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Title

Measurement of antibodies to pneumococcal, meningococcal and haemophilus polysaccharides, and tetanus and diphtheria toxoids using a 19-plexed assay.

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Abstract

The measurement of antibody responses to vaccination is useful in the assessment of immune status in suspected immune deficiency. Previous reliance on enzyme-linked immunoabsorbent assays (ELISA) has been cumbersome, time-consuming and expensive. The availability of flow cytometry systems has led to the development of multiplexed assays enabling simultaneous measurement of antibodies to several antigens. We optimized a flow cytometric bead-based assay to measure IgG and IgM concentrations in serum to 19 antigens contained in groups of bacterial subunit vaccines: pneumococcal vaccines, meningococcal vaccines, *Haemophilus influenzae* b (Hib), and tetanus and diphtheria toxoid vaccines. 89-SF was employed as the standard serum. The assay was used to determine specific antibody levels in serum from 193 healthy adult donors. IgG and pneumococcal IgM antibody concentrations were measurable across 3 log₁₀ ranges encompassing the threshold protective IgG antibody levels for each antigen. There was little interference between antibody measurements by the 19-plexed assay compared with monoplexed assays, and a lack of cross-reactive IgG antibody, but evidence for cross-reacting IgM antibody for 3/19 pneumococcal antigens. 90th centile values for 15/19 IgG concentrations and 12/12 IgM concentrations of the 193 adult sera were within these ranges and percentages of sera containing protective IgG antibody levels varied from 4% to 95% depending on antigen. This multiplexed assay can simultaneously measure antibody levels to 19 bacterial vaccine antigens. It is suitable for use in standard clinical practice to assess the in vivo immune response to test vaccinations and measure absolute antibody levels to these antigens.

Keywords

Antibody
 Multiplex
 Vaccination
 Flow cytometry
 Bacterial
 Immune deficiency

Abbreviations

ELISA enzyme-linked immunosorbent assay
 Hib *Haemophilus influenzae* b
 Pn pneumococcal
 Men meningococcal
 CVPS pneumococcal cell wall polysaccharide
 PS polysaccharide
 MFI mean fluorescent intensity
 NHSBT NHS Blood and Transplant
 ATCC American Type Culture Collection
 NIBSC National Institute for Biological Standards and Control
 NEQAS National External Quality Assurance Scheme
 WHO World Health Organization

1. Introduction

Investigation of the immune response to vaccination is an important part of the immunologist's toolbox when assessing a patient's immunocompetence in the context of suspected primary or secondary immunodeficiency. The immune response to vaccination allows the clinician to determine whether an individual is able to mount an immune response to vaccination and whether protective antibody levels are present. The principle use of this method is in the investigation of patients with suspected antibody deficiency (Paris and Sorensen, 2007), particularly those with possible specific polysaccharide antibody deficiency (Jeurissen et al., 2007). However, the involvement of T cell help in many vaccine responses broadens the usefulness of this method beyond just the assessment of B cell competence. In this respect, the use of a combination of vaccines delivering T-dependent, T-independent and protein-conjugated T-independent antigens will provide the broadest range of information on the competence of an individual's immune system. Test vaccination is included in the guideline algorithms for assessment of immunity both in Europe (de Vries E., 2006; Spickett et al., 1995) and North America (Bonilla et al., 2005; Folds and Schmitz, 2003).

Various studies have used vaccine responses in the assessment of immunity, but these have often focused on the immune response to just one (Lankinen et al., 2004; Wasserman and Sorensen, 1999) or two (McCusker et al., 1997; Raby et al., 1996) vaccines. Measurement of vaccine responses is typically expensive and time-consuming when performed by standard ELISA and may involve sending samples to more than one specialist laboratory. This problem is increased by the need to measure several serotype-specific antibody concentrations in order to assess the response to multivalent vaccines such as the currently-available pneumococcal and meningococcal vaccines. Whole-vaccine ELISA against such vaccines have been employed in a number of laboratories (Hazlewood et al., 1993), but these are unable to distinguish between a high response to one vaccine component and a broad response to all components (Balmer et al., 2003; Huo et al., 2002).

