



Research paper

Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to *Bordetella pertussis*, diphtheria and tetanus

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ARTICLE INFO

Article history:

Received 14 December 2007

Received in revised form 27 February 2008

Accepted 28 February 2008

Available online 26 March 2008

Keywords:

Multiplex immunoassay

ELISA

Bordetella pertussis

Diphtheria

Tetanus

Luminex

ABSTRACT

To increase testing of vaccine induced humoral immunity in immune surveillance studies and vaccine trials, a rapid and simple microsphere-based multiplex assay (pentaplex) was developed for the quantitation of IgG serum antibodies directed against the *Bordetella pertussis* antigens: Pertussis Toxin (Ptx), Filamentous hemagglutinin (FHA), Pertactin (Prn) and to Diphtheria toxin and Tetanus toxin. All individual antigens were covalently linked to carboxylated microspheres. The method was validated with different serum panels ($n=60-78$ samples). With the Multiplex Immunoassay (MIA) no evidence for bead interference between monoplex and pentaplex was found. The specificity of the method was shown by a heterologous inhibition of $<16\%$ and homologous inhibition of $>92\%$. The pentaplex MIA appeared sensitive with lower limits of quantitation (LLOQ) well below those for ELISA (enzyme-linked immuno-sorbant assay). Assay reproducibility was high with intra-assay variability less than 10% and inter-assay variability below 14%. The reproducibility of the bead conjugation was good and beads could be stored up to at least 6 months without quality reduction. Importantly, the correlation of the pentaplex MIA with the individual ELISAs was excellent, $R>0.98$ for the Pertussis antigens and $R=0.95$ for Diphtheria and $R=0.98$ for Tetanus.

Serum IgG antibodies to *B. pertussis*, Diphtheria and Tetanus can be measured easily, specific and reproducible using the pentaplex MIA. The pentaplex MIA shares features of the ELISA with the additional advantages of high sample throughput and small sample volumes and antigen required.

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1. Introduction

The National Immunization Program (NIP) in the Netherlands contains a number of vaccine components and has recently been expanded with the introduction of meningococcal C, 7-valent pneumococcal and 3(-5)-valent acellular pertussis vaccines. To reduce the required number of injections, various combination of vaccines are utilized. One of the most commonly used combo vaccines worldwide for acellular pertussis is DTaP3 which consists of Diphtheria and Tetanus toxoid in combination with the 3 *Bordetella pertussis* com-

ponents Pertussis toxin (Ptx), Filamentous hemagglutinin (FHA) and Pertactin (Prn). In order to assess the immunogenicity of combination vaccines, the immune response to the individual vaccine components must be examined. Not only the antibody response of new candidate vaccines has to be tested but also the response of concomitant vaccines for possible interference.

In the Netherlands the NIP is monitored and evaluated by serological surveillance of the immune status of the population regarding those vaccines included in the program. Following a successful first national survey in 1995/1996 a second survey has started to investigate immune status of the Dutch population (0–79 years) against the vaccines in the NIP and over 8000 serum samples are collected. The conventional enzyme-linked immuno-sorbant assay (ELISA) (Edwards et al., 1995; Meade et al., 1995; von Hunolstein et al., 2000) is a

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widely applied and accepted vaccine antibody detection method. The ELISA is reproducible and specific, but also time consuming when multiple analytes need testing in trials consisting of large numbers of samples. Each different component of the vaccine requires separate assays. Furthermore due to the limited quantity of serum especially from infants and the inclusion of new vaccine components the ELISA technique turns out to be less suitable for large scale immunosurveillance and vaccine trials. The Luminex technology using fluorescent distinct microspheres as a carrier for different antigens enables the detection of multiple analytes in one single serum sample. Several studies have demonstrated the ability to effectively multiplex a range of assays including antibody detection and quantification of vaccination samples (Pickering et al., 2002a,b; Lal et al., 2004; Lal et al., 2005; Prince et al., 2006; Pickering et al., 2007). Without losing the advantages of the conventional ELISA, multiplexing allows a higher sample throughput linked to a strongly reduced sample volume.

Aim of this study was to develop a rapid, reproducible, less time consuming and less serum demanding assay, as compared to ELISA, for determination of serum antibodies against *B. pertussis* (Ptx, FHA and Prn), Diphtheria and Tetanus toxin. In this study the development and validation of a pentaplex microsphere based fluorescent immunoassay (MIA), in which total IgG responses are determined towards the DTaP3 combo vaccine, is described.

2. Materials and methods

2.1. Reagents

Pertussis toxin (Ptx) was obtained from the Netherlands Vaccine Institute, Filamentous hemagglutinin (FHA) was obtained from Kaketsuken (Kumamoto, Japan), P.69 pertactin (Prn) was expressed and purified from an *E. coli* construct as previously described (Hijnen et al., 2005), Diphtheria toxin from *Corynebacterium diphtheriae* (Dtx) and Tetanus toxin from *Clostridium tetani* (Ttx) were obtained from Sigma-Aldrich (St. Louis, MO). R-phycoerythrin (R-PE)-conjugated goat anti-human IgG (gamma chain specific) was obtained from Jackson ImmunoResearch Laboratories Inc. (Westgrove, Pa). Carboxylated microspheres were obtained from Bio-Rad (Bio-Rad Laboratories, Hercules, CA). *N*-hydroxy-sulfosuccinimide (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were purchased from Pierce (Rockford, IL). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (St. Louis, MO). Tween-20 was purchased from Merck (Darmstadt, Germany).

2.2. Reference and control sera

The in-house Pertussis Luminex standard serum was composed of a pool of 28 diagnostic sera submitted to the RIVM for suspected whooping cough for which the diagnosis was confirmed with high IgG Ptx titers (de Melker et al., 2000; Giammanco et al., 2003). Serum values were assigned in EU/ml in both ELISA (Edwards et al., 1995; Meade et al., 1995) and multiplex immunoassay (MIA) for all three individual Pertussis antigens. The in-house references used in the ELISA were calibrated against the U.S. Reference Pertussis Anti-serum Human lot 3 (Ptx IgG and FHA IgG) and lot 4 (Prn IgG) (CBER/

FDA). The in-house Diphtheria-Tetanus (DT) Luminex standard was a control serum obtained from a DTP vaccinated adult. Serum values were assigned in IU/ml in the Toxin Binding Inhibition assay (ToBI) (Hendriksen et al., 1989; von Hunolstein et al., 2000) for both individual toxins. In-house references used in the ToBI were calibrated against the International standards (NIBSC code Di-03 and NIBSC code TE-03). In-house control sera consisted of high anti-Pertussis titrated diagnostic sera, DT diagnostic sera and sera obtained from staff at the Laboratory for Infectious Diseases and Perinatal Screening after routine antibody screening.

2.3. Specimens (of samples)

Panels of 27–78 serum samples (of specimens) were composed for the development and validation of the MIA. These panels included sera from vaccine trials (ISRCTN61378987 and ISRCTN97785537) and routine diagnostic serum samples sent to the RIVM for DTaP antibody screening.

