability due to days, analyst, and microsphere lots, using panel of precision (Panel 1) **(**Table 5**)** and international reference standards **(Supplementary material, Table S1)**. A total of six runs were performed on the sera panel at dilutions of 1:100 to 1:12,800 and 6 runs on IQC panel at dilutions of 1:500 to 1:16,000. Intra-assay precision to determine variability observed for the same analyst was evaluated based on the results obtained from the assays performed on the same day. Inter-assay precision assessed the variability in experiments performed on different days by different analysts using different bead lots. The precision of the assay was reported as the %CV.

4.7.5. Accuracy

Accuracy was estimated from the precision study. A panel of sera samples - Panel 1 (Table 5) and international reference standards (10/262 & TE-3) were used to assess the accuracy. These samples were tested at different concentrations in six assays spread over three days by using three different bead lots and read by two analysts. The estimates were compared to assigned unitages to determine the accuracy. The resulting IgG concentration of each serum samples were calculated and compared with the assigned values with an acceptance criterion of recovery between 70%-130%. The accuracy was evaluated for within run and between run parameters. Dilution accuracy of the assay was evaluated by spiking the reference standard in the blank human serum matrix by calculating the theoretical spiked percentage recovery.

4.7.6. Dilution linearity

Dilution linearity of the assay was evaluated using Panel 5 that spanned over a range of IgG antibody concentrations of five antigens as represented in Table 5. Assay dilutability was evaluated in three independent runs, using 2-fold dilutions from 1:100 until the serum sample was found quantifiable. Similar to the accuracy evaluation, recovery was calculated as percent difference between observed and assigned concentrations. At each dilution, one quantifiable criterion was based on %CV of duplicates and dilution corrected concentrations to be within the range of 70%-130%.

4.7.7. Limit of quantification and limit of detection

Minimum detectable response and minimum quantifiable response for each of the five antigens were determined using MFI at the limits of minimum detectable response and minimum quantifiable response. MFI at minimum detectable response and minimum quantifiable response were curve fitted to calculate the limit of quantification (LOD) and limit of detection (LOQ) of the assay.

4.7.8. Robustness

Five IQCs were used to analyze robustness. Results were used to estimate %CV for each parameter tested. The robustness was evaluated using reference standard and five IQCs. Percentage relative standard deviation (%RSD) was estimated for each of the factor. The following parameters were studied during assessment of robustness: Assay Step-1 and Step-2 incubation times, temperature, different lots of secondary antibodies (Phycoerythrin [PE] lots) and beads. BothStep-1 (incubation with beads; 50–70 min) and Step-2 (Incubation with PE; 20 to 40 min) incubation time assays and primary and secondary incubation temperatures (32–42°C) were evaluated. Two different PE lots and bead lots were also evaluated for robustness.

4.7.9. Stability Study

The stability of the serum samples and IQCs from Panel 6 were monitored at 25°C, and 2–8°C.Serum samples were assessed for stability at 25°C for up to 72 hrs. The stability at 2–8°C were assessed for up to 168 hrs.

Freeze-thaw stability was evaluated at -20°C for serum samples and MRS (Panel 7). The samples were aliquoted and exposed to freeze-thaw cycles wherein the sera samples were thawed for 2 hrs by placing the samples at room temperature (not more than 25°C). Later, sera samples were frozen for 24 hrs at -20°C prior to next thawing. The percent difference of assigned concentrations and observed concentrations were monitored for the stability study. The impact of freeze-thaw cycles was evaluated with an acceptance criterion of ± 30% difference as compared to assigned concentrations.

4.7.10. Solution Stability

The reference standard and the IQCs from Panel 8 were used to evaluate the solution stability. The solution stability of the assay was determined by analyzing the assay plates at predetermined intervals of 0, 6, 8, 12 and 24 hrs. Results obtained at different intervals were compared with the precision study set to determine the hold time of the plate with the acceptance criteria of ≤ 20%.

4.7.11 Edge Effect

Edge effect was evaluated by using the assay control. IQC 3 was placed in each well of the 96-well filter plate. Percentage CV of MFIs from all wells were calculated for the 96-well plate with an acceptance criterion of %CV ≤ 20 for all antigens.

4.7.12 Statistical Analysis

Mean and SD were calculated for continuous variables.

The %CV was calculated for precision and accuracy parameters and the standard was set at 20%. For MRS unitages (IU/ml), the values were rounded off to zero decimal places.

