Development and Evaluation of a Multiplex Microsphere Assay for Quantitation of IgG and IgA Antibodies against *Neisseria meningitidis* Serogroup A, C, W, and Y Polysaccharides

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We developed and evaluated a rapid and simple multiplex microsphere assay for the quantification of specific IgG and IgA antibodies against meningococcal serogroup A, C, W, and Y capsular polysaccharides in serum and saliva. Meningococcal polysaccharides were conjugated to distinct magnetic carboxylated microspheres, and the performance of the assay was assessed using the CDC1992 standard meningococcal reference serum and a panel of serum and saliva samples. The standard curve was linear over an eight 3-fold dilution range in the IgG assay and a seven 3-fold dilution range in the IgA assay. No cross-reactivity was discovered, and the assay showed high specificity with ≥91% homologous inhibition and ≤11% heterologous inhibition for all serogroups and immunoglobulin classes. Lower limits of detections were ≤280 pg/ml for IgG and ≤920 pg/ml for IgA antibodies. The assay was reproducible, with a mean coefficient of variation of ≤5% for intra-assay duplicates, a mean coefficient of variation of ≤20% for interassay repeated analysis with different conjugations of microspheres, and a mean coefficient of variation within 25.8% for interoperator variation. The assay showed good correlation to the standard meningococcal polysaccharide enzyme-linked immunosorbent assay (ELISA) for detection of serum antibodies. This multiplex assay is robust and reliable and requires less sample volume, and less time and workload are needed than for ELISA, making this method highly relevant for serological and salivary investigations on the effect of meningococcal vaccines and for immunosurveillance studies.

Meningococcal disease continues to be a significant public health problem, although vaccines used in national immunization programs or mass vaccination campaigns have reduced the incidence of the disease in several countries (1). The capsular polysaccharide is an important antigen and virulence factor (2), and the most widely used meningococcal vaccines are based on these polysaccharides. Such vaccines have been shown to be effective for serogroups A, C, W, and Y, four of the five major disease-causing meningococcal serogroups (1, 3), and have been available and widely used for nearly half a century.

To evaluate the effect of meningococcal vaccines and determine protection against disease, serogroup-specific serological measures are used. Serum bactericidal activity (SBA) has become the most widely used surrogate of protection and is the basis for licensure of the recent meningococcal vaccines (4). However, this method is highly time consuming and requires specialized laboratories and highly standardized biological reagents. Quantitation of specific anti-meningococcal polysaccharide antibodies, on the other hand, is more suitable for large immunosurveillance studies and contributes to a broader understanding of the immune response. In a vaccine efficacy trial in Finland in the 1970s, a specific immunoglobulin G (IgG) concentration was shown to correlate with clinical protection against serogroup A disease (5). The most common method for antibody quantitation has been enzyme-linked immunosorbent assay (ELISA). ELISA can only measure antibodies against one antigen at a time and is, therefore, labor intensive. In an era where the use of multivalent vaccines is increasing, assays that give the opportunity for multiplexing, that is, testing simultaneously for several analytes within the same sample, provide huge advantages and enhance effectiveness severalfold. Several multiplexing techniques have been developed, but since the first particle-based flow cytometric assays became available in the early 1980s, such methods have become increasingly popular. Multiplex assays substantially reduce the cost, time, and sample volumes required, have a wider analytical range than that of the ELISA, and several studies have shown them to be sensitive, specific, reproducible, and accurate (6–9). Thus, assays based on this technique have been developed for detection of a wide range of antibodies, antigens, genetic material, and etc. (10).

In particle-based assays, antigens are conjugated onto microscopic spheres (beads). Using polysaccharides as antigens in such assays, however, poses a challenge, as they are not able to covalently bind directly with polystyrene microspheres as proteins do. Polysaccharides need a coupling molecule and, thus, an additional step for conjugating them onto the microspheres. Several methods for conjugation to microspheres have been developed using polysaccharides from different bacterial species (6, 8, 9, 11–15). A comparative study of different coupling agents showed that the nontoxic 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM) was the overall preferred coupling agent when conjugating pneumococcal polysaccharides to microspheres (15). We as-

sumed that these findings could be transferred to the development of a meningococcal polysaccharide assay.

Multiplex methods for detection of salivary antibodies have been developed and evaluated for other pathogens ([16]). A multiplex assay developed for measuring IgG antibodies in serum was used for quantification of anti-meningococcal serogroup C antibodies in saliva ([17], [18]). However, to enable investigation of the salivary immune response to multivalent meningococcal vaccines, a multiplex assay for use on saliva as well as serum samples, was developed and evaluated. Additionally, this allowed the possible investigation of the relationship between antibody responses in serum and saliva and the potential for using saliva as a substitute for serum to measure systemic antibody responses ([19]). The method described here detected anti-polysaccharide IgG and IgA antibodies against the four meningococcal serogroups A, C, W, and Y.

