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Development of a Fluorescent-Bead-Based Multiplex Immunoassay To Determine Immunoglobulin G Subclass Responses to *Neisseria meningitidis* Serogroup A and C Polysaccharides▽†

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A fluorescent-particle-based multiplex flow cytometric immunoassay (MIA) for the detection of serum immunoglobulin G (IgG) and two IgG subclasses, IgG1 and IgG2, specific for *Neisseria meningitidis* serogroup A (MenA) and C (MenC) polysaccharides (PS) was developed. The assay comprised three separate duplex assays, one for the detection of the IgG response to MenA and MenC PS, another for the detection of the IgG1 response to MenA and MenC PS, and a third for the detection of the IgG2 response to MenA and MenC PS. Next, the three separate duplex assays were combined and analyzed as a hexaplex assay. No interference between monoplex, duplex, and hexaplex assays was observed, and the assay was found to have low intra- and interassay variation (<9.0% and <27%, respectively). Comparison of the meningococcal subclass MIA to the in-house enzyme-linked inmmunosorbent assays showed a good correlation ($R \geq 0.85$) for each of the subclasses. We conclude that the hexaplex meningococcal subclass MIA is an easy and specific assay for the determination of anti-MenA and anti-MenC PS subclass IgG, requiring minimal amounts of serum to study IgG subclass responses to vaccines.

In human sera the most abundant class of immunoglobulin is immunoglobulin G (IgG; 80%), which is classified into four subclasses (IgG1 to IgG4), each characterized by specific structural and biological qualities. IgG1 and IgG3 antibodies are usually elicited by protein antigens, which are so-called T-cell dependent antigens (8, 22). In contrast, carbohydrate polysaccharide (PS) antigens, T-cell-independent (TI) antigens, preferentially elicit IgG2 in adults (6, 25). In infants and children TI responses are predominantly of the IgG1 subclass (9, 17). The potential functional differences in the abilities of the IgG subclasses to clear bacteria, however, are not always obvious (24).

IgG antibodies directed at the PS capsules of bacteria like *Neisseria meningitidis* are protective against invasive disease. In order to increase the immunogenicity of PS as vaccines, protein carriers are covalently coupled to PS. This changes the original TI response to a T-cell-dependent response (3, 13, 14). This may result in a decrease in the IgG2/IgG1 ratio, as shown previously for pneumococcal-conjugate vaccines in children and adults (6, 25). Nevertheless, the IgG2/IgG1 ratio may be crucial for monitoring after-vaccination and long-term immunity since the subclasses may have different protective properties.

Conventional methods for detection of IgG and the IgG subclasses are based on enzyme-linked immunosorbent assays (ELISA) (2, 7, 12). ELISA is a reproducible and specific assay but time-consuming and often limited by the amount of available serum, particularly when multiple analytes need to be tested. Newly developed methods, such as fluorescent particle-based multiplex flow cytometric immunoassays (MIA), using fluorescent distinct beads as a carrier for different antigens enable the detection of multiple analytes in a single sample (1, 15, 16, 18, 19, 20, 23, 26) with limited amounts of serum.

Unfortunately, existing MIA systems are not able to detect different subclasses of IgG to multiple antigens in a single well. Therefore, we designed a meningococcal subclass MIA according to the same principles as those of the method of Prince et al. (23). A MIA was developed in order to measure IgG and the IgG1 and IgG2 subclass responses to meningococcal serogroup A (MenA) and C (MenC) PS and subsequently to study immune responses to meningococcal PS and PS-conjugate vaccines.

MATERIALS AND METHODS

Specimens. Sera used for evaluation of the meningococcal serogroup A (MenA) and C (MenC) IgG subclass MIA included pediatric and adult sera (n = 22) which were submitted to the National Institute for Public Health and the Environment for antibody screening to determine antibodies to diphtheria, tetanus, and poliomyelitis. In addition, pre- and postvaccination serum samples obtained from healthy adults (n = 32) who were vaccinated with Meningovax A+C (Sanofi Pasteur MSD, Belgium), for reasons of occupational risk, were used.

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Coupling of MenA and MenC PS to carboxylated fluorescent beads. *N. meningitidis* serogroup A and serogroup C capsular PS (National Institute for Biological Standards and Control [NIBSC], Potters Bar, United Kingdom) were conjugated to poly-L-lysine (Sigma-Aldrich, St. Louis, MO) as described previously (10, 15). This conjugate was covalently attached to fluorescent carboxylated beads (Bio-Rad Laboratories, Hercules, CA) using a two-step carbodiimide reaction (15, 27).