2.4. Conjugation of DTaP3 antigens to carboxylated microspheres

Purified Ptx, FHA, Prn, Dtx and Ttx were coupled to activated carboxylated microspheres by using a two-step carbodiimide reaction (Staros et al., 1986). Bead regions were carefully chosen being not directly adjacent to each other: 60 (Ptx), 11 (FHA), 2 (Prn), 45 (Dtx) and 28 (Ttx). A solution of 500 μ l of carboxylated microspheres (12.5×10^6 beads/ml) was activated by addition to 500 μ l phosphate buffered saline (PBS) containing 5 mg of EDC and 5 mg of NHS/ml (pH=6.1). The solution was incubated for 20 min at room temperature (RT), in the dark under constant rotation at 25 rpm. The activated microspheres were washed 2 times with PBS pH 7.2 by centrifugation (12,000 $\times$ g for 4 min) and resuspended in 250 μ l PBS with an antigen to bead ratio of 5 μ g/6.25 $\times 10^6$ activated beads for Ptx, FHA and Prn and 25 μ g/6.25 $\times 10^6$ activated beads for Dtx and Ttx. The beads were incubated for 2 h at RT in the dark under constant rotation at 25 rpm. Subsequently, the beads were washed three times with PBS pH 7.2 and stored in 500 μ l PBS containing 0.05% (w/v) sodium azide and 1% (w/v) BSA at 4 °C in the dark until used (Pickering et al., 2002b; Lal et al., 2004; Prince et al., 2006). The Bio-Rad Amine coupling kit (Bio-Rad Laboratories, Hercules, CA) was used according to the manufacturer's instructions.

2.5. Multiplex immunoassay (MIA) for quantitation of Ptx, FHA, Prn, Dtx and Ttx specific antibodies

Six steps of 4-fold dilutions (1/200 to 1/204,800) of Pertussis in-house reference serum and eight steps of 4-fold dilutions (1/50 to 1/819,200) of Diphtheria-Tetanus in-house reference serum were prepared in PBS pH 7.2 containing 0.1% (v/v) Tween-20 and 3% (w/v) BSA. Sera from the comprised panels and in-house control samples were diluted 1/200 and 1/4000 in PBS containing 0.1% Tween-20 and 3% BSA. Each dilution of the standard and serum samples (25 μ l) was mixed with an equal volume of up to five sets (monoplex, duplex, triplex or pentaplex) of conjugated microspheres (4000 beads/region/well) in a 96-well Multiscreen HTS filter plate (Millipore Corporation, Billerica, MA) and incubated for

45 min at RT in the dark on a plate shaker at 600 rpm. Standard, blank and control were included on every plate. The beads were collected by filtration using a vacuum manifold and washed three times with 100 μ l PBS. To each well 50 μ l of a 1/200 dilution of R-PE conjugated anti-human IgG in PBS was added and the plate was incubated for 30 min with continued shaking. After a second wash, the beads were re-suspended in 100 μ l PBS and shaken before analysis with a Bio-Plex 200 in combination with Bio-Plex Manager software version 4.1.1 (Bio-Rad Laboratories, Hercules, CA). For each analyte, median fluorescent intensity (MFI) was converted to EU/ml or IU/ml by interpolation from a 5-parameter logistic standard curve (log–log) for every bead region/standard. The MIA results were determined from the mean of the two sample dilutions if their MFI signals were on the linear part of the standard curve.

2.6. Assay specificity

A serum with known high concentrations of IgG antibodies directed against all five antigens (Ptx, FHA, Prn, Dtx and Ttx) was diluted 1/2000. Five aliquots of each serum dilution were prepared, and each aliquot was incubated with an equal volume of one of the five purified antigens in a concentration of 5 μ g/ml for 1 h at RT. A 1/4000 dilution of the serum was used as a control (100%). The serum aliquots were assayed as described above in a MIA (pentaplex). Homologous and heterologous inhibition of the MFI compared to the control was calculated as percentage.

2.7. Assay sensitivity

For determination of the assay sensitivity the pertussis (MIA) and Dtx–Ttx (in-house) reference serum was diluted in twelve 4-fold dilution steps, 1/50 to 1/209,715,200 for the pertussis reference and 1/12.5 to 1/52,428,800 for the Dtx–Ttx reference, respectively. From individual blank wells ($n=64$) the average MFI, standard deviation (SD) and $2+SD$ were calculated for each analyte. The lower limit of detection (LLOD) of each analyte was determined by interpolation of the $2+SD$ in the 5-PL from the relevant reference curve and represented as concentration (in EU/ml or IU/ml). From the LLOD the lower limit of quantitation (LLOQ) was calculated as $3 \times LLOD$ (EMEA, 1995).

2.8. Assay reproducibility

Reproducibility of the MIA was assessed by determination of the level of both intra-assay and inter-assay variation. Intra-assay variation within a plate was determined by assaying an independent triplicate of different samples. For each analyte the coefficient of variation (%CV) for each triplicate result per sample was calculated and averaged. Intra-assay variation (plate to plate variation) was further determined by assaying a serum panel ($n=60$) in dependent dilutions between different plates on the same day. The %CV between each of these results for each analyte was calculated and averaged. Inter-assay variation (variation from assay to assay) was assessed by testing a serum panel ($n=60$) in two separate assay runs. The % CV between each of these results for each analyte was calculated and averaged.

The reproducibility of the results generated from separate batches of conjugated microspheres was determined with considerable time intervals. A serum panel ($n=61$) was tested with different batches of conjugated microspheres. The results obtained with the different batches were compared by calculating the correlation coefficient (R).

2.9. ELISA for the quantitation of Pertussis antibodies directed against Ptx, FHA and Prn

The ELISA was performed essentially according to the procedure previously described by Edwards and Meade (Edwards et al., 1995; Meade et al., 1995). Purified Ptx, Prn and FHA used for coating were identical to the fractions used for the MIA. High binding 96-well microtiter plates (Greiner Bio One, Germany) were coated with 1.5 μ g/ml Ptx and 2.5 μ g/ml Prn in 0.04 mol/l carbonate buffer pH 9.6 and 0.4 μ g/ml FHA in PBS pH 7.2. The plates were incubated overnight at 22 °C and successively washed 4 times with 320 μ l PBS containing 0.05% (v/v) Tween-20. For each serum eight steps of 2-fold dilutions (1/60 to 1/7680) in PBS pH 7.2 containing 0.05% (v/v) Tween-20 and 0.5% (w/v) BSA were tested. On the same plate an in-house reference serum (per analyte) and control serum were included and tested in eight steps 2-fold dilutions optimized per antigen in the same buffer. Plates were incubated for 2 h at 28 °C. After a second wash step the plates were incubated with alkaline phosphatase-conjugated goat anti-human IgG (Sigma, St. Louis, US) in a dilution of 1/10,000 in PBS pH 7.2 containing 0.05% (v/v) Tween-20 and 0.5% (w/v) BSA for 2 h at 37 °C. After the final wash step the assay was developed with *p*-nitrophenyl phosphate (pNPP) substrate (1 mg/ml solution) and optical density was read at 405 nm with a Biotek powerwave plate reader (Biotek systems, Winooski, VT). Data acquisition was performed using Gen5 secure (Biotek systems, Winooski, VT). For each analyte, optical density at