MATERIALS AND METHODS

Test and reference samples. Standard human reference serum CDC1992 (National Institute of Biological Standards and Control [NIBSC] code 99/706, pooled sera from 14 adults vaccinated with a meningococcal A, C, W, and Y polysaccharide vaccine) was obtained from NIBSC, and the serogroup-specific concentrations of IgG and IgA antibodies previously assigned to it were used for quantification of samples ([20], [21]). To develop and evaluate the assay, a control panel of 15 serum and 15 saliva samples were established. The samples in the panel were chosen to cover a wide range of different concentrations of specific anti-meningococcal IgG and IgA antibodies to cover the dynamic range of the assay. These samples were obtained from employees at the Norwegian Institute of Public Health (NIPH) before and after routine preventive meningococcal vaccination with a tetravalent conjugate vaccine (Menveo; Novartis Vaccines) due to occupational risk. Additional samples used for the correlation assays were obtained from healthy Norwegian individuals, either vaccinated with Menveo meningococcal vaccine or unvaccinated, whose samples were submitted to the NIPH for anti-meningococcal antibody screening, and randomly selected saliva samples from a study in 10- to 14-year-old patients before and after vaccination with a serogroup A conjugated vaccine (MenAfriVac) in Burkina Faso ([22]). The study obtained all necessary ethical approvals. All samples used for development of the assay were deidentified.

Conjugation of *Neisseria meningitidis* capsular polysaccharide to microspheres. Meningococcal polysaccharides were conjugated to magnetic microspheres (MagPlex COOH microspheres, size 6.5 ± 0.2 μm; Luminex Corp., TX, USA) based on the method developed for pneumococcal polysaccharides by Schlottmann et al. ([15]). *N. meningitidis* purified capsular polysaccharides were obtained from NIBSC (United Kingdom), serogroup A (NIBSC code 98/730), serogroup C (07/318), serogroup W (01/428), and serogroup Y (01/426), and reconstituted in sterile water to a concentration of 1 mg/ml. A volume of 875 μl of each polysaccharide was treated with 70 μl of a 200 mg/ml solution of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) (Sigma-Aldrich, MO, USA) in sterile water. The polysaccharide/DMTMM mixture was incubated in the dark, on a rotator, for 1 h at room temperature (RT). After incubation, the mixture was added to a Sephadex G-25M PD10 column (GE Healthcare, United Kingdom) equilibrated with phosphate-buffered saline (PBS) (in-house; containing 5.53 mM disodium hydrogen phosphate, 1.13 mM sodium dihydrogen phosphate, and 138.6 mM sodium chloride; pH 7.4) and eluted with 3.5 ml of PBS to separate the DMTMM-modified polysaccharides from free DMTMM, giving a calculated final polysaccharide concentration of 0.25 mg/ml. Different polysaccharide concentrations were tested individually for each serogroup, and, showing only small differences in all serogroups, a final concentration of 0.25 mg/ml for each polysaccharide was found to give the overall best standard curves, with regard to range and fluorescent intensity. Five hun-

dred microliters of modified polysaccharide was added to 500 μl magnetic microspheres (1.25 × 10^7 microspheres/ml), vortexed, sonicated, and incubated in the dark on a rotator at RT overnight. The following day, using a magnetic holder, the conjugated microspheres were rinsed twice with 0.5 ml of PBS-Tween 0.05% (PBST) to remove noncovalently bound modified polysaccharides before adding 500 μl of blocking buffer (PBS with 10% bovine serum albumin [Gibco; Thermo Fisher Scientific, Inc., MA, USA], 0.05% sodium azide). Conjugated microspheres were stored at 4°C in the dark.

Multiplex microsphere assay for quantification of anti-meningococcal polysaccharide IgG and IgA antibodies. Conjugated microspheres were vortexed, sonicated, and diluted to a final concentration of 2,500 microspheres per well of each serogroup in 50 μl dilution buffer (PBS with 10% antibody-depleted human serum [Valley Biomedical, VA, USA]), which was the volume added to each well of a 96-well Nunc flat-bottomed plate (Bio-Rad, United Kingdom). Different concentrations of antibody-depleted human serum in the dilution buffer were tested to find this optimal concentration. Standard meningococcal reference serum CDC1992 was 3-fold diluted (1:50 to 1:109,350). From the control panel, two in-house control serum samples, one at the upper end and the other at the lower end of the standard curve, were selected and diluted 1:1,000 or 1:100, respectively. These controls were included in each run to evaluate the run. Serum samples were diluted in the range from 1:100 to 1:1,600, and 50 μl of each prediluted serum sample was added to the wells. Saliva samples were diluted from 1:1 to 1:20 directly in wells. Samples and microspheres were incubated for 1 h. All incubation steps were done on a plate shaker set to 600 rpm at RT and in the dark. The plates were washed three times with PBST using a magnetic washer. For the IgG assay, 50 μl of R-phycoerythrin (PE)-conjugated anti-human IgG (Sigma-Aldrich, MO, USA) diluted to 1:200 in PBS was added and incubated for 25 min. For the IgA assay, 50 μl of a 1:1,000 dilution of monoclonal mouse anti-human IgA (Sigma-Aldrich, MO, USA) was added and incubated for 25 min before adding 50 μl of a 1:200 dilution of R-PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Inc., PA, USA) and subsequently incubated for another 25 min. Various concentrations of all secondary antibodies were tested. After incubation with secondary antibodies, plates were washed and microspheres resuspended in 125 μl of PBS. Results were read on a Bio-Plex reader (Bio-Rad, United Kingdom) detecting fluorescent intensity, and Bio-Plex Manager 6.1 software (Bio-Rad, United Kingdom) was used to generate a 5-parameter log-log standard curve for analyzing mean fluorescent intensity (MFI) of unknown samples against the known concentrations of reference serum CDC1992 to determine Ig concentrations in the samples.

Assay sensitivity. The sensitivity of the multiplex assay was determined by comparing the values from blank wells to the standard curve, as described previously ([8]), with slight modifications using 24 blank wells in three different runs with different batches of conjugated microspheres for the calculations. The lower limit of detection (LLOD) in picograms per milliliter was calculated from the relevant standard curve, based on the averaged MFIs, with two positive standard deviations. This was done for each serogroup and for IgG and IgA.

