Methods 158 (2019) 33–43

Contents lists available at ScienceDirect

Methods

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

Development and validation of magnetic bead pentaplex immunoassay for simultaneous quantification of murine serum IgG antibodies to acellular pertussis, diphtheria and tetanus antigens used in combination vaccines

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ARTICLE INFO

Keywords:
Mutiplex ELISA
Acellular pertussis
Magnetic beads
Combination vaccines
Immunogenicity

ABSTRACT

We describe here a magnetic bead-based multiplex (pentaplex) immunoassay (MIA) platform developed as an alternative to enzyme-linked immunosorbent assays (ELISA) used in immunogenicity testing of DTaP/TdaP vaccine in animals. MIA simultaneously measures the concentration of serum (IgG) antibodies against *B. Pertussis antigens*; pertussis toxin, filamentous hemagglutinin (FHA), pertactin (PRN) and tetanus (T) and diphtheria (D) toxoid in the Tdap vaccine immunized animals. Assay validation experiments were done using a panel of serum samples. The results are expressed in IU/ml using WHO reference mice serum. The standard curve was linear with 4PL logistic fit over an eight 2-fold dilution range with LOQ of 0.003, 0.022, 0.005 IU/ml for PT, FHA and PRN and 0.016 U/ml for T and D antigens indicating sensitivity. No interference was observed in monoplex versus multiplex measurements. Specificity was demonstrated by ≥90% homologous and ≤15% heterologous inhibition for all the antigens. The assay was reproducible, with a mean coefficient of variation (CV) of ≤10% for intra-assay duplicates and ≤25% for interassays using different lots of beads and analyst. Accuracy was demonstrated wherein the ratio of observed vs. assigned unitages were within 80–120%. The study suggests that the Pentaplex (MIA) platform meets all the criteria for the serological assay combination vaccines with additional advantages of high throughput, reduced sample volumes, faster analysis with reduced manpower in contrast to conventional monoplex ELISA.

1. Introduction

Combination or multivalent vaccines are the cornerstones of paediatric and adult immunization programs. Diphtheria (D), pertussis (whole cell; WP) and tetanus (T) antigens combined into a single product with an adjuvant represent the first successful example of combination vaccine to achieve public health benefits [1–3]. The substitution of whole cell pertussis antigens with acellular pertussis (aP) antigens has further paved the path for even more complex combination vaccines [4]. The compositions of available acellular pertussis vaccines vary widely, yet most of them contain detoxified pertussis toxin, alone or in combination with filamentous haemagglutinin (FHA), pertactin 69 kDa, PRN and fimbriae antigens (fim 2, 3) [5].

The preclinical evaluation of aP antigens to predict their clinical efficacy is a major challenge. WHO and European Pharmacopeia recommends a non-lethal immunogenicity test in mice for monitoring lot to lot consistency during the manufacturing. The assay involves immunization of mice with serial dilutions of the vaccine and serum IgG antibody response against each pertussis vaccine component is then measured by enzyme-linked immunosorbent assay (ELISA). Consequently, a large number of different ELISAs have to be performed to estimate antibody levels for all the antigens in the vaccine, which involves higher costs, logistics and most importantly requires considerable serum volumes [6,7].

The Multiple Analyte Profiling technology (xMAP®; Luminex Corp., Austin, TX) is a flow cytometry-based system (19) based on the use of distinct fluorescent microspheres as the carrier of different antigens enables the simultaneous detection of up to 100 analytes in a single well of a 96-well flat-bottom plate. Several studies have previously described the accuracy and high-throughput advantage of this platform for evaluating vaccine-elicited binding antibodies [8–11]. For example, Pavliakova D et al reported a 13-Plex Luminex assay for quantification of

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https://doi.org/10.1016/j.ymeth.2019.01.015
Received 18 September 2018; Received in revised form 27 December 2018; Accepted 23 January 2019
Available online 25 January 2019

Table 1
Description of serum panels used in the validation. Assigned unitages to panel sera are also provided, which was used for precision and accuracy studies.

Panel	Validation parameter	Description	Details				
1	**Specificity**	**Following panel of sera samples was used for establishing specificity**					
1A	Specificity	Sera obtained by immunization of whole cell pertussis vaccine. The sera will be positive for only pertussis antigens	Pertussis vaccine manufactured at SII. was immunized on day 0, and 14, 28 days. The animals were bled on day 42 for the sera. Sera of 30 mice were pooled to develop the panel			1A	
1B	Specificity	Sera containing antibodies to Diphtheria antigens only	Sera was obtained by immunization of NIH Harlan mice with SII manufactured Pneumo conjugate vaccine. The vaccine used diphtheria protein as carrier protein. Animals were immunized on day 0, 14 and 28. Sera were collected on day 42. (Serum of 30 mice was pooled to develop the panel)				
1C	Specificity	Sera containing antibodies to Diphtheria and Tetanus antigens only	Sera were obtained by immunization of Harlan mice with a commercially available Pneumoconjugate vaccine (Synflorix vaccine). This vaccine uses carrier protein of tetanus and diphtheria in the conjugate vaccine. The immunization protocols were similar of what was followed for above panel. (Pool of N = 30)				
1D	Specificity	Negative control sera	The sera pool is obtained by immunization with placebo buffer (Tdap vaccine). The immunization protocol is similar to above. (Pool of N = 30)				
2	**Precision**	**Post vaccinated sera samples**					
2A	Precision	Obtained by immunization 1/2.5 of human dose of Tdap vaccine	Single dose immunization.				
			IU/ml			**U/ml**	
			PT	FHA	PRN	DT	TT
			37	50	10	13	99
2B	Precision	Obtained by immunization 1/5.0 of human dose of Tdap vaccine	Single dose immunization.				
			IU/ml			**U/ml**	
			PT	FHA	PRN	DT	TT
			18	26	5	3	56
2C	Precision	Obtained by immunization of 1/10th of human dose of Tdap vaccine	Single dose immunization.				
			IU/ml			**U/ml**	
			PT	FHA	PRN	DT	TT
			8	22	1	1	24
2D	Precision	High titer sera obtained by immunization of 1/2.5 of human dose Tdap vaccine on day 0, 14 and 28. Sera collection on day 42	Multiple immunizations.				
			IU/ml			**U/ml**	
			PT	FHA	PRN	DT	TT
			40	159	33	44	244

human serum antibodies to *Streptococcus pneumoniae* capsular Polysaccharides used in conjugate vaccine [12]. In another study, using Luminex-based mPlex-Flu assay, influenza -specific IgG antibody mediated cross-reactivity to adjuvanted recombinant influenza hemagglutinin (rHA) was studied in ferrets and mice [13]. Further in another application to Human papiloma Virus (HPV) quadrivalent vaccine, a multiplexed luminex assay was used to assess long term antibody to responses to serotypes 6, 11, 16 and 18 in humans [14]. A number of studies are also available for multiplexed analysis of diphtheria, tetanus and acellular pertussis antigens. However, a majority of these reports are for quantification of human antibodies with applicability to seroprevalence or evaluating pre and post vaccination samples [15,16]. Very few reports on MIAs are described for applicability to the preclinical assessment of DTaP or Tdap based combination vaccines in animals, as required by regulatory agencies [17].