MIA for the quantification of MenA and MenC PS antibodies. The MenA and -C PS subclass MIA was performed as described previously (15) with modifications. Reference serum sample CDC1992 (NIBSC code 99/706) was serially diluted in phosphate-buffered saline (PBS), pH 7.2, containing 50% antibody-depleted human serum (Valley Biomedical, Winchester, VA); unknown and control serum samples (control serum 89S-2 [FDA, Frederick, MD] and in-house control serum obtained from Wyeth, Collegeville, PA) were diluted 1:100 in the same buffer. Previously assigned concentrations of meningococcal IgG, IgG1, and IgG2 antibodies in CDC1992 were used in this assay (11, 12). Standard and serum samples were added to an equal volume of MenA- and -C-conjugated beads (4,000 beads/antigen/well) in a 96-well MV Multiscreen filter plate (Millipore, MA) and incubated for 20 min at room temperature (RT) on a plate shaker at 650 rpm in the dark. After three washes with PBS using a vacuum manifold, wells containing beads for determination of IgG received *R*-phycoerythrin-conjugated goat anti-human IgG (gamma chain specific) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1:200 in PBS. Wells for detection of IgG1 and IgG2 received a mouse anti-human monoclonal antibody solution for detection of human IgG1 (clone HP6069; dilution, 1:100) and IgG2 (clone HP6002; dilution, 1:200) (Zymed Laboratories, Invitrogen, Paisley, United Kingdom), respectively, both diluted in sample buffer. After another incubation of 20 min at RT on a plate shaker in the dark and three washes, wells for detection of the IgG subclasses received *R*-phycoerythrin-conjugated goat anti-mouse IgG (gamma chain specific) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) diluted 1:200 in PBS and wells for detection of IgG on the same plate received PBS. After a 20-min incubation in the dark at RT on a plate shaker and three washes, each duplex of a given sample was suspended in PBS and carefully mixed with the other duplexes. Samples were analyzed as a hexaplex using a Bio-Plex system in combination with the Bio-Plex Manager software, version 4.1.1 (Bio-Rad Laboratories, Hercules, CA). For each analyte, mean fluorescent intensity (MFI) was converted to μg/ml by interpolation from a five-parameter logistic standard curve.

Assay specificity. The MenA and -C PS-specific IgG, IgG1, and IgG2 specificities were determined as described previously (15), with the modification that different serum specimens were tested: CDC1992, 89S-2, and an in-house reference serum.

Assay sensitivity. To determine the sensitivity of the subclass MIA, we used a method described previously (15) with the exception that 40 blank wells were used. The means plus 2 standard deviations were calculated, and minimal levels of detection (MLD) were determined. The lower limit of quantitation (LLOQ) was calculated as three times the MFI of the MLD (5).

Assay reproducibility. The reproducibility of the meningococcal subclass MIA was determined by measuring intra-assay variation and interassay variation. Intra-assay reproducibility was calculated as the mean percentage of coefficients of variation (CVs) for 10 samples determined in duplicate within one plate. The interassay variation was assessed by testing samples ($n = 54$) in duplicate on two different days, and the CV percentages were calculated.

ELISA for determination of meningococcal serogroup A and C PS IgG1 and IgG2 antibodies. MenA- and -C-specific IgG, IgG1, and IgG2 ELISA were performed as described previously (2, 7, 12), with the modification that different secondary conjugated antibodies were used: a goat anti-human IgG alkaline phosphatase-labeled antibody (Biosource, Camarillo, CA) and a rabbit anti-mouse Ig horseradish peroxidase-labeled antibody (DAKO, Glostrup, Denmark).

RESULTS

Development of a MIA for detection of IgG and the different IgG subclasses. (i) Interference and cross-reactivity. Two different duplex assays were developed and performed in parallel, the first to detect IgG1 and the second to detect IgG2. Both subclass duplex assays were combined with the duplex assay for detection of IgG. To confirm the ability of the combination of beads to detect IgG, IgG1, and IgG2 and to exclude interference or migration of conjugate from one bead type to another, MFIs generated by each bead set (monoplex) were compared with the MFIs generated by two bead sets together for both meningococcal serogroups (duplex), which were in turn compared to the MFIs generated when all three duplex assays were combined (hexaplex). No cross-reactivity or crossover of conjugate was detected, indicating that the antigen-antibody complexes formed were stable. In addition, sera ($n = 10$) were measured in the monoplex, duplex, and hexaplex MIA. Again, no differences in calculated antibody concentrations were found (data not shown).