Assay specificity. To assess assay specificity, we tested reference serum CDC1992 in an inhibition assay, adding free polysaccharides. Homologous free polysaccharides were expected to cause close to 100% inhibition, whereas heterologous free polysaccharides were not expected to cause any inhibition. Reference serum was diluted 1:100 in dilution buffer and incubated for 1 h with 2.5 μg/ml of each polysaccharide, as described by Lal et al. ([8]). Reference serum with no added polysaccharides was used as a control and all dilutions were run as regular samples in either the IgG or IgA multiplex assay.

Assay precision. For determination of intra-assay precision, duplicates of different serum (*n* = 15) and saliva (*n* = 15) samples were used. Coefficient of variation (CV) was calculated for each duplicate and then averaged.

For interplate precision, 15 serum and 15 saliva samples were analyzed

in three different runs on different days and with three different lot preparations of conjugated microspheres and other reagents. The percent CV across these three assays was calculated for each sample and averaged.

Interoperator precision was determined from assays run by three different operators, of whom two had limited experience with this assay. The assays were performed on different days, using different lots of conjugated microspheres. The percent CVs of serum ($n = 14$) and saliva ($n = 16$) samples were calculated and averaged.

Stability. The stability was assessed by comparing MFIs of standard curves generated in assays performed within 1 week after polysaccharide conjugation to microspheres to standard curves generated in assays performed with microspheres that had been stored for 3 and 6 months after conjugation, as well as comparing percentages of aggregated microspheres ([26]). In addition, Ig concentrations in serum and saliva samples were analyzed at these same time points and compared. Stability of the DMTMM-modified polysaccharides was assessed by comparison of microspheres conjugated with polysaccharides freshly modified with DMTMM and after storing the modified polysaccharides for 4 months at $-20°C$. Furthermore, the stabilities of the test samples were assessed by reading plates at different times (1 h, 3 h, 1 day, and 2 days) after the assay was finished, storing the plates at 4°C, and shaking them for 5 min prior to reading.

ELISA for quantification of anti-meningococcal polysaccharide IgG and IgA antibodies. The IgG and IgA ELISA procedure was performed as previously described ([23]) with some modification. In brief, Nunc Maxi-Sorp 96-well microtiter plates (Thermo Fisher Scientific, Inc., MA, USA) were coated with serogroup A, C, W, or Y meningococcal polysaccharides in complex with methylated human serum albumin at a final concentration of 10 μg/ml of each component. Coated plates were incubated overnight and stored up to 14 days at 4°C. The day of analysis, plates were washed in PBS with 0.1% Brij and 0.02% sodium azide and incubated for 1 h at RT with 3% fetal calf serum (FCS) in PBS with 0.02% sodium azide. Samples were set up in two dilutions, serum samples diluted from 1:100 to 1:1,000 and saliva samples diluted at 1:5 to 1:20, in dilution buffer containing PBS with 3% FCS, 0.1% Brij, and 0.02% sodium azide. Serial 2-fold dilutions of reference serum CDC1992 were prepared in the range from 1:100 to 1:12,800. Test sample or reference serum was added in duplicate, 100 μl to each well. The microtiter plates were then incubated overnight at 4°C. After washing, secondary alkaline phosphatase antibodies, goat anti-human IgG (gamma chain specific), or goat anti-human IgA (alpha chain specific) from Sigma-Aldrich (MI, USA), were added and plates were further incubated at 37°C for 2 h. After being washed, 100 μl of 1 mg/ml *p*-nitrophenyl phosphatase substrate (Sigma-Aldrich, MI, USA) prepared in an in-house 10% diethanolamine buffer was added to each well. Optical density (OD) values were read at 405 nm on an Emax plate reader (Molecular Devices, United Kingdom) when the reference serum at dilution 1:100 had reached an OD of approximately 2. IgG or IgA antibody concentrations were calculated against the standard reference curve using a 4-parameter logistic curve-fitting analysis in SoftMax 2.35 computer software.

Statistical analysis. Statistical analyses were done using Stata SE/13, GraphPad Prism 5, and Microsoft Excel 2010 software.

RESULTS

Development of a multiplex microsphere assay for quantification of meningococcal serogroup-specific IgG and IgA antibodies. Each polysaccharide was conjugated onto distinct microspheres and initially run in monoplex to evaluate the range of fluorescent intensity and the linearity of the standard curve. The standard reference serum showed good linearity over an eight 3-fold dilution range in the IgG assay and a seven 3-fold dilution range in the IgA assay. We used a 3-fold dilution ranging from 1:50 to 1:109,350, as dilutions of <1:50 appeared to be above the point of saturation for serogroup A and C in the IgG assay and dilutions of >1:109,350 reached the values of the blank for serogroup W

and Y in the IgA assay. When analyzing serum samples at different dilutions from 1:100 to 1:1,600, parallelism with the standard curve was observed for all dilutions ([24]). When analyzing saliva samples, on the other hand, a dilution of 1:1 appeared to be too concentrated, giving lower values (prozone), whereas a 1:20 concentration seemed to overestimate antibody levels, and linearity was shown only in the dilution range from 1:5 to 1:10.

To ensure that only those antibodies directed against the polysaccharide originally conjugated to the distinct microspheres were detected and to rule out migration of conjugate between microspheres or interference when the microspheres were mixed into a multiplex, averaged MFIs generated from three runs in the monoplex assay were compared to averaged MFIs generated from three runs in the multiplex assay. For all serogroups, there was virtually no difference in MFIs of the standard reference serum between the monoplex and multiplex for IgG (Fig. 1) and IgA (Fig. 2).