We report here development and validation of a magnetic bead based pentaplex immunoassay for combined quantification of mouse serum antibodies against pertussis components (Ptx, FHA and PRN), diphtheria and tetanus toxoid. The immunoassay is intended for direct applicability to mouse immunogenicity test as recommended in European, British and Indian pharmacopeia for quality control testing of aP antigens in Tdap or DTaP vaccine. The described Penta-Plex magnetic microsphere based fluorescent immunoassay (MIA) measures total IgG levels in Tdap immunized animals against an international reference standard. The study indicates that assay exhibits a wide dynamic range for all the five antigens. The assay was validated as per the international regulatory guidance provided by US FDA, EMEA and ICH on validation of bioanalytical methods and assessed key characteristics: quantifiable range, precision, specificity and ruggedness and dilutional linearity. The multiplex assay satisfies all the requirements for a quality control assay and will be useful for measuring immune responses to Tdap or DTaP combination vaccines.

2. Materials and methods

2.1. Purified antigens, reagents, vaccines

Pertussis toxin, Filamentous hemagglutinin, Pertactin, diphtheria and tetanus toxoid was supplied by Serum Institute of India Pvt Ltd. Protein content of antigens were estimated by a validated BCA assay. The antigens are QC tested to meet all the purity and integrity requirements. R-phycoerthyryn (R-PE) - conjugated anti-mouse was obtained from Jackson Immunoresearch. Beads (carboxylated microspheres) were obtained from Bio-Rad laboratories. Sulfo-NHS was procured from Thermofisher and EDC was purchased from Bio-Rad. BSA was obtained from Sigma Aldrich. Tween-20 was purchased from Merck. Tdap Vaccine manufactured by Serum Institute of India Ltd comprising of following antigens; Tetanus toxoid not less than 2.5 Lf, diphtheria toxoid- not less than 5 Lf; Pertussis Toxoid 8 mcg, detoxified filamentous hemagglutinin- 8 mcg and Pertactin- 2.5 mcg in 0.5 ml dose was used in the study.

2.2. Standard sera

WHO international reference mouse serum (NIBSC 97/642) was used as reference standard. The international reference sera were developed by pooling mouse serum samples following vaccination with DTaP vaccine. The sera have following assigned unitages: 17 units; anti-PT, 143 units' anti-FHA, 30 units of anti-PRN and 32 units of anti-FIM 2 and 3 per vial. One lyophilized vial was reconstituted with 500 µL of 1X

PBS. Aliquots were prepared and stored at −20 °C till further use. The concentrations of anti-diphtheria and anti-tetanus IgG were unknown and so these were subjectively set at 100 U/ml for validation purpose in the study.

2.3. Animals

NIH mice (male or female) were housed and used for experiment according to the institutional ethics guidelines for animal experiments at Serum Institute of India Pvt Ltd. Mouse immune sera were generated by immunization of groups of 6–8 week old mice (N = 10/group). The retroorbital bleeding method was used for sera collection on on respective day after immunization. Details of serum panel development with respect to animal experiment is given in Table 1. All the experiments were regulated under National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.4. Sera panel for validation testing

The QC control sera were used for validation testing. Separate serum panels for specificity and precision studied were developed. The details of the development of these serum panels are given in Table 1 and Fig. 1.

The study also included testing ofn serum samples obtained by immunization of Tdap vaccine at four dilutions of 2.5, 5, 10 and 20 on day 0 and sera collection on day 35. For each dilution (N = 10) animals were used. Antibody concentrations for all the five antigens were determined for each mice and geometric mean concentrations (GMC) were reported.

2.5. Coupling of purified antigens to polystyrene beads

Purified Ptx, FHA, PRN, Dtx and Ttx were coupled to activated microspheres (beads) of regions; 35, 42, 14, 44 and 48 respectively following a procedure according to Van Gageldonk et al. [18]. Briefly, 2.5 to 12.5 × 10^6 beads were activated by incubating it with 100 ug of EDC (1-ethyl-3 dimethyl amino propyl carbodiimide hydrochloride) and N-hydroxysulfosuccinmide (sulfo-NHS) (pH = 6.1) for 20 min. This was followed by washing steps using magnetic separator. Respective antigens were added to wash activated beads and kept in dark for 2 h under constant mixing (15–30 rpm). The resulting mixture is washed and the supernatant is discarded. After three steps of pelleting and washing, coupled beads are blocked using 1% BSA buffer for 30 min and are finally kept in storage buffer (0.1% w/v BSA in PBS containing 0.05% sodium azide and 0.02% tween 20). Final count of beads is enumerated using neubauer chamber.

2.6. Pentaplex assay

Eight steps of 2 fold dilutions of NIBSC reference standard serum were prepared in luminex assay buffer. Sera samples were diluted in assay buffer containing PBS (1X), BSA (0.2%), tween 20 (0.1%) and sodium azide (0.01%). Each dilution of reference and sample were mixed with 1:1 (50 µl) conjugated beads to attain 4000 beads/region/well in a 96 well multiscreen HTS filter plate (Millipore Corporation, USA) and incubated for 1 h at 37 degree celcius in the dark on the plate shaker at 150 rpm. Suitable blanks (negative control, sera obtained from un-immunized animals) and assay blanks (without serum) were included in every plate. The beads were washed three times with PBS by filtration using magnetic plate assembly. Subsequently, beads were further washed three times using luminex assay buffer. This was followed by addition of PE labeled secondary anti mouse antibody (recommended dilution of 1:250) and followed by incubation at 37 °C for 30 min with 150 rpm shaking. The beads were washed and read on the flow cytometer (Bioplex-200). The events were acquired using Bio-plex manager software. The system classifies beads on the basis of its unique spectral pattern analyzed as median fluorescent intensity (MFI) of the signal of the reporter antibody. For each analyte (5 plex), MFI is converted to U/mL by interpolation from a 4 PL standard curve for every bead region/standard. Each sample/standard dilution is read in duplicates and % CV is monitored.