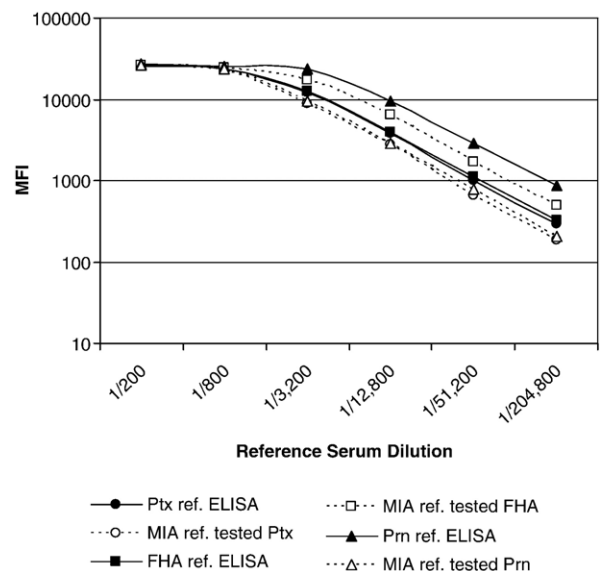


Fig. 1. MFI generated from 4-fold serum titration of the in-house (ELISA) reference sera for Ptx, FHA and Prn and one MIA reference serum for Ptx, FHA and Prn combined.

OD405 was converted to EU/ml by interpolation from a 4-parameter logistic standard curve of the in-house reference sera for Ptx, FHA and Prn. The LLOQ for both Ptx and FHA was determined 2 EU/ml and for Prn 4 EU/ml. For statistical analysis titers below the LLOQ were assigned half the LLOQ (respectively 1 EU/ml for Ptx and FHA and 2 EU/ml for Prn) (Berbers et al., 1999).

2.10. Toxin Binding Inhibition assay (ToBI) for the quantitation of Diphtheria and Tetanus anti-toxin antibodies

Diphtheria and Tetanus anti-toxin antibodies were measured with the Toxin Binding Inhibition assay (ToBI) as described earlier (Hendriksen et al., 1989). Low affinity binding 96-well microtiter plates (Greiner Bio One, Germany) were blocked with 300 μ l PBS containing 0.5% (w/v) BSA for 1 h at

37 °C. After the blockbuffer was discarded the plates were filled with 100 μ l PBS pH 7.2. Subsequently, twelve steps of 2-fold dilutions (1/2 to 1/4096) of the mixed Diphtheria and Tetanus in-house reference serum (prediluted), in-house control serum (prediluted) and serum samples were made in the plate. The serum dilutions were preincubated with 140 μ l of a fixed amount of toxin (a mix of both diphtheria and tetanus toxin) overnight at 22 °C. A volume of 100 μ l of these serum dilution series was transferred to high binding 96-well microtiter plates (Greiner Bio One, Germany) coated with 10 μ g/ml anti-Dtx monoclonal Dim 9 (Metz, 2005) or polyclonal horse anti-Ttx serum. The plates were incubated 2 h at 37 °C and successively washed 4 times with 320 μ l PBS containing 0.05% (v/v) Tween-20. The remaining free toxin was determined with toxin specific (Dtx and Ttx) conjugated polyclonal horse anti-serum diluted in PBS containing 0.1% (v/v)

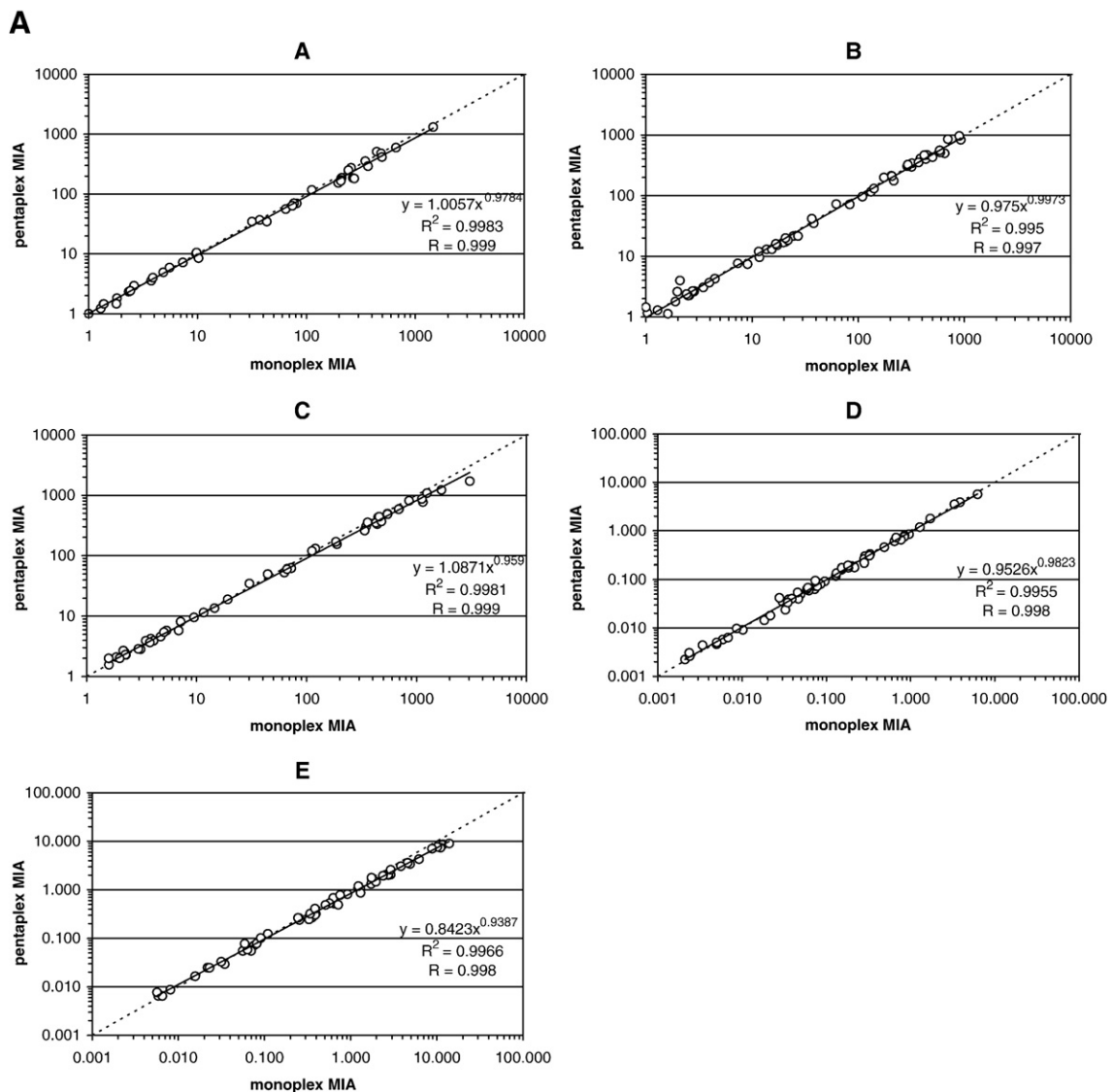


Fig. 2. (A) Comparison of IgG concentrations obtained from the monoplex MIA and pentaplex MIA with a broad range serum panel ($n=60$): (A) Ptx, (B) FHA and (C) Prn in EU/ml and (D) Dtx and (E) Ttx in IU/ml. (B) Comparison of MFI signals generated from the monoplex and pentaplex MIA with the reference serum titration for (A) Ptx, (B) FHA and (C) Prn in mEU/ml and (D) Dtx and (E) Ttx in μ IU/ml.