Log/log linear regression model was used to fit the reference standard curve. Calibration curves were generated using the 4-PL logistic fit and the values for back fitted recoveries

were set between 70%-130% and the %CV values at ≤ 20%. At least 75% of the calibration standards of minimum six standards had to meet these criteria.

The percentage recovery for selectivity assessment was calculated as:

$$(Observedconcentrationofspikedsample - Observedconcentrationofunspike$$

Following equations were used to calculate MFI for determination of LOD and LOQ:

Minimum Detectable Response = Avg. blank human serum MFI + 3 * (SD of blank serum MFI).

Minimum quantifiable response = 5* (MFI at minimum detectable response). The lowest quantifiable response was further multiplied with 200 (minimum two sera dilutions i.e., 100 and 200) to obtain LOQ in IU/ml.

The percent agreement between bead-based assay and ELISA was calculated as:

$$(Concentrationobtainedbybeadbasedassay \div ConcentrationobtainedbyELIS$$

Statistical analyses were performed using Microsoft Office Excel 2019 and statistical software Graphpad prism 7.05.

Declarations

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Author contributions

V.R, L.K, P.D.G, M.G, H.S, US, HR, SG, S.P, K.M, C.A, A.DB and L.H participated in (a) conception, design, development and validation of the assay, analysis and interpretation of the data; (b) drafting the article and revising it critically for important intellectual content; and (c) approval of the final version.

Competing interests

The authors declare no competing interests.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article.

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Figures

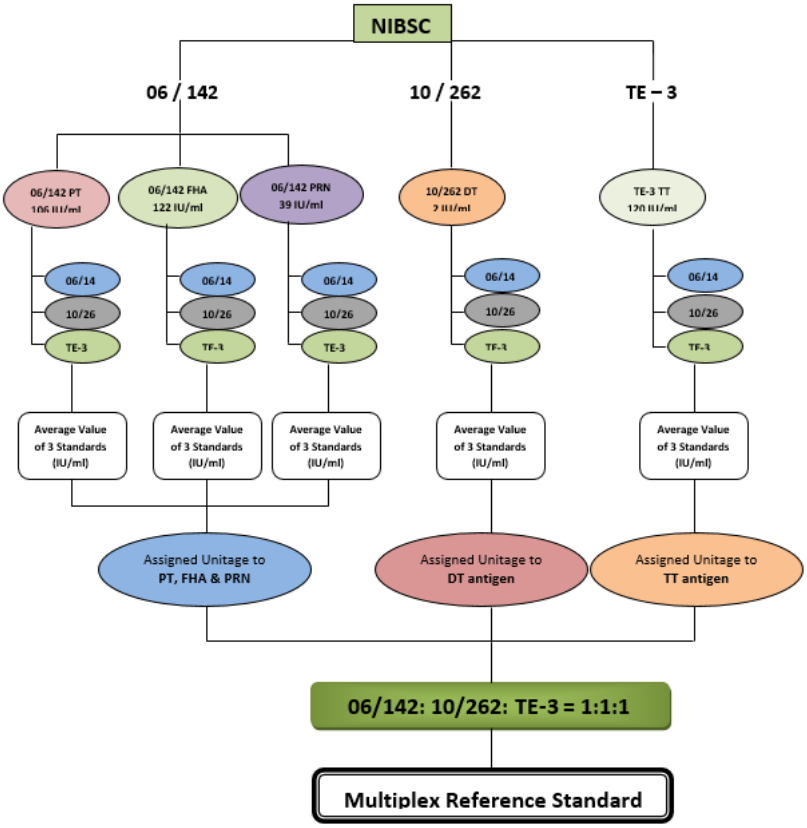

Figure 1

MRS reference unitage assignment approach

DT: Diphtheria Toxoid, FHA: Filamentous Hemagglutinin; IU/ml: International Units per milliliter; MRS: Multiplex Reference Standard; NIBSC: National Institute for Biological Standards and Control; PRN: Pertactin, PT: Pertussis Toxin; TE-3: NIBSC reference standard for tetanus toxoid; TT: Tetanus Toxoid.