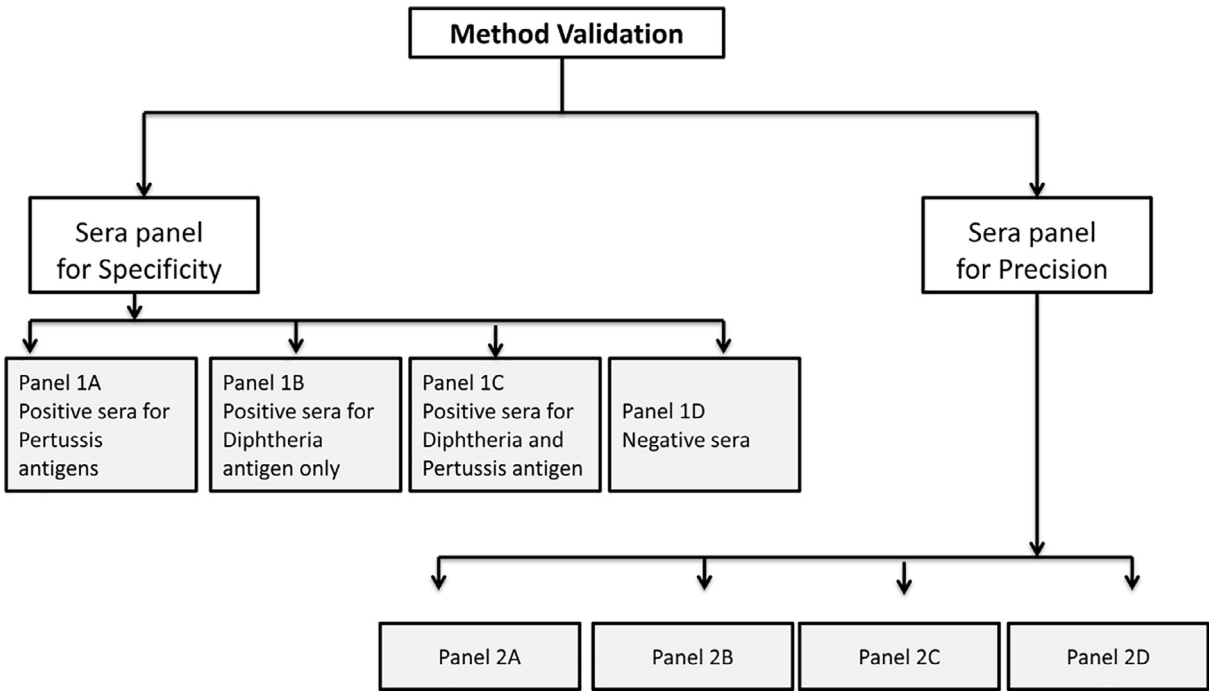


Fig. 1. Sera panels used for validation testing. The panels were designed to demonstrate assay specificity and precision. The specificity panel was designed to demonstrate assay specificity and selectivity for all the five antigens. Precision panel had five different sera samples with different unitages of all the five antigens.

2.7. Assay development

The assay optimization experiments focused on determining the optimal condition of major controllable parameters. The major assay conditions such as concentration of the blocking buffer, secondary antibody as well as optimization of incubation time for the secondary antibody and samples was evaluated. Positive control serum with assigned unitage was used in the optimization experiments. The experimental conditions involving using BSA at the more than 0.1%, PE conjugated antibody concentration at 1/250 and incubation time of 30 min for both secondary antibody and serum samples resulted in significant improvement in assay performance.

2.8. Assay validation parameters

2.8.1. Assay specificity

Inhibition experiments were performed to establish the specificity of the method. A high titre serum with known high concentrations of IgG antibodies specific to all the five antigens (Ptx, FHA, PRN, Dtx and Ttx) were serially diluted and homologous and heterologous inhibition was determined by addition of one of the antigens to the reaction mixture. Homologous and heterologous inhibition is reported as the percentage compared to the control. The assay specificity was also demonstrated using a panel of serum samples, which were obtained by immunization of one or two of the antigens used in the assay. Details of the panel are given in Table 1.

2.8.2. Assay linearity and range

Mean fluorescent intensity (MFI) in response to the anti-mouse reference standard serum (NIBSC 97/642) serially diluted in 2 folds and were analyzed for the quantifiable range. The data obtained from linearity data sets were analyzed using 4 PL curve fitting using Bioplex software. Curve constants such as 'a' (estimated response at zero concentration), 'b' (denotes slope factor), 'c' (denotes mid range concentration) and'd' (denotes estimated response at infinite concentration) were used for trending and 95% CI. ere calculated. The fit was carried out using Bioplex-200 system manager software. The quantifiable range is the concentration range of the standard curve over which back fits (residuals) is within the predetermined acceptance criteria. Acceptance criteria of back fits (70–130%) were used [19,20].

2.8.3. Assay sensitivity

The lower and upper limits of the quantification (LOQs) were determined using a negative serum sample and positive serum controls respectively. Using negative serum, readings from individual wells (n = 50), mean MFI, standard deviation (SD) and mean ± 2SDs MFI values for all the five antigens are calculated. The lower limit of detection (LLOD) for each analyte was determined by interpolation of mean ± 2SD target values from the reference curve and represented as concentration in IU/mL for PT, FHA and PRN and U/mL for T and D antigens. From the LLOD; the lower limit of quantification (LLOQ) is calculated is 3 × (LLOD) following guidance from EMEA [16].

2.8.4. Assay precision

Precision of the assay was assessed by determination of repeatability and ruggedness. Repeatability was assessed by evaluating variability within a single bead lot and analyst. Ruggedness was performed to assess variability at different analysts and different lots of antigen coupled beads. Panel 2 as per Table 1 was used for the assessment. Interassay precision was estimated by testing the panel samples across multiple assay runs by analyst-1 and intra-assay precision was estimated on the basis of runs carried out by analyst 2 using different bead lot and on a different day. % CV of results for each analyte was calculated and presented.

2.8.5. Dilutional integrity

Dilutability depicts the equivalence of the dilution corrected concentrations across the test sample through a series. The minimum required test sample dilution for serum sample is 1:100 and 1:50 for standard serum and positive control serum. Dilutability of the assay was evaluated using 2 fold dilutions until the serum sample was found quantifiable. The quantifiable criterion was based on % RSD of duplicates and dilution corrected concentrations to be within 70–130% criterion.

2.8.6. Accuracy

Accuracy is a measure of closeness of agreement of test results obtained by the analytical method to an assigned value. Four control serum samples were used for determination of accuracy. These four serum samples were designed to cover range of IU/mL values and obtained by immunizing animal at three different dilutions of TdaP vaccine. 40 animals for each dilution (1/2.5. 1/5 and 1/10 of human dose of Tdap vaccine) were immunized on day 0 and sera was collected on day 35. Sera samples were pooled dilution wise and three control samples representing the three respective dilutions were used for accuracy studies. These sera samples were assigned unitages against international reference serum using a pharmacopeia method. 4 sera panel samples were run across six runs (3 runs by analyst 1 and 3 runs by analyst 2) and observations of concentrations were compared against assigned values and assay was considered accurate if the values were within 70–130% of assigned values.

2.9. Data analysis

Pearson coefficient analysis and linear regression were used to analyze relationship between data sets. For pearson coefficient analysis, statistical software (JASP version 0.9.0.1) was used. Variability analysis of precision and accuracy sets was done using % CV values which is calculated using Microsoft Excel functions. Geometric CVs were used to represent variability among animals in applicability studied. Geometric CV is calculated using ($GCV = (10^{s} - 1) \times 100\%$). S is the standard deviation of the $\log_{10}$ transformed potency estimates.