TABLE 1. Calculated MLD and LLOQ for each of the individual IgG subclasses

Serogroup and IgG subclass	MLD (pg/ml)	LLOQ (pg/ml)
MenA		
IgG	410	1,230
IgG1	8,600	25,800
IgG2	1,810	5,430
MenC		
IgG	120	360
IgG1	1,270	3,810
IgG2	1,040	3,120

(ii) Standard curves. Previously assigned antimeningococcal IgG, IgG1, and IgG2 concentrations for reference serum CDC1992 where used. As shown earlier (15), the standard curves for detection of MenA and -C IgG showed a good linearity over six fourfold dilutions of the reference serum. Standard curves for MenA and -C IgG1 showed good linearity over four steps of a fourfold dilution of the reference serum and were also parallel with the corresponding IgG line according to the principles of Plikaytis et al. (21). The same result was found for the IgG2 subclass of MenA and -C antibodies in five steps of a fourfold dilution of the reference serum.

Assay validation. (i) Specificity. The specificity of the meningococcal subclass MIA was assessed by preabsorption with free PS. Good specificity was observed for MenA and -C in serum samples CDC1992 and 89S-2 and an in-house reference serum. The percent reductions of MFIs after homologous serogroup PS was added exceeded 85%, 75%, and 80% for IgG, IgG1, and IgG2, respectively, for both serogroups. Addition of heterologous PS resulted in inhibition of less than 20% (IgG), <25% (IgG1), and <10% (IgG2) for both serogroups.

(ii) Sensitivity. For the determination of the sensitivity of the meningococcal subclass MIA, values obtained from blank wells were compared to the relevant standard curve (Table 1). The LLOQ for detection of anti-MenC IgG in the subclass MIA is approximately 50 times more sensitive than that for detection of anti-MenC IgG in the ELISA (data not shown).

(iii) Reproducibility. The meningococcal IgG subclass MIA showed a high intra-assay reproducibility, with CVs of 6.3% for MenA IgG and 5.1% for MenC IgG. For the IgG1 and IgG2 subclasses CVs of 8.3% and 9.0% were found in the case of MenA and 5.1% and 7.6% for MenC, respectively. Interassay variation for the meningococcal IgG subclass MIA and ELISA was calculated by comparison of antibody concentrations obtained from a panel of serum samples ($n = 54$) analyzed in duplicate on two different days (Table 2). In general the CVs found with the MIA were lower than the CVs for the ELISA.

TABLE 2. Interassay variability for detecting antimeningococcal IgG subclass responses using ELISA and MIA

Serogroup and IgG subclass	CV (%) by:	
	ELISA	MIA
MenA		
IgG	17	16
IgG1	28	23
IgG2	30	27
MenC		
IgG	15	9
IgG1	31	19
IgG2	21	18

Comparison of multiplex subclass MIA with ELISA. To evaluate the MenA and -C PS subclass MIA, a panel of pediatric and adult serum samples (n = 54) was composed. IgG, IgG1, and IgG2 antibodies from the samples of this panel were measured with meningococcal ELISAs and the developed meningococcal subclass MIA in duplicate on two different days. Results were compared by linear regression and by calculating correlation coefficients (R^2 values). The correlation between the ELISA and meningococcal subclass MIA was good for all antibody types directed against MenA and -C PS (Fig. 1A, D, and E and 2A, D, and E, respectively). The sums of IgG1 and IgG2 antibody concentrations correlated very well with the total amount of IgG determined by MIA (Fig. 1B and 2B) and by subclass ELISA (Fig. 1C and 2C). Even the sums of IgG1 and IgG2 antibody concentrations as determined by ELISA versus MIA still revealed acceptable correlations (Fig. 1F and 2F).