Different concentrations of microspheres added to each well were tested with no difference in results, although an increase in reading time was observed when using <1,500 microspheres/well. A concentration of 2,500 microspheres/well was chosen, as it was considered practical to avoid using excessive amounts of expensive microspheres but still resulted in reasonable reading times (approximately 45 min for a full 96-well plate) and gave the opportunity to reanalyze the assay if some unexpected error occurred.

Assay sensitivity. Sensitivity of the IgG and IgA assays was determined by calculations from blank wells. The LLOD was calculated from the corresponding standard curves and was <280 pg/ml for IgG and <920 pg/ml for IgA (Table 1). Although low in each assay, there was a significant difference in sensitivity for IgG and IgA antibodies in all serogroups.

Assay specificity. To ensure that the assay measured serogroup-specific antibodies only and that the polysaccharides had not been altered in the conjugation process, an inhibition specificity assay was performed. The MFIs of the standard reference serum generated in the different assays are shown in Fig. 3. Very good specificity was observed for all serogroups, with homologous inhibition for all serogroups of ≥92% in the IgG assay and ≥91% in the IgA assay and heterologous inhibition of ≤11% in the IgG assay and ≤6% in the IgA assay. The same inhibition assay was performed with in-house serum controls showing the same good specificity, but when analyzing in-house saliva controls, some cross-reactivity between serogroup W and Y was observed (data not shown).

Assay precision. The intra-assay CV was <5.1% for the IgG and IgA assays against all four serogroups (Table 2).

The interassay precision was assessed with different conjugations of microspheres and preparations of reagents to ensure maximal variability and was overall very satisfactory, with mean percent CV values for all serogroups, sample materials, and Ig classes <20% (Table 2). This suggested low assay to assay and batch to batch variation of the conjugated microspheres. The interoperator precision with different lots of microspheres was somewhat higher, but all precision measurements were within 25.8% CV.

Stability. When assessing stability, by comparing MFIs of the standard reference serum in assays using newly conjugated microspheres and assays using microspheres conjugated 3 months and 6 months prior, there was a reduction in the MFI of ≤45% after 3 months and ≤75% after 6 months (only analyzed for IgG). However, when quantifying serum and saliva samples using the refer-
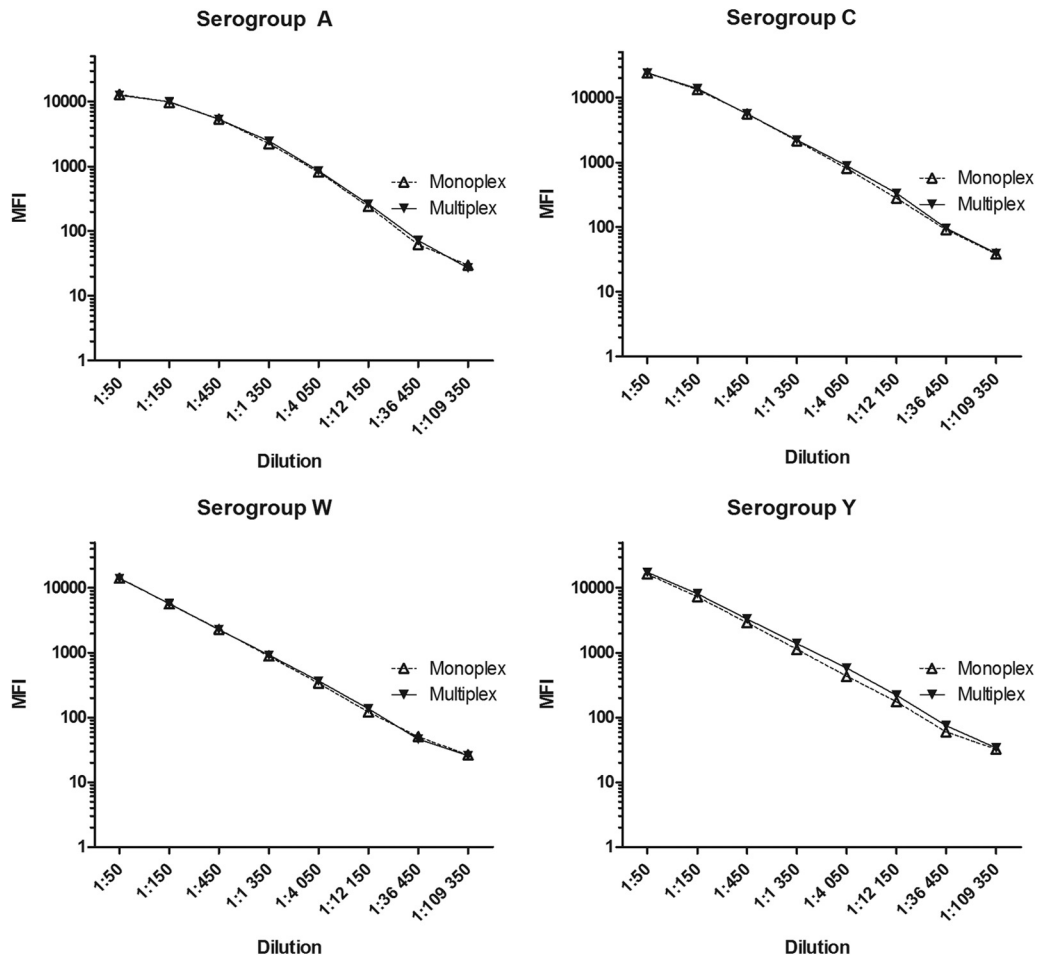
FIG 1 Mean fluorescent intensity (MFI) values generated with standard reference serum CDC1992 (NIBSC 99/706) when run as IgG monoplex and multiplex assays.

ence serum, there was no difference in Ig levels above normal interassay variation. Additionally, there was no actual loss in range and no increase in bead aggregation during performance of the assay (data not shown).