3. Results

3.1. Pentaplex MIA assay development and optimization

Five antigens i.e. diphtheria, tetanus, pertussis toxin, Filamentous hemagglutinin and pertactin antigen were coupled to respective beads using a reported procedure [15]. Preliminary experiments were performed for confirmation of bead to antigen ratio for all the five antigens. The reactivity in the negative control wells was monitored for each bead lot and signal-to-noise ratios were then determined for each coupling condition. The one that gave the best signal to noise ratio with the minimum amount of antigen was selected. Bead to antigen ratio of 5 μg of antigens per $6.25*10^{6}$ activated beads gave the best results which was further in agreement with findings of Prince et al. [21]. Different lots of antigens were evaluated in order to demonstrate the robustness and consistency of the coupling process. Purity (more than 95%) of antigens was found to be critical with respect to performance of the beads in the assay. The coupling method was further standardized with respect to vortexing and rpm speeds to minimize aggregation. It was observed that rpm speeds up to 100 were suitable for uniformity and critical to maintaining aggregation below 5%. Table 2 provides the overall performance of conjugation method with respect to recoveries. The effect of different dilution buffers and blocking agents were also studied. A minimum concentration of 0.2% BSA and tween 20 was found optimum for blocking for all the antigens. The assay protocol is guided by recommendations from Luminex Cook book [22] wherein the 4000 beads/antigen were incubated with the minimum volume of diluted serum (50 μl) for 60 min. NIBSC reference serum, which is

Table 2
Performance of bead coupling method. Bead counts were counted on hemocytometer pre and post coupling and percent recovery is reported. The percent recoveries are representative of 4 coupling reactions.

Sr.No	Antigen	Bead region no.	Initial Beads Taken	Bead count post coupling	% Recovery
1	Pertussis Toxin (PT)	35	3.75×10^6	3.52×10^6	95
2	Filamentous Hemagglutinin (FHA)	42	3.75×10^6	3.12×10^6	83
3	Pertactin (PRN)	14	3.75×10^6	3.30×10^6	88
4	Diphtheria Toxin (PT)	44	$3.75 \text{ X } 10^6$	$3.40 \text{ X } 10^6$	91
5	Tetanus Toxin (TT)	48	3.75×10^6	3.72×10^6	99

positive for all the five antigens, was used for evaluation of linearity. Excellent linearity was observed for all the five antigens in the reference serum (Fig. 1). Assay specificity was also established by studying possible interference among bead sets by comparing the reference standard curves generated from monoplex MIA versus the multiplex MIA in three different sets of experiments. Fig. 3 shows the correlation ($r > 0.99$) between monoplex versus multiplex measurements using NIBSC reference serum.

3.2. Assay specificity and selectivity

3.2.1. Assay specificity and selectivity was studied using two methods

Method 1: Specific panels of sera were designed to establish the antigen specificity on the beads (Table 1). A panel of four sera samples was used to demonstrate the specificity. The panel details are mentioned in Table 1. Panel 1A showed the positive reaction for PT, FHA and PRN coupled beads only and no reaction was observed at D and T bead regions. Sera B is generated by using Diphtheria toxoid and assay showed positive response for only D antigen, while a negative response was observed for all the other bead regions. Sera C is specific to D and T antigens and assay showed positive correlations wherein no response was detected for pertussis antigens and positive response was observed for D and T antigens. Sera D is negative serum control and assay showed near to baseline response for all the five antigens confirming the assay specificity and selectivity (Table 3).

Method 2: Homologous and heterologous inhibition experiment: Inhibition experiments were also performed in order to demonstrate selectivity and specificity. Homologous and heterologous binding of reference serum to beads were evaluated by pre-incubating 25 µg each of the antigens with the fixed dilution of serum (1:50). Homologous inhibition of more than 90% was observed for all the antigens, while heterologous inhibition was below 20%. Table 4 provides the results of the inhibition experiment.

3.3. Standard curve quantifiable range

The assay aims to determine antibody response to all the five antigens of Tdap vaccine in a single well. International reference serum NIBSC (97/642) was used for establishment of assay range. Each vial of mouse reference serum (NIBSC code; 97/642) contains 17 units of anti-PT, 143 unit of anti-FHA, and 30 units of anti-PRN. International reference serum is positive for T and D antibodies, a concentration of 100 U/mL was given for both the antigens due to non-availability of pre-assigned unitages. The standard curve was fitted using 4 PL fit. During the assay development, curve fitting was evaluated both using 4 PL and 5 PL logistic fits. Both the fitting models were found equally good to meet pre-determined criteria on curve residuals (back fits) and precision of replicates. Four parametric curve fitted back-calculated concentrations of the standard in the defined range met the acceptance criteria of mean accuracy within the range of 80–120% and imprecision less than 20%. LOQ of the assay was determined as the lowest calibration point for which the concentration can be back-calculated on the regression curve with 70–130% accuracy and a CV below 25%. The ULOQ is the upper calibration point that meets these criteria. The dynamic range thus extends from the LOQs to ULOQs. The standard curve ranges for all the five antigens are shown in Table 5 and Fig. 2.

Table 3
Method Selectivity. Specificity/selectivity of Pentaplex MIA: Panel sera samples representing antibodies positivity for combinations among PT, FHA, PRN, DT & TT antibodies. Values in tables are representative of Mean fluorescence intensity (N = 3) which was observed for target and non-target bead regions for the respective serum panel.

Panel Sera	PT	FHA	PRN	DT	TT
Panel 1A (PT, FHA PRN)	826	3942	329	129	89
Panel 1B (DT)	18	34	10	7249	11
Panel 1C (DT, TT)	15	42	7	5426	12,482
Panel 1D (Negative serum)	55	42	47	26	44

Table 4
Method specificity of pentaplex MIA: percentage inhibition on addition of homologous and heterologous inhibitor. Concentration used: 25 µg/ml for all the antigens.

Inhibitor/Beads	PT	FHA	PRN	DT	TT
PT	87	0	0	0	0
FHA	0	90	4	0	0
PRN	13	0	99	1	15
DT	0	1	5	99	0
TT	10	7	2	5	95

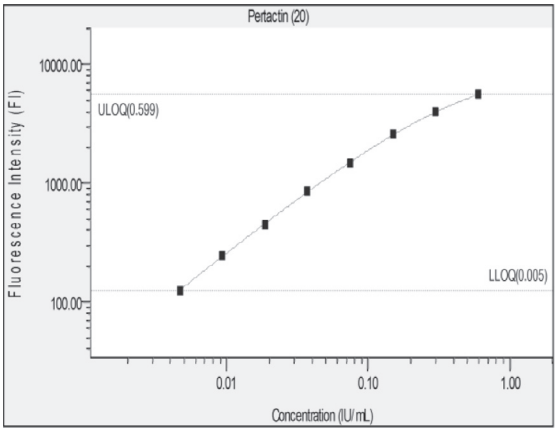
3.4. Assay reproducibility

Tables 6 and 7 summarizes the intra and inter-assay variability data. Mean % CV of intra-assay variations obtained from analysis of serum panel samples in independent runs by a single analyst and using a single bead lot ranged between 3 and 15% for all the analytes. The mean % CV for inter-assay variations (different day, different analyst and different bead lot) for all the different antigens ranged between 3 and 20%. The assay displayed good repeatability and intermediate precision for all the antigens.