DISCUSSION

In this study a hexaplex meningococcal subclass MIA was developed and validated against the conventional ELISA. The multiplexing capability of flow cytometric systems was used to simultaneously determine quantitative levels of IgG and the IgG subclasses for MenA and -C PS. The developed MIA was found to be rapid and reproducible and less time-consuming

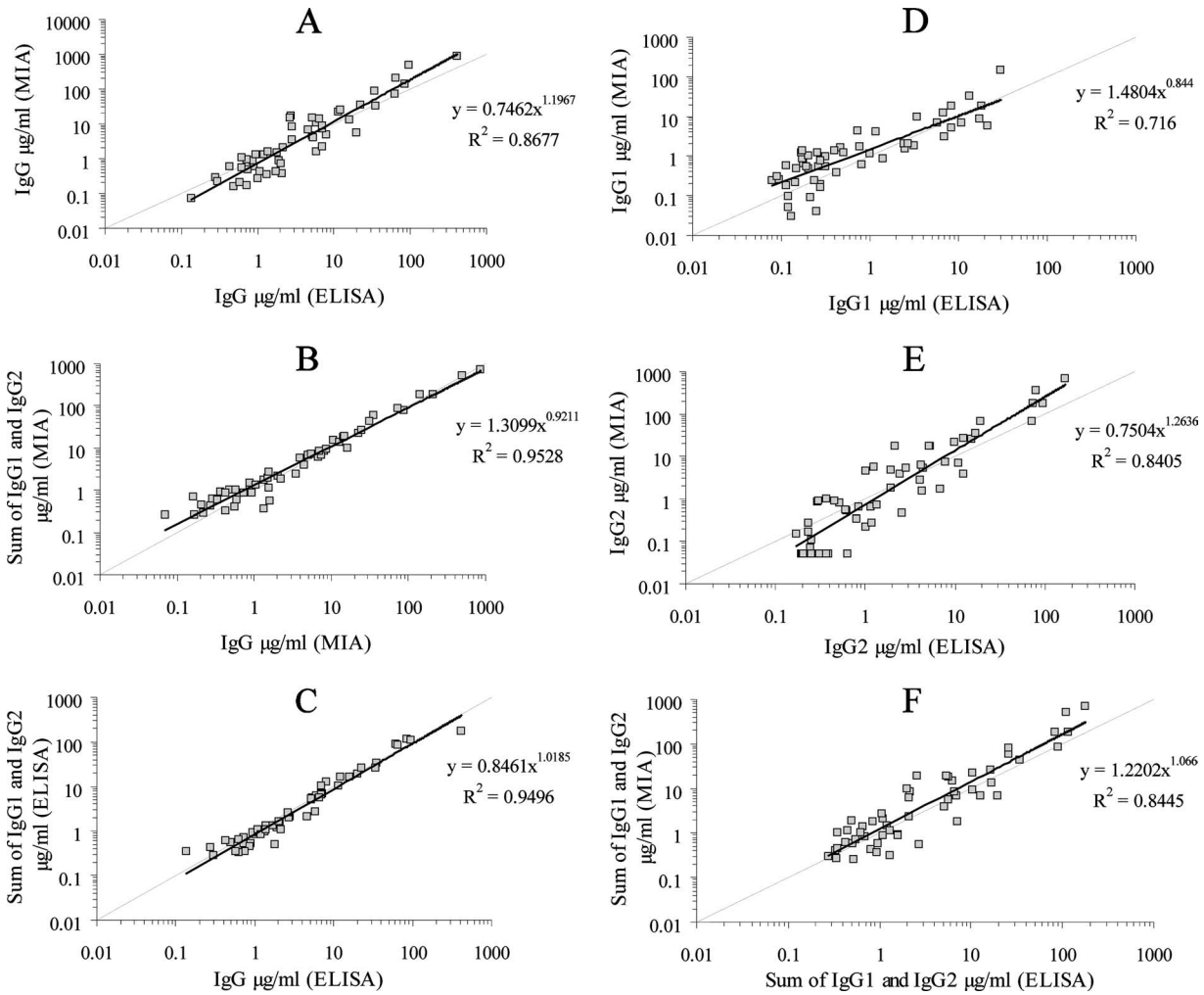

FIG. 1. Comparison of IgG, IgG1, IgG2, and sums of IgG1 and IgG2 obtained by the meningococcal serogroup A ELISA and MIA. (A) Comparison of IgG between ELISA and MIA. (B) Comparison of the sum of IgG1 and IgG2 with IgG obtained by MIA. (C) Comparison of the sum of IgG1 and IgG2 with IgG obtained by ELISA. (D) Comparison of IgG1 between ELISA and MIA. (E) Comparison of IgG2 between ELISA and MIA. (F) Comparison of the sum of IgG1 and IgG2 between ELISA and MIA.