The ability to freeze and store the DMTMM-modified polysaccharides was investigated after storage for 4 months at −20°C. This revealed no difference above normal interassay variation when comparing standard curves generated from microspheres conjugated with fresh or stored modified polysaccharide (data not shown).

Reading of plates at different time points after completing the assay showed no decline in MFI values and no difference in either IgG or IgA concentrations in serum and saliva, although an increase in the percentage of aggregated microspheres was observed after 1 and 2 days: 4.3% in the original assay compared to 13.3% and 8.3%, respectively, when averaged across the IgG and IgA assay. When reading the same plates >4 times, the required time to detect sufficient amount of microspheres and read the MFI increased but no difference in outcome was measured.

Comparison of the multiplex microsphere assay with ELISA. For comparison of the multiplex microsphere assay to the standardized meningococcal polysaccharide ELISA, a panel of 54 serum samples and 16 saliva samples was analyzed with the two

methods, and concentrations of serogroup-specific IgG and IgA antibodies were determined. The results were analyzed by linear regression and correlation coefficients were calculated. Highly significant correlations were found for IgG (Fig. 4) and IgA (Fig. 5) antibodies in serum. When analyzing saliva samples, we did not detect any consistent correlation between the two methods, with correlation coefficients ranging from 0.02 to 0.8 (data not shown).

For further evaluation of the assay's performance and suitability to analyze salivary samples, a selection of saliva samples from individuals before and after vaccination with a monovalent meningococcal serogroup A conjugate vaccine, MenAfriVac, was analyzed. These results showed an increase in specific anti-meningococcal serogroup A IgG ($P = 0.023$) and IgA ($P = 0.002$) antibodies after vaccination but no increase in anti-serogroup C, W, and Y antibodies (Fig. 6).

DISCUSSION

We developed a meningococcal polysaccharide multiplex assay to measure specific IgG and IgA antibodies and compared the results with those obtained in conventional ELISA. The assay showed a wide dynamic range, and the standard reference serum CDC1992 generated a linear standard curve over a ≥21-fold dilution range. When analyzing serum samples, parallelism with the standard
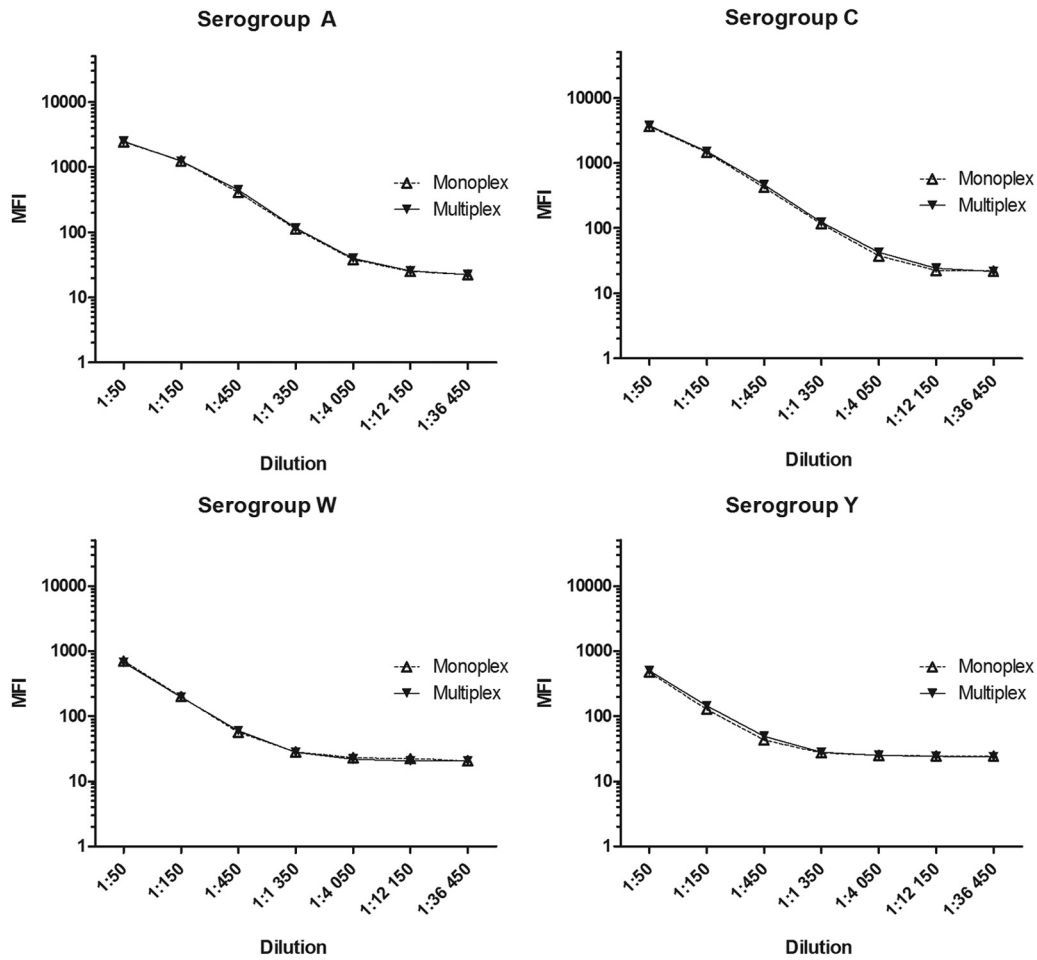
FIG 2 Mean fluorescent intensity (MFI) values generated with standard reference serum CDC1992 (NIBSC 99/706) when run as IgA monoplex and multiplex assays.