Assay ruggedness was also studied with respect to assessing variations due to use of different lots of antigen coupled beads. Four different lots of beads were used for assessment for all the antigens. The lot to lot variability was studied using Pearson correlation analysis (Fig. 4). The

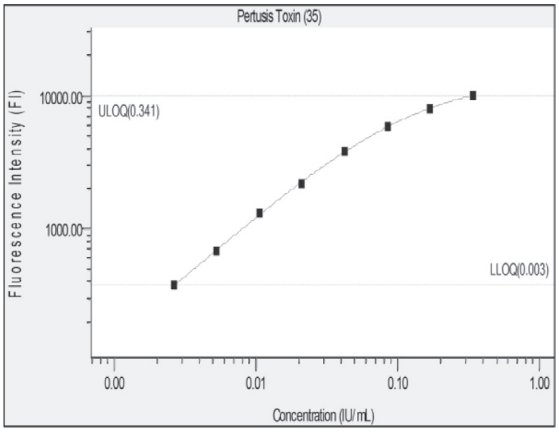
Table 5
Standard Curve ranges for all the analyte giving the limits of quantification. The curve fitting was evaluated with both 5 and 4 PL logistic curves. 4 PL logisitic curves were selected for further studies. Correlation coefficients observed for 4 and 5 PL fits are also presented.

Analyte/ Assay	Penta-Plex Assay		R^2 value obtained using 4PL curve fit	R^2 value obtained using 5PL curve fit
	IgG (ULOQ)	IgG (LLOQ)		
PT	0.34	0.003	0.995	0.995
FHA	2.86	0.022	0.997	0.998
PRN	0.6	0.005	0.999	0.999
DT	2	0.016	0.995	0.998
TT	2	0.0016	0.998	0.999



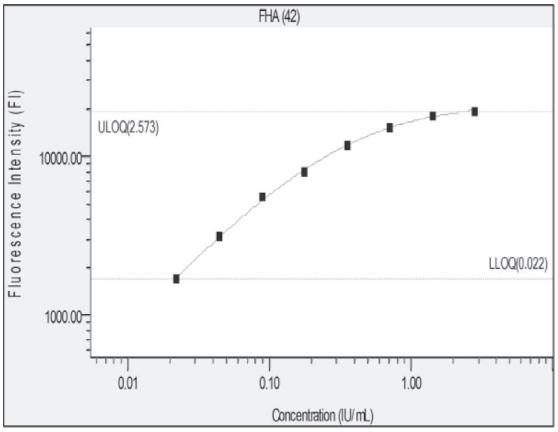
Regression type: Logistic - 4PL

Std Curve: -5.03916 + (10209.1 + 5.03916)
/ (1 + (Conc / 0.472973)^-0.938901)
FitProb. = 0.9560, ResVar. = 0.1653



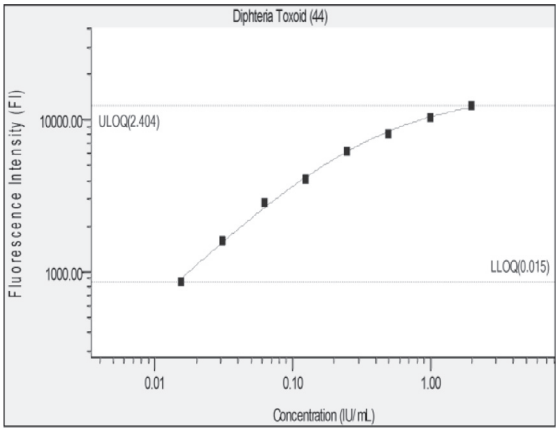
Regression Type: Logistic - 4PL

Std. Curve: FI = 21.1876 + (13701.2 - 21.1876)
/ (1 + (Conc / 0.113708)^-0.962804)
FitProb. = 0.8610, ResVar. = 0.3256



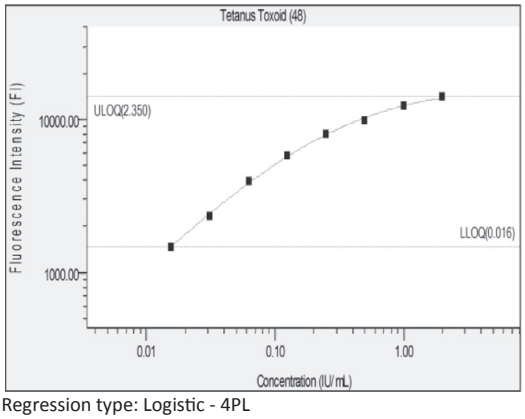
Regression type: Logistic - 4PL

Std Curve: -16.498 + (22254 + 16.498)
/ (1 + (Conc / 0.305051)^-0.936422)
FitProb. = 0.6486, ResVar. = 0.6195



Regression Type: Logistic - 4PL

Std. Curve: FI = -10.9987 + (15138 + 10.9987)
/ (1 + (Conc / 0.369441)^-0.865262)
FitProb. = 0.2532, ResVar. = 1.3376



Regression type: Logistic - 4PL

Std Curve: -12.4986 + (16850 + 12.4986)
/ (1 + (Conc / 0.279298)^-0.806481)
FitProb. = 0.6289, ResVar. = 0.6471

Fig. 2. Pentaplex assay reference curves for all the five antigens. Dashed lines represent lower and upper LOQs.