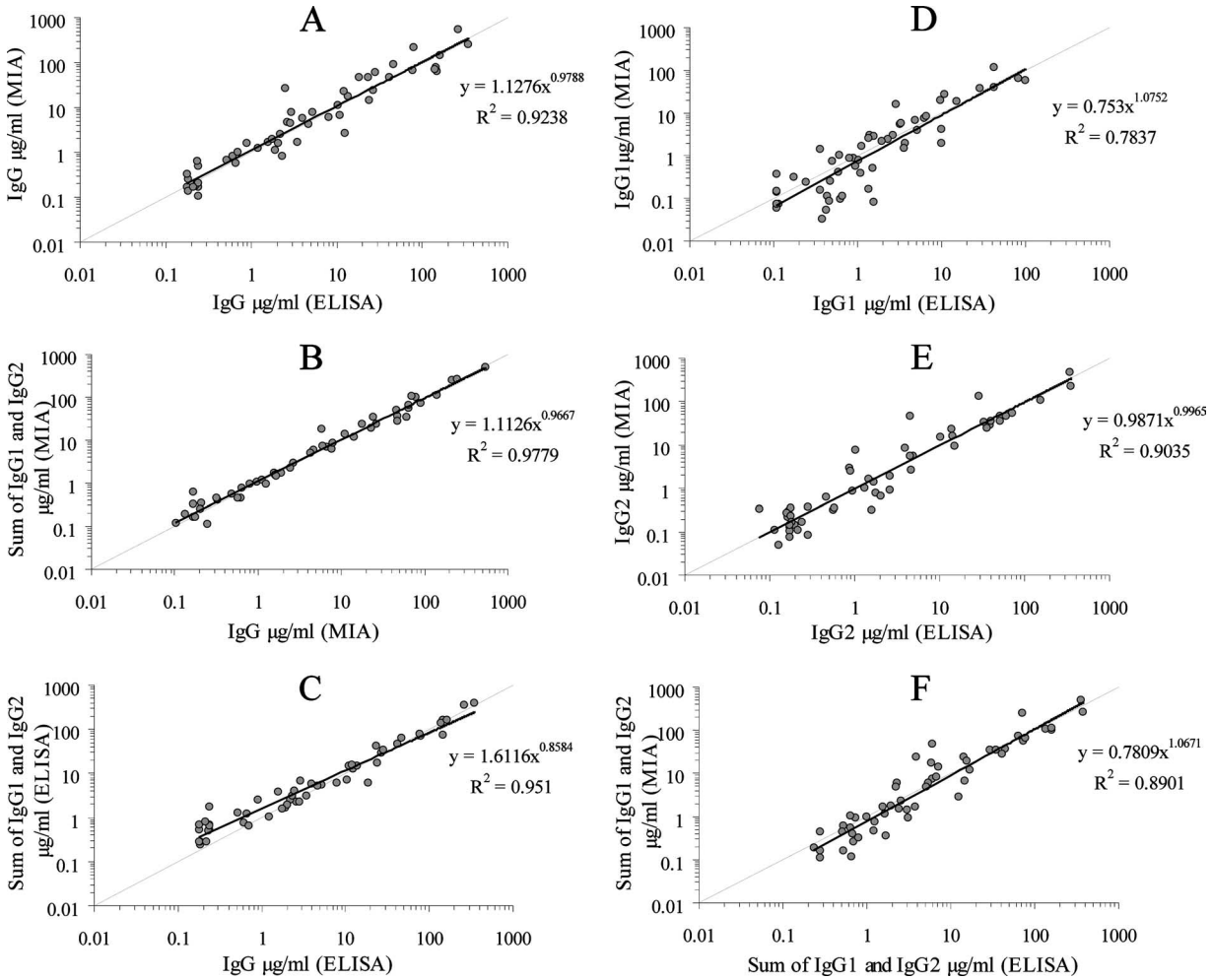

FIG. 2. Comparison of IgG, IgG1, IgG2, and sums of IgG1 and IgG2 obtained by the meningococcal serogroup C ELISA and MIA. (A) Comparison of IgG between ELISA and MIA. (B) Comparison of the sum of IgG1 and IgG2 with IgG obtained by MIA. (C) Comparison of the sum of IgG1 and IgG2 with IgG obtained by ELISA. (D) Comparison of IgG1 between ELISA and MIA. (E) Comparison of IgG2 between ELISA and MIA. (F) Comparison of the sum of IgG1 and IgG2 between ELISA and MIA.

and serum demanding than the ELISA, and it correlated well with the ELISA. Several points of attention will be discussed below.