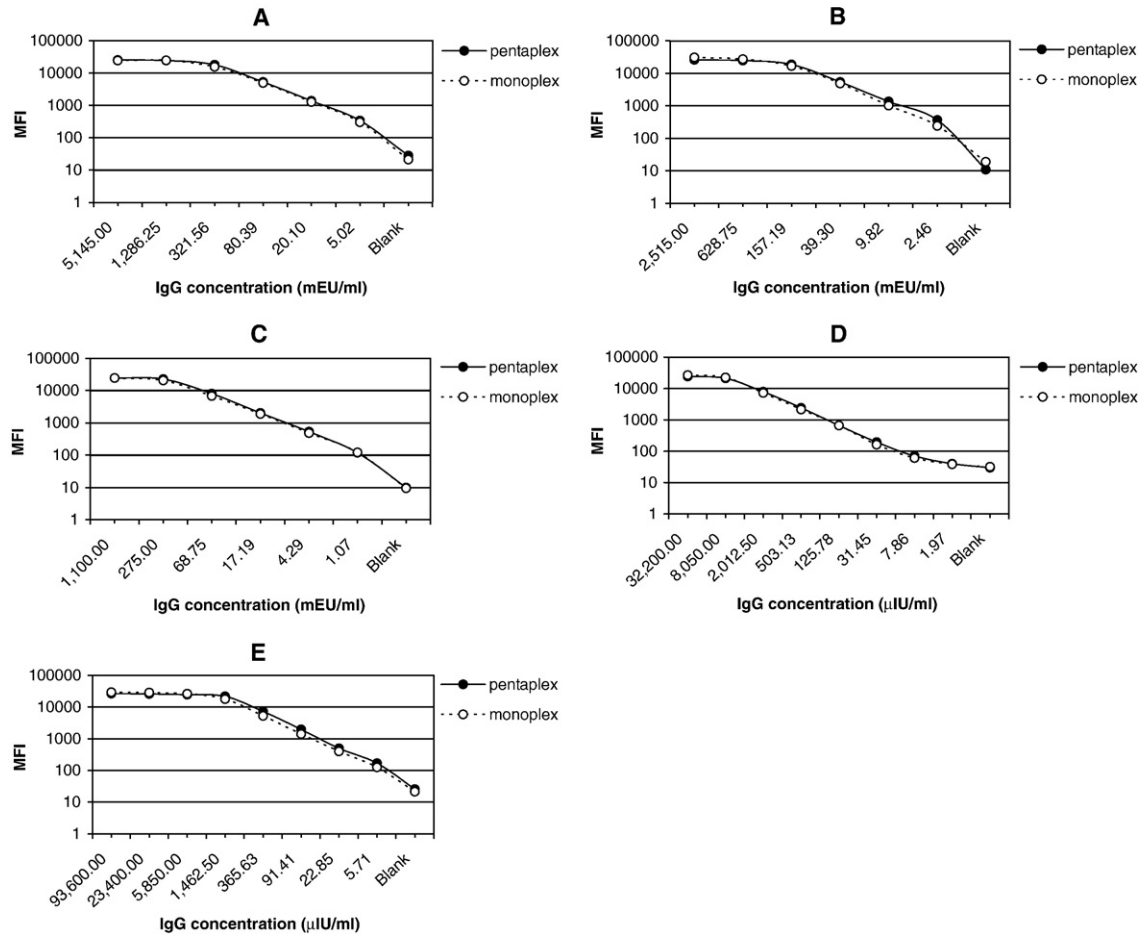
B

Fig. 2 (continued).

v) Tween-80 and 0.5% (w/v) BSA in a 2 h incubation at 37 °C. After the washstep the assay was developed with TMB substrate and optical density was read at 450 nm. For each analyte, optical density at OD450 was converted to IU/ml by interpolation from a 4-parameter logistic standard curve of the in-house reference sera for diphtheria and tetanus. The LLOQ for both analytes was 0.01 IU/ml. For statistical analysis titers below the LLOQ were assigned half the LLOQ (0.005 IU/ml) (Berbers et al., 1999; von Hunolstein et al., 2000).

3. Results

3.1. Development of a pentaplex microsphere immunoassay (MIA) for quantification of Ptx, FHA, Prn, Dtx and Ttx specific antibodies

The pentaplex MIA was a combination from the parallel development of a triplex MIA for Ptx, FHA and Prn of *B. pertussis* and a duplex MIA for Diphtheria and Tetanus toxin. Initial experiments with the pertussis antigens mono- and triplex MIA showed an optimal conjugation ratio of 5 μ g antigen per 6.25×10^6 activated beads which was in accordance with previously reported results (Prince et al., 2006).

MIA results did not improve with a twice as high amount of antigen. By optimizing of the DT duplex and pentaplex the best results were obtained with a 25 μ g antigen to 6.25×10^6 activated beads ratio, thus improving the MIA results as well as the correlation with the ToBI. For the triplex pertussis MIA the bead conjugation time was investigated. Regardless an elevated incubation time from 2 to 5 h, no differences in MFI signals generated for the individual reference serum curves were found. Furthermore the results of a serumpanel ($n=60$) assayed with both 2 and 5 h incubated beads showed excellent correlation coefficients (R), 0.990 (Ptx), 0.998 (FHA) and 0.997 (Prn), respectively. The influence of different Ptx antigens for conjugation was investigated with material obtained from the Netherlands Vaccine Institute and from Kaketsuken (Kumamoto, Japan) which were similar on SDS-PAGE. A good correlation was found between titers generated with the monoplex MIA of both conjugated bead sets in two independent experiments, $R=0.973$ ($n=32$) and $R=0.975$ ($n=27$), respectively. For the DT duplex we found that the purity of the antigen for conjugation was critical. In contrast to the use of in-house purified toxins, the use of commercially available toxins from Sigma Chemical Company showed excellent results in the MIA. Bead aggregation of the pentaplex MIA was low, generally

Table 1

Specificity of the pentaplex MIA: percentage inhibition on addition of heterologous and homologous inhibitor in a concentration of 2.5 µg/ml

Inhibitor	Ptx	FHA	Prn	Dtx	Ttx
Ptx (2.5 µg/ml)	93.6	5.8	5.0	15.7	3.5
FHA (2.5 µg/ml)	10.6	88.5	5.9	5.6	5.9
Prn (2.5 µg/ml)	4.7	5.6	99.3	1.0	0
Dtx (2.5 µg/ml)	6.0	3.8	4.2	97.6	4.6
Ttx (2.5 µg/ml)	0	2.0	5.6	5.7	85.8

between 3 and 6% and never exceeded 10%. With respect to the DT coupling procedure we compared our conjugation method with the Biorad Amine coupling kit and no indication was found that this commercial kit improved coupling results.