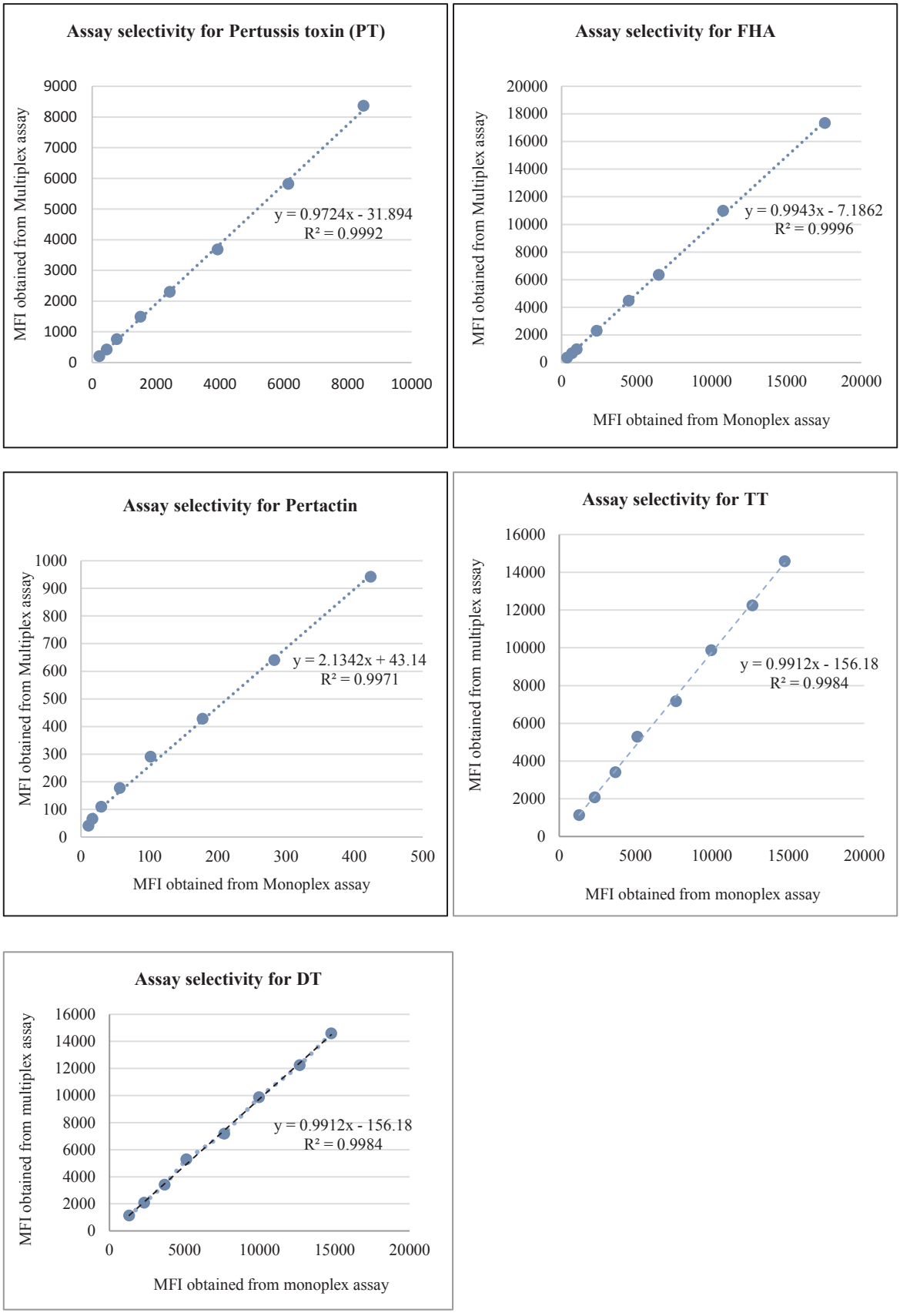


Fig. 3. Comparison of MFI signals generated from monoplex and pentaplex MIA with reference serum titration for Ptx, FHA, PRN in IU/ml and Dtx and Ttx in U/ml.

Table 6
Precision and accuracy estimates of the MIA assay for PT, FHA and PRN. Intra-assay (with same bead lot and analyst-1) and inter-assay variation (different day, different analyst and different bead lot). Accuracy represents concordance with assigned value in both inter and intra-assay formats.

Pertussis toxin		Precision		Accuracy	
Panel Sera used for study	Assigned potency in (IU/ml)	Intra assay variative (Mean % CV)	Inter assay variative (Mean % CV)	% Interassay accuracy (% agreement with assigned value)	% Intrassay Accuracy (% agreement with assigned value)
2A	37	10	17	117	85
2B	18	9	16	106	84
2C	8	6	13	104	88
2D	40	9	19	100	81
Filamentous hemaagglutinin (FHA)	**Assigned potency (IU/ml)**	**Intra assay variation (Mean % CV)**	**Inter assay variation (Mean % CV)**	**% Interassay accuracy**	**% Intrassay Accuracy**
2A	50	4	9	106	109
2B	26	4	17	106	103
2C	22	4	18	95	90
2D	159	1	0	99	83
Pertactin	**Assigned potency (IU/ml)**	**Intra assay variation** (Mean % CV)	**Inter assay variation** (Mean % CV)	**% Interassay accuracy**	**% Intrassay Accuracy**
2A	10	5	0	107	100
2B	5	3	3	96	104
2C	1	0	0	100	100
2D	33	3	11	105	86

results indicated that four bead lots were consistent and an excellent correlation coefficient was observed amongst all the bead lots for all the four antigens (Fig. 4) demonstrating the ruggedness of coupling method.

3.5. Assay accuracy

Tables 6 and 7 depicts the assay accuracy for all the antigens. The estimated values were compared with assigned concentrations and % accuracy was calculated for the sera panel. The % accuracy estimates were found within 80–130%, thereby meeting the criteria for concordance and accuracy.

3.6. Dilutional integrity

The effect of dilution was studied on high titre sera wherein dilutions ranging from 6.25 to 20,0000 to determine any bias. No significant bias was observed with studied dilutions and the estimate at each dilution was found within the acceptance criteria of 70–130% of expected value (Fig. 5).

3.7. Assay applicability

The applicability of the developed MIA in an mouse immunogenicity test was confirmed by confirming the dose response in Tdap immunized animals. Mouse Immunogenicity test involves the dose response analysis of reference and test vaccine. For routine batch analysis, a minimum of three dilutions of vaccine are chosen. Animals were immunized with three test dilution of Tdap vaccine and sera samples were analyzed on day 35 for IgG responses using pentaplex assay. The assay was able to predict the dose wise trend in the geometric mean concentrations of antibodies against all the antigens in the vaccine. Dose dependent responses for the antigens in the vaccine is presented in Fig. 6.

4. Discussion and conclusion

The objective of quality control testing of acelluar pertussis antigens is the laboratory evaluation of their immunological efficiency to protect against human disease. The mouse intracereberal challenge assay, which is accepted assay for whole cell pertussis vaccines have challenges in interpreting the potency of acellular pertussis antigens. There are reports from clinical trials which demonstrated that acellular

Table 7
Precision and accuracy estimates of the MIA assay for DT and TT: Intra-assay (with same bead lot and analyst-1) and inter-assay variation (different day, different analyst and different bead lot). Accuracy represents concordance with assigned value in both inter and intra-assay formats.

Diphtheria		Precision		Accuracy	
Panel Sera used for study	Assigned potency in (IU/ml)	Intra assay variation (Mean % CV)	Inter assay variation (Mean % CV)	% Interassay accuracy (% agreement with assigned value)	% Intrassay Accuracy (% agreement with assigned value)
2A	13	6	15	100	85
2B	3	13	20	130	70
2C	1	0	0	100	100
2D	44	15	5	80	105
Tetanus	**Assigned potency (IU/ml)**	**Intra assay variation** (Mean % CV)	**Inter assay variation** (Mean % CV)	**% Interassay accuracy**	**% Intrassay Accuracy**
2A	99	6	9	100	103
2B	56	6	11	95	90
2C	24	3	14	103	101
2D	244	13	10	82	100