Previously described MIA all use different buffers as serum diluents. The buffers described are PBS with various concentrations of Tween 20 (16, 19, 20), fetal calf serum (15, 18), bovine serum albumin (1, 23, 26), or antibody-depleted human serum (1, 4). We found that antibody-depleted human serum at a concentration of 50% in PBS could substantially reduce nonspecific signals in comparison with buffers containing fetal calf serum or Tween 20 (see the figure in the supplemental material). By using antibody-depleted human serum, no nonlinearity between samples and reference serum was detected; therefore, one serum dilution was sufficient for the determination of specific antibodies.

No interference or cross-reactivity between different duplex assays was observed, indicating that antigen-antibody complexes were stable, which is comparable to results from Prince et al. (23). In addition, combining the duplex assays to form a hexaplex MIA ensures identical measuring conditions and reduces the analysis time by at least a factor of 2. The linearity and range of standard curves for MenA and -C IgG are similar to the results of Lal et al. (15). These were found to be lower for the individual IgG subclasses, possibly due to lower levels of subclass antibodies present in reference serum CDC1992. The specificity of the MIA for the detection of IgG and IgG subclasses for MenA and -C was found to be comparable to those of other MIA (1, 15, 16).

Intra- and interassay variation revealed the good reproducibility of the assay. The MIA proved to be more reproducible than ELISA, with low day-to-day variation. Daily variation is higher for the IgG subclass concentrations than for IgG because IgG subclass concentrations can be very low; therefore, higher variations between days will be found. Moreover, more variation is also introduced in the subclass MIA by the use of mouse monoclonal antibodies for the detection of the IgG subclasses.

IgG and IgG subclass concentrations determined by the meningococcal subclass MIA showed a good correlation with those from individual ELISA for the same antigens. The num-

ber of serum samples tested is comparable to those from other studies (15, 19, 26), and the samples span a wide range in concentrations for all types of IgG. The levels of MenA IgG1 antibodies in sera with low titers (<1 μg/ml) in the meningococcal subclass MIA were in general higher than those in ELISA. It is unclear why this dissimilarity between the two assays occurs, especially because anti-MenA IgG2 and anti-MenC IgG1 and IgG2 antibody levels in sera with low IgG subclass concentrations (<1 μg/ml) were in general lower in the meningococcal subclass MIA than in ELISA (Fig. 1E and 2D and E). This indicates that less nonspecific signal is observed when IgG subclass concentrations are determined with the meningococcal subclass MIA, with the exception of IgG1 for MenA. These discrepancies in serum samples with an anti-PS IgG concentration of less than 1 μg/ml were also observed by Lal et al. (15) and Pickering et al. (20) for several other bacterial PS capsules.

A major advantage of the meningococcal subclass MIA described here in comparison with the ELISA is that substantially less volume of the specimens is needed for the assay. The MIA requires 5 μl of serum, while at least 60 μl is needed for ELISA. Besides the reduction of specimen volume, shorter incubation periods and incubation at RT are benefits. Furthermore, the use of antigen and reagents is extremely reduced. The amount of antigen needed to determine IgG, IgG1, and IgG2 in a single serum sample using the meningococcal subclass MIA is up to 65-fold less than for ELISA. Additionally, due to the flexibility of the system the assay can be extended in time without increasing specimen volume or assay time (100 different bead regions are available, and systems that can measure up to 500 analytes in a single well are under construction). For instance, the assay was expanded for the subclass responses to meningococcal serogroups W-135 and Y. By adding beads conjugated with MenY and MenW-135 PSs to the three different duplex assays, IgG, IgG1, and IgG2 responses to these PSs could be measured simultaneously. R^2 correlations between IgG and the sum of IgG1 and IgG2 for W-135 and Y PS were at least 0.90 (data not shown).

In conclusion, our results indicate that the meningococcal subclass MIA described here is an assay in which the IgG subclass responses to MenA and MenC PS can be easily and specifically measured. There is a good correlation between the meningococcal subclass MIA and in-house ELISA for the detection of IgG subclasses. The meningococcal subclass MIA is an excellent alternative to existing ELISA, which would simplify and speed up the evaluation of the immunogenicity of meningococcal PS and conjugate vaccines.

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