A variety of serum dilution buffers was used to investigate the influence on the results of the MIA: ADHS (Antibody Depleted Human Serum (Valley Biomedicals, Winchester, VA)), PBS, PBS–0.1% (v/v) Tween 20, PBS–0.05% (v/v) Tween 20–1% (w/v) BSA, PBS–0.05% (v/v) Tween 20–2% (w/v) BSA and PBS–0.1% (v/v) Tween 20–3% (w/v) BSA. Best results were obtained with serum dilution buffer PBS–0.1% (v/v) Tween 20–3% (w/v) BSA. Elevated concentrations of Tween-20 and BSA clearly improved the results especially in the low concentration area of the MIA. The effect was substantial for toxin antigens, Ptx, Dtx and Ttx. A considerable decrease in the ELISA correlation and MFI signals was obtained using ADHS, especially for the toxin antigens.

Sample dilutions were optimized to cover a broad concentration range for all five antigen bead sets in the pentaplex. The MIA should cover a concentration range which includes antibody titers of pre-vaccination, post-vaccination and natural infection. With a minimum of two single dilutions (1/200 and 1/4000) over 99% of the pertussis antibody titers could be covered with high reproducibility. The CV for mean sample results generated with the 1/200 and 1/4000 dilution was <20% for all analytes. This is comparable to the %CV obtained for ELISA and ToBI with their 2-fold serial dilutions over 8 or 12 wells and meets the criteria for determination of parallelism and non-parallelism in bioassay dilution curves (Plikaytis et al., 1994). With respect to the DT titers a 1/16,000 dilution especially for tetanus titers might only occasionally be necessary.

A great advantage of the MIA is the possibility to determine multiple antigens in only one single well. To quantitate these results a standard reference serum is needed for the different antigens of interest, but a reference serum covering all antigens is preferable. Linearity was demonstrated between the MIA reference serum and the individual in-house reference sera for Ptx, FHA and Prn over the seven 4-fold dilution range in a triplex MIA, as illustrated in Fig. 1. The possible interference between the different bead sets was investigated by comparing IgG concentrations generated from the monoplex MIA with the pentaplex MIA. Fig. 2A shows the correlation for all five bead sets determined individually in a monoplex and pentaplex MIA with a broad range serum panel ($n=60$). For all bead set combinations the correlation coefficient was very high ($R>0.996$). The MFIs generated from the 4-fold diluted MIA reference sera in the monoplex and pentaplex MIA for all combinations of bead sets revealed identical reference curves for monoplex and pentaplex (Fig. 2b). Also, no interference of

various antigen coupled bead regions was found between monoplex and duplex or triplex MIA.

3.2. Assay specificity

Inhibition experiments were performed in order to confirm the antigen specificity of the pentaplex MIA. The percentage homologous and heterologous inhibition of the MFI of a high IgG titered serum following addition of one of the individual antigens to the reaction mixture was determined (Table 1). Addition of homologous antigens resulted in >90% inhibition of the signal for Ptx, Prn and Dtx and >85% for FHA and Ttx. When the presented antigen concentration of the inhibitor was increased to 25 µg/ml the homologous inhibition of FHA and Ttx increased to 92% and 99%, respectively. Upon addition of heterologous antigens the inhibition was <10% for all variables except for Dtx inhibition with 2.5 µg/ml Ptx which resulted in 16% heterologous inhibition. However, using other high IgG titered sera showed heterologous inhibition of <10% for all variables. Therefore heterologous inhibition seems serum dependent.

3.3. Assay sensitivity

The sensitivity of the pentaplex MIA was assessed for each analyte by collecting MFI values from 64 blank wells of several independent assays. By interpolation of 2 standard deviations from the mean MFI in the relevant reference curve the lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were expressed in mEU/ml or mIU/ml for each analyte. The LLOD and LLOQ for each analyte are presented in Table 2. The calculated LLOD and LLOQ indicate that compared to the Pertussis ELISA and the ToBI the pentaplex MIA is much more sensitive.

3.4. Assay reproducibility

The reproducibility of the pentaplex MIA was determined by obtaining both intra- and inter-assay variation data (Table 3). The mean %CV for the intra-assay variation, determined from independent triplicates of several different samples within one plate ranged from 7 to 9% for all analytes. Intra-assay variation from plate to plate was calculated from the %CV obtained from dependent duplicates assayed on different plates with a broad range serum panel ($n=60$). The mean %CV for these 60 sera ranged from 5 to 10% for all analytes. Both intra-assay results indicate that variation within plate and between plates is very low for all analytes. Inter-assay variation was determined from comparison of the IgG concen-

Table 2

Calculated lower limit of detection and -quantitation for each of the five individual analytes in the pentaplex MIA

Analyte	LLOD (mEU/ml)	LLOQ (mEU/ml)	LLOQ ELISA (mEU/ml)
Ptx	0.26	0.78	2000
FHA	0.10	0.30	2000
Prn	0.22	0.66	4000
	LLOD (mIU/ml)	LLOQ (mIU/ml)	LLOQ ToBI (mIU/ml)
Dtx	0.02	0.06	10
Ttx	0.01	0.03	10

Table 3

Reproducibility of the pentaplex MIA: inter-assay variation (within plate and between plates) and inter-assay variation (between assays) represented as the mean percentage CV

Analyte	Intra-assay variation (within plate)	Intra-assay variation (between plates)	Inter-assay variation (between assays)
	mean %CV (n)	mean %CV (n)	mean %CV (n)
Ptx	7 (9)	5 (60)	10 (60)
FHA	8 (11)	9 (60)	10 (60)
Prn	8 (10)	7 (60)	13 (60)
Dtx	9 (7)	7 (60)	10 (60)
Ttx	7 (8)	10 (60)	11 (60)

trations obtained from a broad range serum panel ($n=60$) in two separate assay runs. The mean percentage CV ranged from 10 to 13%.

Furthermore the variation between results generated by different preparations of conjugated microspheres was as-

essed by determination of the correlation coefficient for all analytes with a panel of serum samples. As presented in Fig. 3 the correlation coefficient for batch to batch comparison was high for all analytes ($R \geq 0.979$). Different assay runs over time with identical bead sets for all analytes showed that conjugated microspheres could be stored at 4 °C for at least 6 months without significant reduction in MFI signal, increased aggregation of the beads or loss of reproducibility.