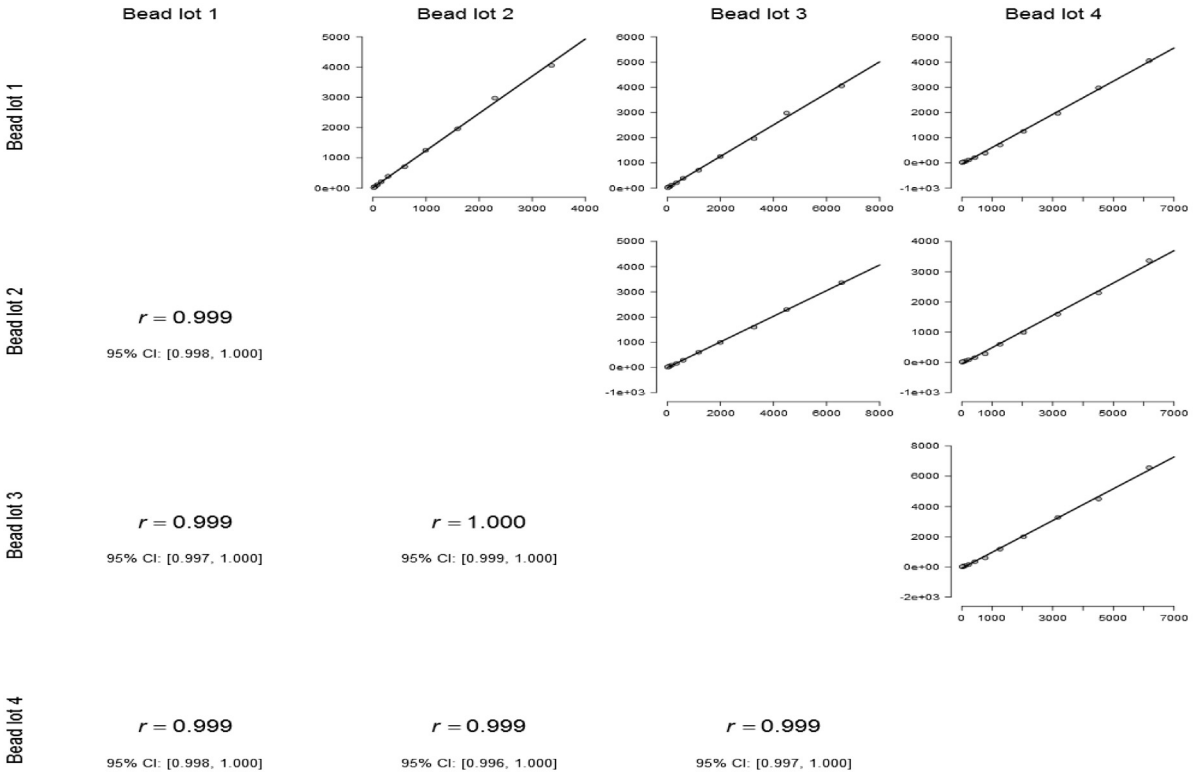


Fig. 4. Reproducibility of bead coupling methods. Four different bead lots were studied for comparability and correlation analysis using Pearson correlation analysis. Excellent correlation was observed for all the four bead lots. Data is representative of Ptx. Similar results were observed for all the antigens. Data of FHA, Prn, DT and TT antigens are presented in supplementary information.

pertussis vaccines may have significant clinical efficacy without acceptable activity in the mouse intracereberal challenge test [23]. WHO and European Pharmacopeia recommend an immunogenicity test in mice, designed to demonstrate consistency in the vaccine by measuring total IgG responses against all the antigens in the vaccine claimed to contribute to vaccine efficacy. An international mouse serum containing antibodies to five antigens is available to determine IgG concentrations. An immunogenicity test consists of two stages; induction of antibodies with a preselected-test dose of vaccine followed by measurement of induced antibody response. Immunogenicity test being the critical release assays needs well characterized and validated serological assay to demonstrate lot to lot consistency against a clinically proven batch. We report here development and validation of a magnetic microsphere based multiplex serological immunoassay for simultaneous determination of mouse serum antibodies against B. *pertussis* antigens (PT, FHA and PRN), diphtheria and tetanus antigens. Several laboratories have reported multiplex immunoassay to measure IgG antibodies against pertussis, D and T antigens in human samples [24]. Very few reports on development and validation of MIA for use in the mouse immunogenicity test are available. Stingers et al. [17] reported a mouse hexaplex assay for simultaneous estimation of IgG isotypes and avidity against diphtheria, tetanus, pertussis toxin, pertactin, FHA and Fim 2/3 antigens. The assay utilizes a 96 well format and direct binding format to that followed in conventional ELISA, thus allows easy bridging.

The most critical component of the assay is antigen coupled beads and the resulting assay specificity and selectivity. The surface of Luminex microspheres contains 100 million carboxy groups on the beads which facilitate covalent attachment to the proteins during a coupling reaction with the aid of EDC and sulfo- NHS. The protein antigens are thus covalently linked through the amide bond with the activated beads. Assay makes use of magnetic beads, thus allowing excellent recoveries and specificity to the assay. Table 2 reports the conjugation efficiency results using the assay optimized conditions. This is further in accordance with the previously published reports on higher MFI signals and excellent recoveries with magnetic beads compared to non-magnetic beads [25]. Robustness of coupling method was also demonstrated by evaluating multiple bead lots. Excellent lot to lot consistency was observed for all the bead lots as evaluated by correlation analysis (Fig. 3).

The assay is quantitative in function and designed for simultaneous estimation of antibodies to all the five antigens. NIBSC international reference standard was used as the reference standard as it contains antibodies to all the five antigens. Using dilution curves of international reference standard, values were assigned to serum panels used in the validation. The analytical range of the assay consisted of 8 fold dilution curve using NIBSC reference sera and fitted using 4 PL parametric logistic covered 0.34–0.003 IU/mL for PT, 2.86–0.022 IU/mL for FHA and 0.60–0.005 IU for pertactin. For D and T antigens, analytical range was established in the concentration of 2–0.016 U/mL. The analytical ranges for all the antigen were found suitable for detection of prevaccinated or placebo group vaccination titres (Table 5). Analytical specificity or selectivity in multiplex assays is demonstrated by assessing the possible cross reaction of antibodies to non-target beads. Specificity was demonstrated by three approaches in the study. In the first approach performance in monoplex versus multiplex assays were compared for all the five antigens. Results showed the excellent correlation analysis suggestion no cross reactivity among the coupled beads (Fig. 2). In the second approach, panel of sera samples, which is positive for antibodies to one to two antigens out of five antigens were used. Table 3 indicates the assay selectivity wherein the assay was able to identify the panel sera samples with good accuracy. In the third approach, specificity was demonstrated by homologous and