curve was observed for a wide range of dilutions. However, when analyzing saliva samples, this was true only in the dilution range from 1:5 to 1:10. Thus, each sample was applied in one dilution only for antibody determinations but with a much wider dilution range for serum samples than that of saliva samples. The MFI and concentration levels in the 1:1 dilution of saliva, lower than those of the more diluted samples, may be explained by interference due to the viscosity or matrix effect of the saliva at a low dilution. Despite this, a 1:1 dilution did not result in more variability, and the results were consistent over several runs.

Cross-reactivity and interference were not observed when comparing IgG monoplex and multiplex assays, suggesting that

only serogroup-specific antibodies were detected and that the coupling of the modified antibodies to the microspheres was stable with no crossover of conjugate.

The inhibition assay showed high homologous inhibition and low heterologous inhibition, suggesting that the polysaccharides were not structurally altered by the DMTMM modification and conjugation process and remained intact after coupling to the microspheres. The specificity was even better than what has been found by others using poly-L-lysine (PLL) as a coupling agent (8), which is consistent with findings by Schlottmann et al. (15) when comparing the conjugation methods for pneumococcal polysaccharides. When analyzing saliva controls (*n* = 2) in the same inhibition assay, on the other hand, there appeared to be some cross-reactivity between serogroup W and Y polysaccharides. As this was not seen in serum, we believe it is not a reflection of poorer specificity of the assay when analyzing saliva but a result of physiological cross-reactivity of salivary antibodies induced by the very similar structures of the W and Y polysaccharides (25).

The precision of the assay was good, with very low intra-assay variation and limited lot to lot variation of coupled microspheres. The interassay precision was all below <20% CV, whereas the interoperator precision was, as expected, somewhat higher. How-

TABLE 1 LLOD for IgG and IgA antibodies against serogroup A, C, W, and Y meningococcal polysaccharides

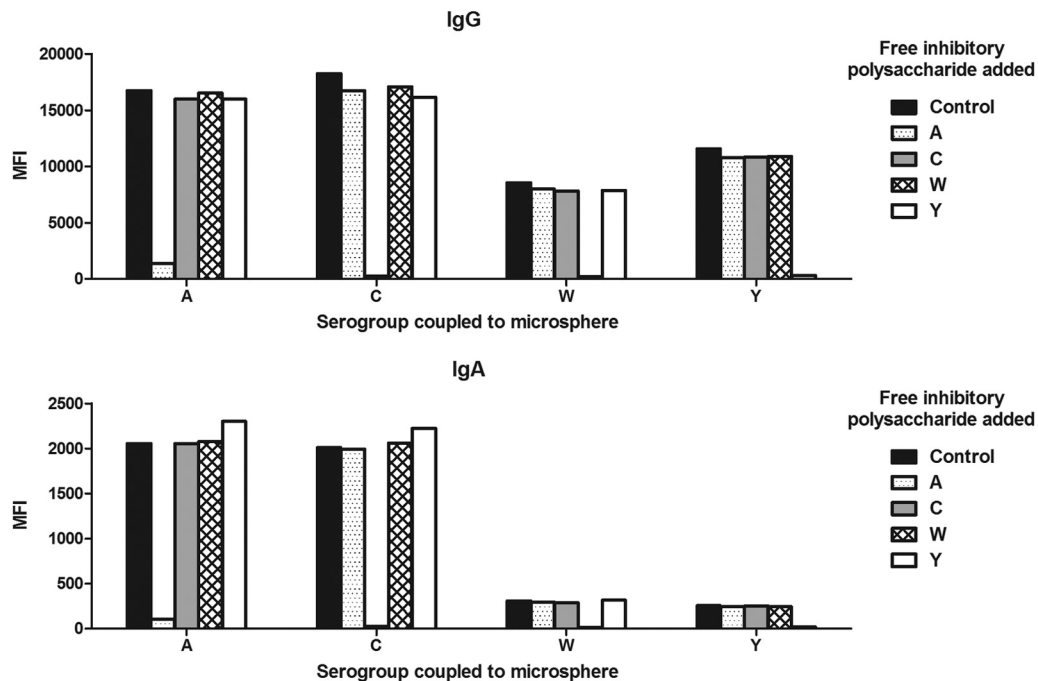| Serogroup	LLOD (pg/ml) for:	
	IgG	IgA
A	280	410
C	70	220
W	50	577
Y	100	920

FIG 3 Mean fluorescent intensity (MFI) values generated with standard reference serum CDC1992 (NIBSC 99/706) for IgG and IgA following individual incubation with either A, C, W, or Y free inhibitory polysaccharides. Incubation with dilution buffer only was used as a control.

ever, even when performed by operators with limited experience with this assay, which is likely to increase the variation, the interoperator precision was acceptable.