3.5. Comparison of the pentaplex MIA with the ELISA and the ToBI

The performance of the pentaplex MIA compared to the standardized ELISA methods was evaluated with a panel consisting of sera with a broad range of titers ($n=60$) from vaccine trials and routine diagnostic analysis. Comparison of IgG concentrations obtained from both methods was analysed by linear regression and determination of the correlation coef-

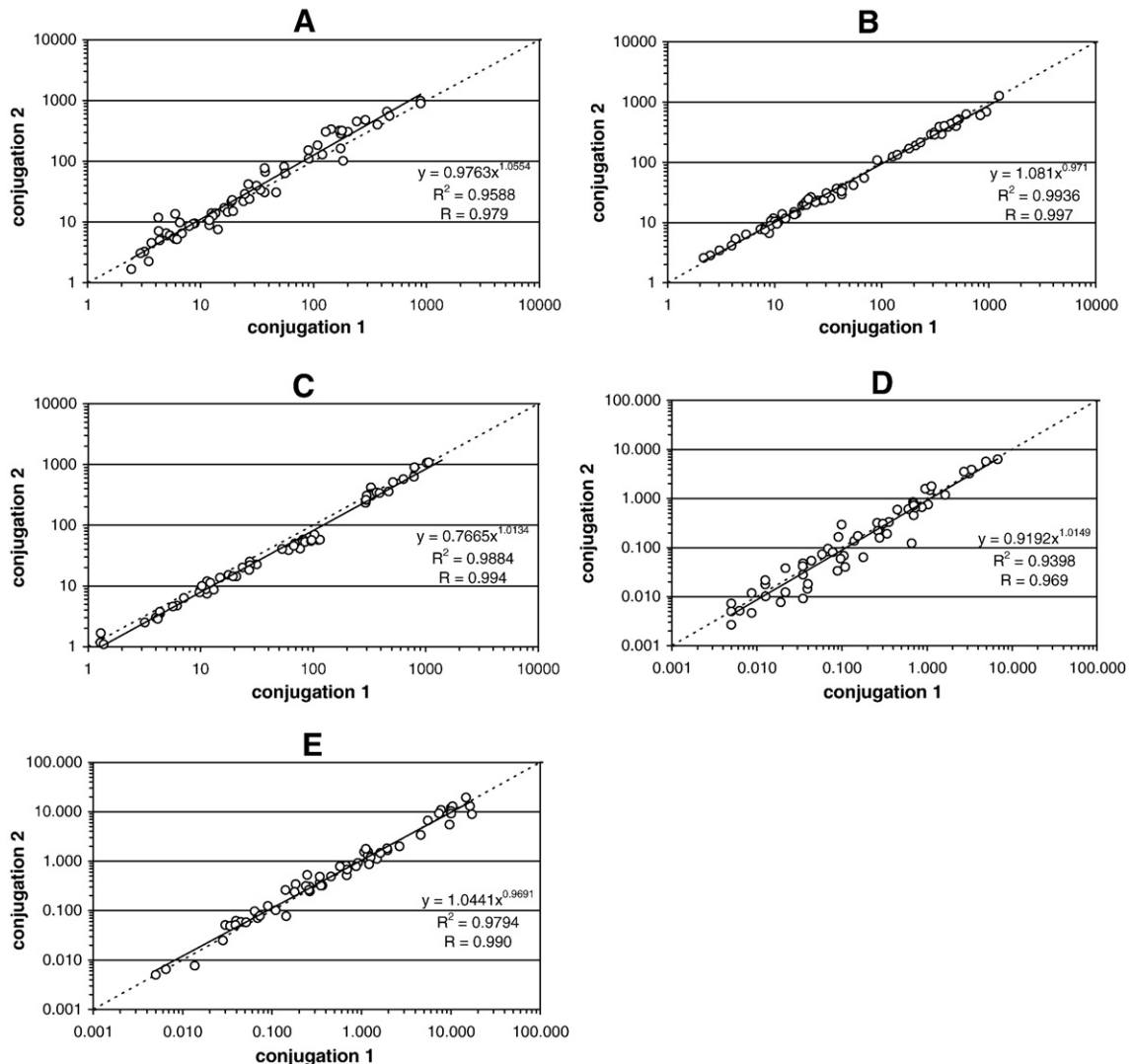


Fig. 3. Comparison of IgG concentrations obtained from separate preparations of conjugated microspheres with a broad range serum panel ($n=61$): (A) Ptx, (B) FHA and (C) Prn in EU/ml and (D) Dtx and (E) Ttx in IU/ml.