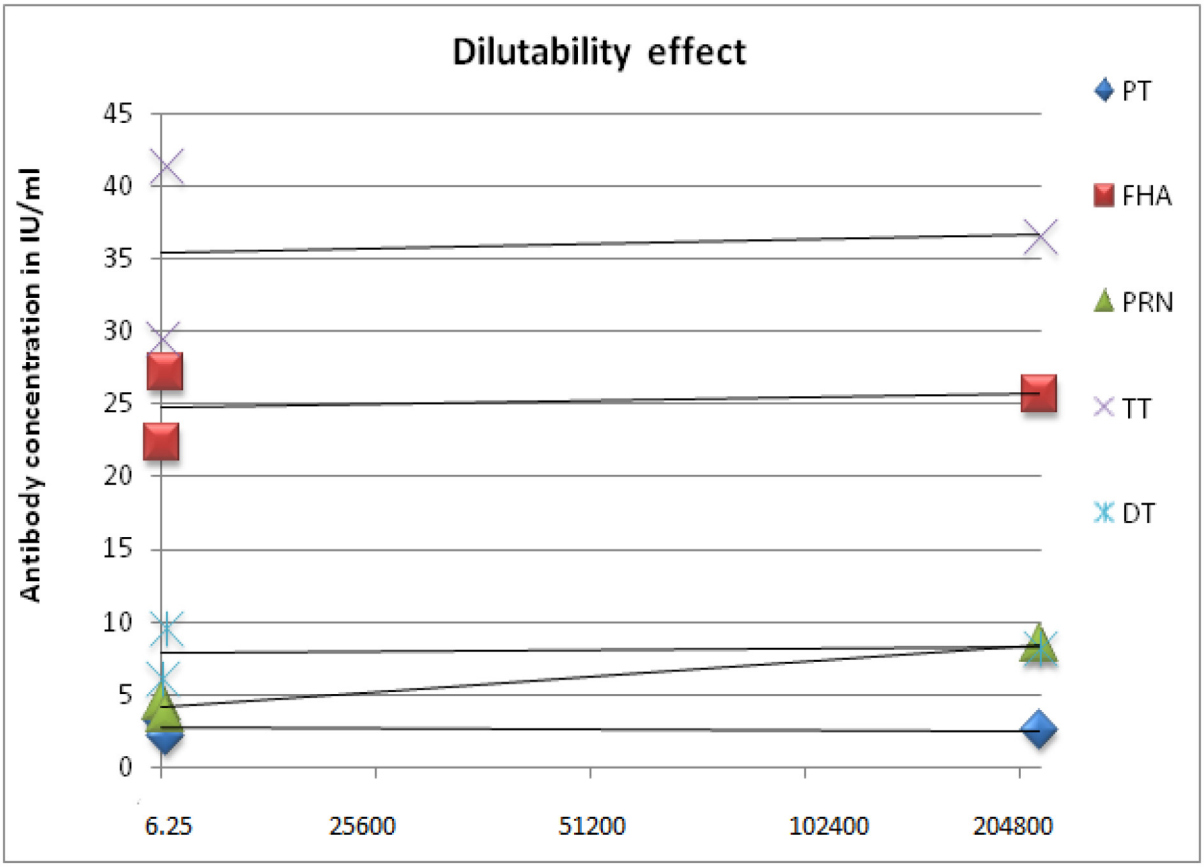


Fig. 5. Dilution integrity assessment. Positive control serum with assigned value was used for the assessment. Graph is representative of assessment for all the five antigens. The dilution wise estimate of concentrations was within ± 30% criteria.

heterologous inhibition method (Table 4). Excellent specificity can be attributed to the quality of the antigens used in the coupling and magnetic beads in the assay. Chemically well defined and purified antigen batch for each analyte is necessary for a an optimum coupling process. There are reports which have shown that use of the aceullar pertussis purified toxins significantly improves the assay performance and concordance with the values obtained by conventional ELISA methods. The study made use of purified toxins (PT, FHA and PRN) and toxoids (D and T) for the coupling process. Commercial availability of purified D and T toxins is always a challenge and have batch to batch variability. As the intended objective was to evaluate the immune responses against vaccines which use toxoids as antigens, the study focused on validating the assay with use of D and T toxoids as capture antigens. Specificity results clearly suggest that results were comparable to performance reported in published results of similar multiplex assays which used purified toxins of D and T for the coupling [18]. The validation studies also assessed the precision and analytical accuracy of the assay. Inter-assay and intra-assay variability was found to be below 20% and excellent concordance was observed between assigned and predicted values.

Towards applicability and implementation to mouse immunogenicity test, the assay was applied to analysis of sera samples obtained from animals which were immunized with different dilutions of Tdap vaccine. The assay was able to predict the dose dependent trend in IgG responses against all the five antigens, which will be important to define the potency and quality of vaccine against a reference vaccine

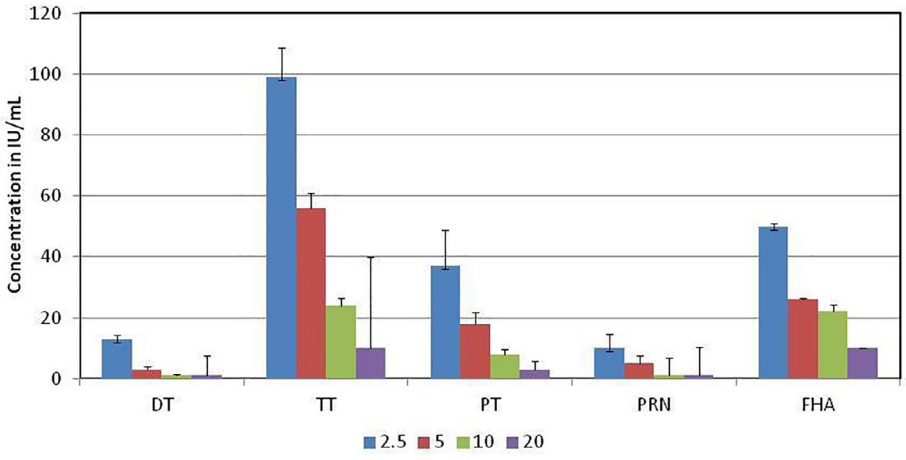


Fig. 6. Mouse Immunogenicity Test. Effect of immunizing Tdap vaccine dose on mice antibody responses against DT, TT, PT, PRN and FHA as analyzed by pentaplex MIA. Animals (N = 10/dilution) were immunized with different dilutions of Tdap vaccine (1/2.5, 1/5. 1/10 and 1/20 dilution of human dose) on day 0 and sera was collected on day 35. Geometric mean concentrations (IU/ml) were calculated using pentaplex MIA. The error bars are representative of respective group standard deviations.

batch of proven clinical efficacy (Fig. 5). The multiplex immunoassay described here thus meets the necessary criteria for implementation as quality control tool for Tdap or DTaP containing vaccine. The assay is able to simultaneously quantify antibodies to all the five antigens using the minimum serum volumes and very small amounts of antigens of the five antigen as compared to conventional ELISA platforms. The multiplexed measurements further reduced the time and labor to assay large number of sera samples. For example, 100 samples can be assayed for 5 antigens on five different plates in a single day. This will be a significant cost and time savings in quality control laboratory setting where multiple vaccine batches are manufactured and each batch needs to be analyzed for its immunogenicity potential.

In conclusion, the assay described here meets all the requirements of specificity, selectivity and reproducibility and accuracy and is thus a viable alternative to conventional ELISA for detection of DTaP3 antibodies in mouse serum samples. The robustness and assay format also offers opportunities to include additional antigens of DTaP combinations such as Hib and Hepatitis B or other pertussis antigens such as adenylate cyclase and Fim 2/3. The study reports development, qualification and validation of multiplex assay. For further use in the quality control testing of vaccines, detailed characterization and validation studies are required

Declaration of interests

None.

Acknowledgement

Authors thanks Serum Institute of India Ltd for all the funding support. All the authors are employees of Serum Institute of India Pvt Ltd and declare no conflict of interest. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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