Among the major advantages of multiplex assays is the reduction in time and labor due to analyzing four serogroups simultaneously, which is not possible in ELISA. For example, the time spent analyzing 15 samples for all four serogroups was reduced from 1.5 days in the ELISA to about 4 h in the multiplex assay. Despite the fact that some of the reagents used in the multiplex, especially the microspheres, are expensive, the reduction in time

and labor results in an overall reduction in costs. Performance of the entire multiplex assay within 1 day, as well as no difference seen if the plate is read repeatedly even after 2 days, permits flexibility in the planning and execution of the laboratory work.

Maybe the most important advantage of the multiplex assay is the reduction in required sample volume compared to that of the ELISA. Minimizing the need for repeated analyses for each serogroup and thereby conserving sample material is the most important factor, but additionally, less sample volume in each assay is required. This is highly relevant especially for saliva samples,

TABLE 2 Precision of the multiplex assay

Parameter	Sample type	Serogroup A	Serogroup C	Serogroup W	Serogroup Y
Intra-assay (mean % CV of duplicates)[a]					
IgG	Serum	3.3	3.4	3.9	4.5
	Saliva	1.4	2.4	2.3	2.0
IgA	Serum	3.4	5.1	5.0	4.5
	Saliva	1.6	1.4	3.9	1.9
Interassay (mean % CV of samples)[b]					
IgG	Serum	12.6	16.2	19.7	16.7
	Saliva	18.9	15.7	16.6	17.7
IgA	Serum	13.2	10.7	12.4	16.2
	Saliva	12.0	11.8	9.1	10.2
Interoperator (mean % CV of samples)[c]					
IgG	Serum	19.7	20.8	23.1	21.2
	Saliva	15.1	23.9	25.8	25.6
IgA	Serum	16.0	12.6	19.1	16.4
	Saliva	14.6	19.9	22.6	17.0

[a] $n = 15$.
[b] $n = 15$; 3 different runs.
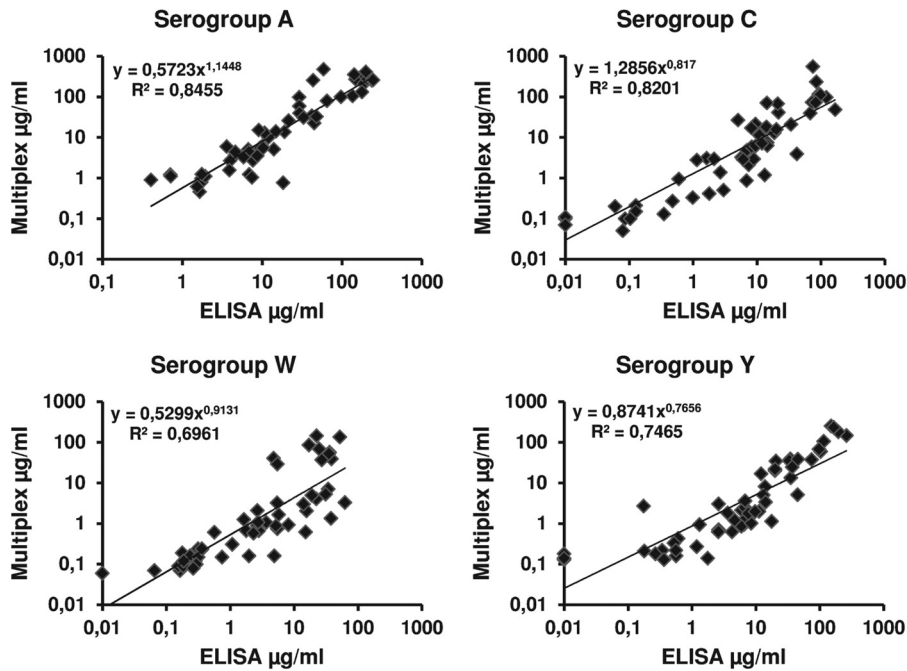[c] $n = 14$ (serum) and 16 (saliva); 3 different operators.

FIG 4 Correlation of serogroup-specific anti-meningococcal polysaccharide IgG antibody concentrations in serum samples ($n = 54$) measured by ELISA and multiplex assays.

which may be difficult to obtain in large enough quantities to analyze in multiple assays, as they cannot be diluted to the same extent as serum samples. In order to analyze salivary IgG and IgA antibodies against all four serogroups, one only needs 1/30 to 1/60 of the volume necessary for the same analysis in ELISA.

Two other important features of the multiplex assay also favor

the use of such an assay when analyzing samples with low levels of antibodies, i.e., saliva: greatly increased sensitivity and dynamic range. The LLOD is <280 pg/ml for IgG and <920 pg/ml for IgA and shows the good sensitivity of the assay. The assay was reliable, with acceptable interassay CVs for samples with antibody levels as low as 1.5 ng/ml. However, samples with values <1 ng/ml had
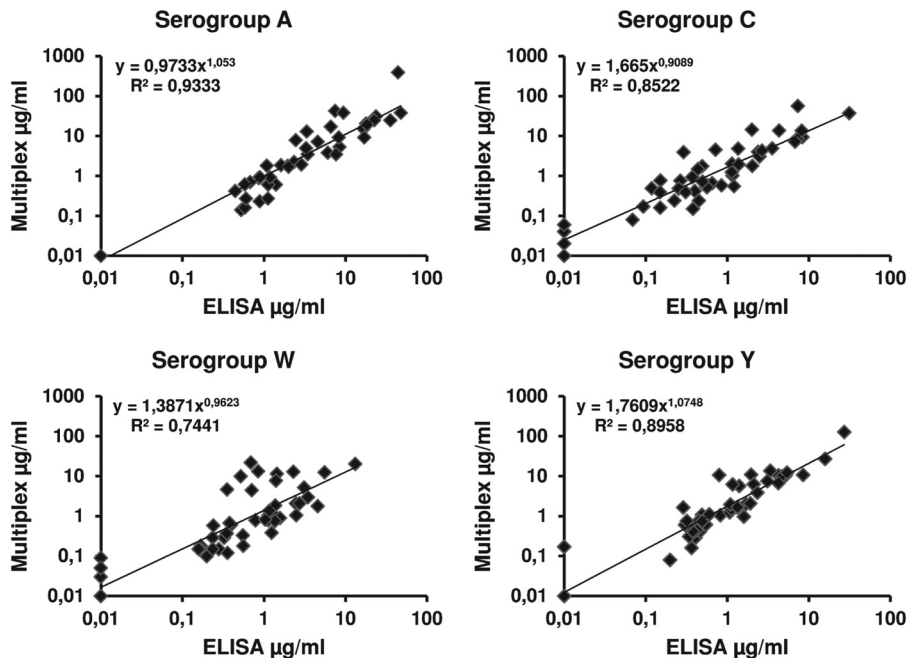