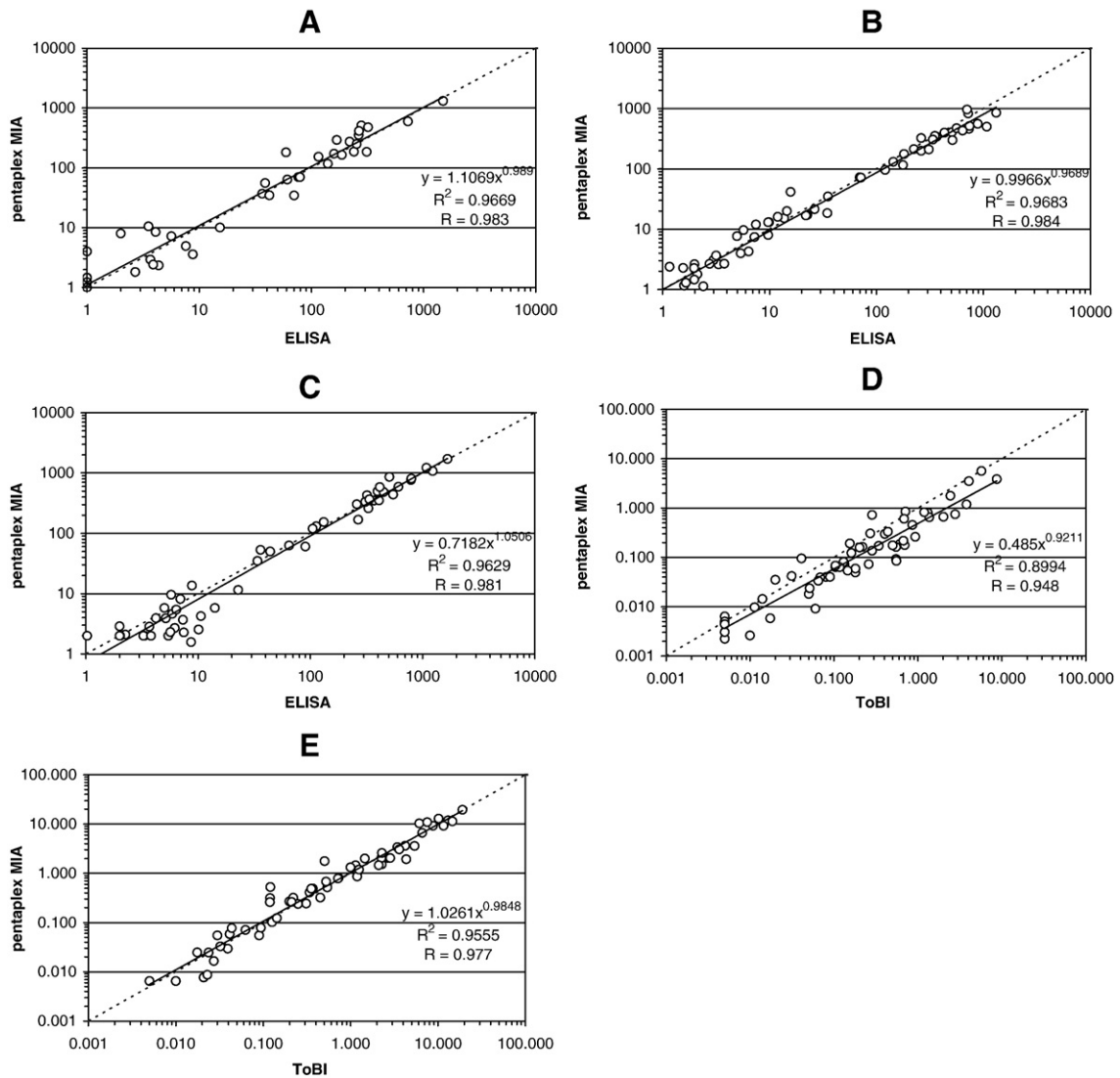


Fig. 4. Comparison of IgG concentrations obtained from the pentaplex MIA and the Pertussis ELISA and DT ToBI (Ig total) with a broad range serum panel ($n=60$): (A) Ptx, (B) FHA and (C) Prn in EU/ml and (D) Dtx and (E) Ttx in IU/ml.

ficients (R) between pentaplex MIA and ELISA or ToBI (Fig. 4). For all analytes good correlations were found with R values that ranged between 0.948 (Dtx) and 0.984 (FHA). The slopes ranged between 0.92 (Dtx) and 1.05 (Prn). These correlation results were comparable to previously generated data during the development of the pentaplex MIA. The triplex MIA for Pertussis antigens and duplex MIA for DT were also compared with the ELISA and the ToBI using different serum panels ($n=60$ Fig. 5A and $n=78$ Fig. 5B, respectively).

4. Discussion

In this study we describe the development and validation of a microsphere based multiplex immunoassay for the simultaneous determination of serum antibodies against *B. pertussis*, Diphtheria and Tetanus.

The optimal conjugation concentration for Ptx, FHA and Prn was identical to the one previously described for the

tetraplex assay for Ptx and FHA (IgG and IgA) from *B. pertussis* (Prince et al., 2006). The MFI signals and assay results for Dtx and Ttx were optimal with an increased conjugation ratio as compared to the pertussis ratios. Even more important, a well defined and purified antigen batch for each analyte is necessary for a successful conjugation. This not only improved the conjugation efficiency of the proteins to the microspheres, but also lowered the percentage of aggregated beads. The necessity of using pure antigen was further confirmed by the high correlation between different bead couplings with these antigen batches. To secure antigen availability for future testing we have used worldwide commercially available antigens with a very high purity as much as possible for the development of the pentaplex MIA. Batch to batch antigen changes or introduction of a new antigen from a different source vs. the old one should be validated in the MIA in common with the ELISA. Using internationally accepted standard reference sera for this antigen validation makes it possible to bridge old and

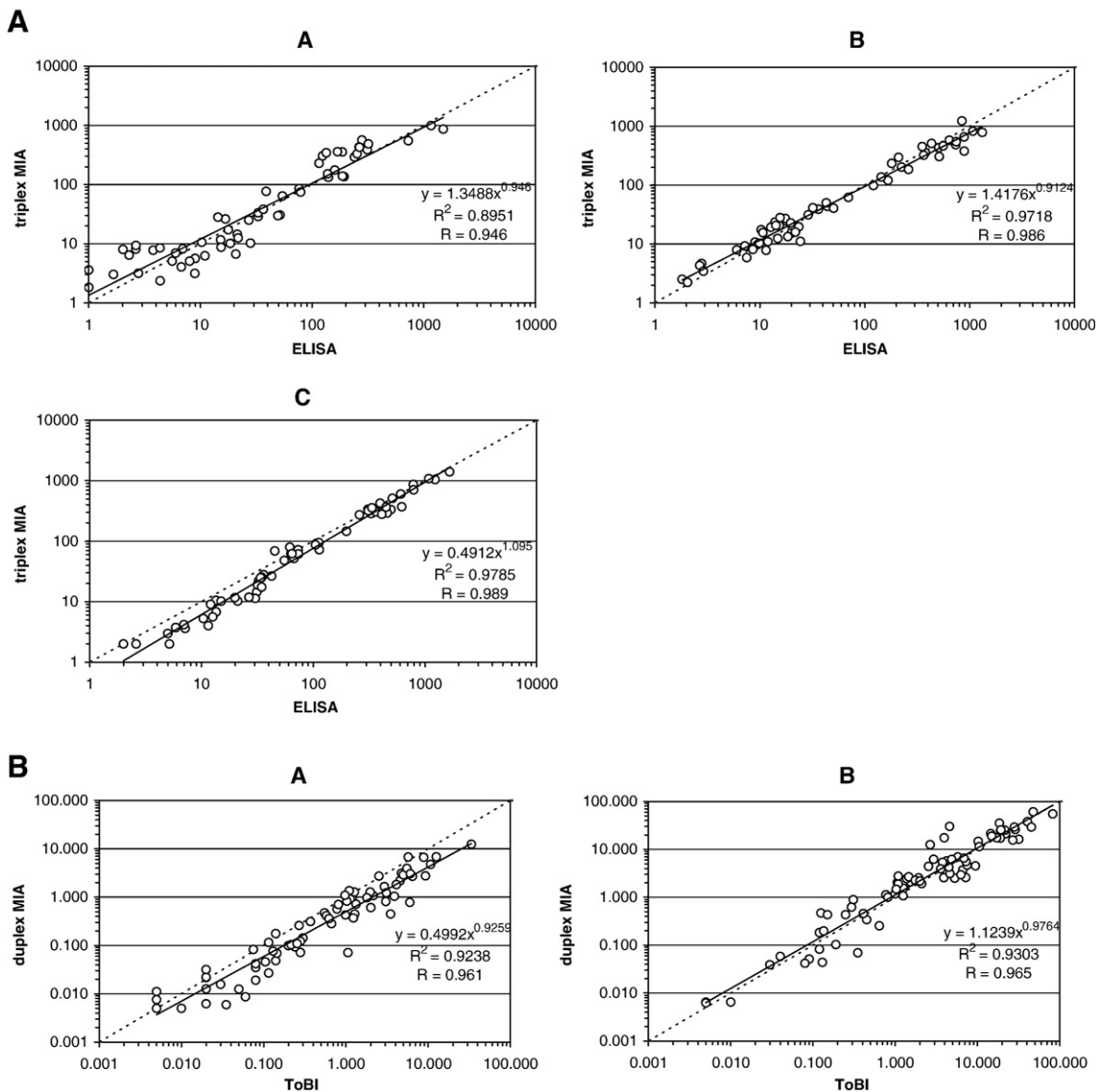


Fig. 5. (A) Comparison of IgG concentrations obtained from the tripex MIA and the Pertussis ELISA with a broad range serum panel ($n=60$): (A) Ptx, (B) FHA and (C) Prn in EU/ml. (B) Comparison of IgG concentrations obtained from the duplex MIA and Ig total concentrations obtained from the DT ToBI with a broad range serum panel ($n=78$): (A) Dtx and (B) Ttx in IU/ml.