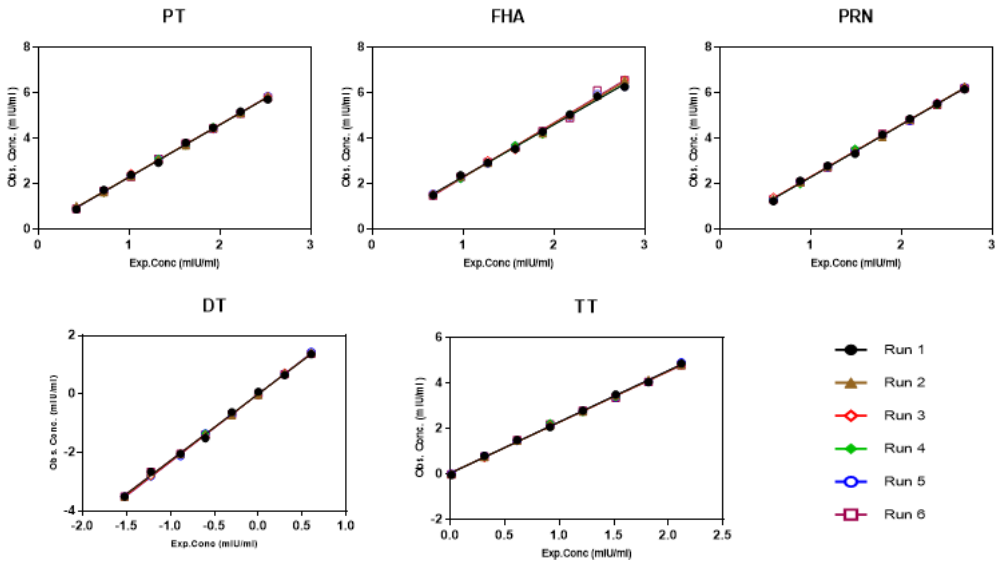

Figure 2

Performance of in-house developed multiplex reference standard (MRS)

The observed concentration (mIU/ml) data for the reference standard curve are on the y axis and Expected IgG antibody concentration data are on the x axis. The calibration curve graphs for A) Pertussis (PT), B) Filamentous Haemagglutinin (FHA), C) Pertactin (PRN), D) Diphtheria Toxoid (DT), E) Tetanus Toxoid (TT)

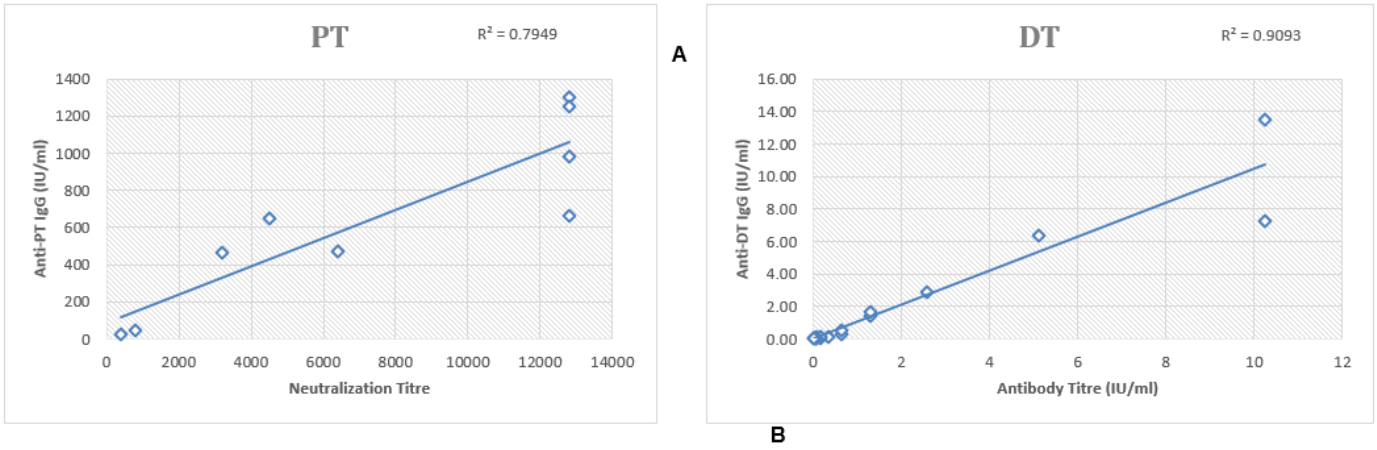

Figure 3

Correlation study between bead-based multiplex assay and toxin-neutralization assay for (A) PT and (B) DT

Correlation coefficient was determined using regression analysis. R^2 values were determined for agreement between assays. DT: Diphtheria Toxoid, PT: Pertussis Toxin

Supplementary Files

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