FIG 5 Correlation of serogroup-specific anti-meningococcal polysaccharide IgA antibody concentrations in serum samples ($n = 50$) measured by ELISA and multiplex assays.

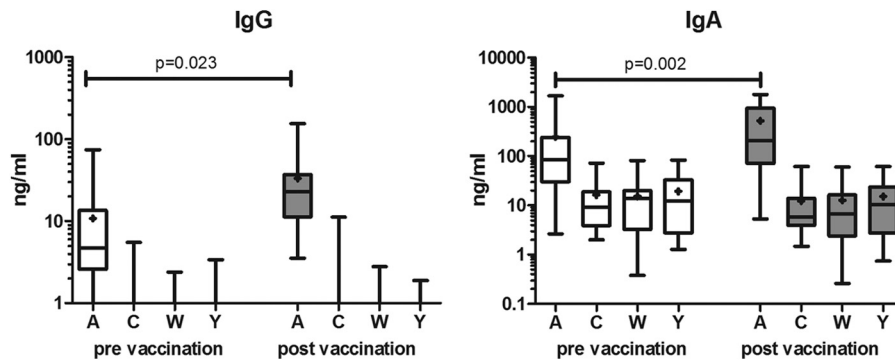FIG 6 IgG ($n = 31$) and IgA ($n = 21$) concentrations in saliva before and after vaccination with a monovalent serogroup A meningococcal conjugate vaccine (MenAfriVac) in Burkina Faso. Boxes extend from the 25th to the 75th percentile with a horizontal line at the median. Whiskers represent ranges, and plus symbols (+) represent means.

high increased interassay CVs (≥70%), which may suggest that the lower limit of quantitation for the assay may be around 1,000 pg/ml. Nonetheless, an LLOD of 1 ng/ml is considered to be very good and is >50 times lower than what has previously been shown for the standard meningococcal ELISA (8). We did observe that antibody concentrations determined by the multiplex assay were lower than those determined by the ELISA for samples with very low antibody concentrations (data not shown). This has been seen by others (8) and is believed to be due to the increased dynamic range of the multiplex, which is better at differentiating between samples with low levels of antibodies. All in all, this makes the multiplex assay preferable and highly suitable for use when analyzing saliva or other types of samples where comparison of very low antibody levels is relevant.

The difference in sensitivities between the IgG and the IgA assays might be explained by the different secondary detection antibodies used, especially by the fact that in the IgA assay the detecting antibodies are like a sandwich made up of two different secondary antibodies. However, the sensitivities of the two assays are well below the likely practical limit for quantitation of 1 ng/ml, and each is considerably lower than the standard meningococcal ELISA.

IgG and IgA antibody levels in serum determined by the multiplex assay and ELISA showed good correlation. When analyzing saliva, however, no clear correlation was observed. This can be due to the low number of saliva samples analyzed and because of the difference in the two methods' abilities to analyze samples with low antibody levels. As previously discussed, the sensitivity and reproducibility of the ELISA are poorer than for the multiplex assay, and half of the saliva samples analyzed had antibody levels below the limit of detection in the ELISA. This may explain the observed lack of correlation when analyzing saliva samples. The development and evaluation of the assay developed here, measuring IgA antibodies and analyzing saliva samples, are valuable additions to previous work investigating IgG antibodies in serum only. The reliability, simplicity, and robustness of our assay are comparable to other meningococcal multiplex assays (8, 11), which use PLL as a coupling agent instead of DMTMM. In addition, the method described here avoids the use of toxic chemicals, such as cyanuric chloride, which is used in the PLL coupling method (15).

Our multiplex assay takes valuable advantage of the multiplexing possibility of the microsphere based flow cytometric technol-

ogy, but as 100 distinct microspheres are available, there is a great possibility of expanding the assay. Additionally, with replacement of the monoclonal anti-human IgA secondary antibody only, the assay will be suitable for detection of other Ig classes; work on this is in progress. In conclusion, we have developed a simple, specific, sensitive, and reliable multiplex microsphere assay for the simultaneous detection of specific IgG and IgA antibodies against meningococcal serogroup A, C, W, and Y capsular polysaccharides. The assay shows good correlation to the standard meningococcal ELISA in serum and is applicable to use for analyzing saliva samples. It is a convenient alternative to ELISA when investigating the immune response against multiple meningococcal serogroups. The method is highly relevant for investigating the effects of meningococcal vaccines, carriage, and disease, as well as for studying the potential for salivary antibodies as a surrogate for serum antibody levels and for immunosurveillance studies.

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