new data and even data between Luminex and ELISA. Specificity experiments demonstrated that only analyte specific immunoglobulins were detected with the pentaplex MIA suggesting that the conjugation reaction did not adversely affect some antigenic epitopes in particular during the bead coupling. Heterologous and homologous inhibition was comparable or even better than previously published results of similar multiplex assays (Biagini et al., 2003; Lal et al., 2004; Lal et al., 2005).

Previously described multiplex assays use various buffers especially for sample dilution. Most buffers are based on PBS with addition of various concentrations Tween-20 (Pickering et al., 2002a,b; Lal et al., 2005), bovine serum albumin (BSA) (Biagini et al., 2003; Prince et al., 2006), fetal calf serum (FCS) (Lal et al., 2004; Martins et al., 2006) or antibody depleted

human serum (ADHS) (Biagini et al., 2003; Dias et al., 2005). Experiments in this paper with various serum dilution buffers clearly showed the importance of the serum dilution buffer for the pentaplex. Buffer effects were found to influence the level of the MFI signal as well as possible overestimation of the IgG concentration especially in the lower area of the concentration range. This possible non-specific binding effect was found most frequently for the toxin antigens. With freshly prepared PBS and addition of Tween-20 (0.1% (v/v)) and BSA (3% (w/v)) the overestimation was reduced to a level where correlation with ELISA was also good in the lower concentration range. In addition Tween-20 was not critical during the wash procedure or incubation with the secondary antibody. In the lower concentration range we found that the Prn titers measured with the MIA were lower compared to ELISA,

for the pentaplex as well as the triplex. This could possibly be caused by a higher non-specific binding in the ELISA in this area. In contrast to the other antigens, Dtx titers from the pentaplex and duplex MIA were persistently lower than in the ToBI over the whole concentration range. This phenomenon could not be explained by the use of Diphtheria toxin in the MIA because toxin is also used in the ToBI. We prefer to use toxin in our assays instead of the vaccine component toxoid because antibody levels against toxin reflect the natural situation although we almost never observed any differences between the anti-toxin and anti-toxoid titers in the ToBI.

The luminex reference sera for quantification were 4-fold serially diluted in the MIA at least covering the LLOQ of the ELISA up to the maximum MFI signal of the Bio-Plex. Serum samples measured in two optimal dilutions of 1/200 and 1/4000 showed high linearity with the 5-PL reference curve. In practice, these two dilutions were sufficient to cover most of the anti-DTaP3 titer range. Depending on the expected level of the antibody titers for study samples the serum predilution can be easily adjusted to obtain reproducible results from one single Multiplex assay. Furthermore, the use of only two sample dilutions strongly contributes to the high throughput of the developed MIA in contrast to the ELISA using a 2-fold serial dilution series for reference and serum samples.

The theoretically determined LLOD and LLOQ indicated an increased sensitivity of the MIA compared to the ELISAs especially for the pertussis antigens. However, the biological significance of Pertussis titers well below the ELISA LLOQ of 2 EU/ml is open for debate. For Diphtheria and Tetanus the developed MIA offers the possibility to reproducibly measure IgG antibody titers not only between 0.1 and 0.01 IU/ml but also below the LLOQ of the ToBI (0.01 IU/ml) which is equal to the minimum protective level for DT antitoxin antibodies in serum samples according to the WHO (von Hunolstein et al., 2000). With the described eight-step diluted reference curve it is feasible to reproducibly measure antibody titers in pre-vaccination samples below 0.01 IU/ml. The data we found for intra- and inter-assay variability for the MIA were similar or better compared to ELISA, indicating a higher reproducibility of the MIA. The IgG concentrations determined with the MIA demonstrated an excellent correlation with both the *B. pertussis* ELISA and DT ToBI, albeit the fact that the ToBI is based on the antigen inhibition principle, measuring Ig total antibody concentration rather than direct IgG antibody detection.

The multiplex immunoassay for the simultaneous determination and quantification of IgG serum antibodies to *B. pertussis*, Diphtheria and Tetanus toxin described herein makes optimal use of the characteristics of the Luminex technology. The development and validation showed the ability to specifically measure five different analytes in one single assay with small sample volumes using very small amounts of antigen as compared to conventional ELISA techniques. The flexibility and robustness of the microsphere assay makes it possible to include more new target antigens to the pentaplex such as Fimbrae 2 and 3. Furthermore, the simultaneous measurement greatly reduced the amount of time and labor to assay large quantities of sera. For example, using the pentaplex MIA 100 samples can easily be assayed for 5 different antigens on three plates within one single day. Alternatively, with conventional ELISA techniques, 3 × 10 ELISA plates for the 3

pertussis antigens and 2 × 17 ToBI plates for DT are necessary. In this example 40–250 µg of antigen is needed for the pertussis ELISAs compared to only 1 µg in the pentaplex MIA ensuring much less batch to batch changes.

Implementation of luminex technology in a laboratory introduces a new technique but does not require a much higher investment in equipment compared to ELISA (reader, washer). The MIA technology is easily adapted and the use of in house reference sera calibrated against international standards for multiple antigens is preferred. Obviously, as no kits are available for vaccine antigens one needs more time to develop the optimal procedures for conjugation of these vaccine antigens and validation of the assay compared to ready to use kits like for cytokines. Despite the fact that the running costs per plate of the MIA are higher, this technology will be cost-effective in the long-run due to the increased throughput and sample and antigen saving properties as well as reduction in man labor. We estimate that the break-even point for the MIA compared to the ELISA is reached if two different antigens are measured simultaneously, mainly due to reduced labor time. Thus the MIA will be cost-effective if more than two antigens are measured. Furthermore, results will be much sooner available especially in large scale studies.

In conclusion, the multiplex immunoassay we described is specific, sensitive, reproducible, sample and antigen saving and above all it is a fast method to detect a variety of antibodies in human sera. The correlation with ELISA and ToBI is high and therefore the MIA is a viable alternative method for the detection of DTaP3 antibodies in large scale immunosurveillance studies.

Acknowledgements

We thank Dr. Gerard Loeber and Dr. Ger Rijkers for critical review of the manuscript and Debbie van Rooijen for analytical support.

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