There has been a recent increase in the development and use of multiplexed bead-based assays for assessing antibody responses to vaccination. Such assays are able to simultaneously measure antibody levels to more than one monovalent vaccine (Pickering et al., 2002b) or a number of serotype antigens in multivalent vaccines, such as quadrivalent meningococcal vaccines (Lal et al., 2004) and different multi-valent pneumococcal polysaccharide vaccines (Borgers et al., 2010; Elberse et al., 2010; Lal et al., 2005; Pickering et al., 2002a). Standardization of these assays has been possible with the assigning of antibody concentrations in internationally available reference sera, such as 89-SF, to a range of components in such vaccines (Holder et al., 1995; Joseph et al., 2004; Quataert et al., 1995; Quataert et al., 2004). Advantages of multiplexed assays include speed, reduced cost and smaller sample volume requirements compared with conventional ELISA, while good agreement with equivalent ELISA has been maintained (Elberse et al., 2010; Lal et al., 2004; Lal et al., 2004; Pickering et al., 2002b; Pickering et al., 2002a). Increased throughput and lower cost in such assays facilitates the possibility of measuring other antibody isotypes in addition to IgG, such as IgM. IgM is the first isotype to appear in acute infection and the appearance of other antibody classes is dependent on normal class-switching.

In the present study we describe the development and use of a 19-plexed antibody assay that simultaneously measures antibody concentrations against five vaccines: two multivalent polysaccharide/polysaccharide conjugate vaccines (23-valent/13-valent pneumococcal and quadrivalent meningococcal vaccines), one monovalent polysaccharide conjugate vaccine (Hib) and two protein vaccines (tetanus and diphtheria toxoid). The assay was then used to measure specific IgG and IgM antibody concentrations in 193 healthy adult sera.

2. Materials and Methods

2.1. Ethical approval

Ethical approval for the use of anonymous blood samples for the optimization of the 19-plexed assay from healthy adults was granted by the Life and Health Sciences Ethical Review Committee of the University of Birmingham. Informed written consent was obtained from participants.

2.2. Serum

89-SF (Food and Drug Administration, Maryland, US) was used as the standard serum. Published IgG and IgM absolute concentrations for 89-SF for pneumococcal (Quataert et al., 1995; Quataert et al., 2004) and IgG concentrations for the four meningococcal serotypes (Joseph et al., 2004) are available. International reference sera 96/536 (anti-Hib Human Reference Serum), TE-3 (1st International Standard for Tetanus Immunoglobulin, Human) and 00/496 (Diphtheria Antitoxin Human Serum) (all National Institute for Biological Standards and Control; NIBSC, Potters Bar, UK) were used to assign anti-Hib, tetanus and diphtheria toxoid IgG concentrations to 89-SF. Serum samples from 193 adult donors for determining adult ranges for the assay were from NHS Blood and Transplant, Birmingham, UK (NHSBT; age range 17-65 years). These sera were all obtained in 2007 within 72 hours of venesection.

2.3. Antigens

Thirteen *Streptococcus pneumoniae* (pneumococcal; Pn) capsular polysaccharides (Pn serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F) were obtained from the American Type Culture Collection (ATCC; Virginia, USA). Pn 22F was used as an absorbant to remove to remove cross-reactive antibodies and not as an analyte in the assay. Tetanus toxoid from Quadrantech (Epsom, UK) and all other antigens were from NIBSC: four *Neisseria meningitidis* (meningococcal; Men) capsular polysaccharides (Men serogroup A, C, W-135 and Y), capsular polysaccharide from Hib (Hib PRP), and diphtheria toxoid.

Pneumococcal and Hib polysaccharides were reconstituted in sterile water at 5 mg/ml, meningococcal polysaccharides at 2 mg/ml, and tetanus and diphtheria toxoid at 50 µg/ml. All polysaccharide antigens (except for Pn 22F), but not the two toxoids, were conjugated to poly-L-lysine (PLL; Sigma) using cyanuric chloride as described elsewhere (Lal et al., 2005). The polysaccharide-PLL conjugate was purified using G25 PD-10 Sephadex desalting columns (GE Healthcare, Chalfont St Giles, UK).

2.4. Conjugation of antigens to microspheres

Conjugation of antigens to carboxylated microspheres (BioRad, Hertfordshire, UK) specific for different bead regions on a Luminex instrument was performed using a two-step carbodiimide reaction as described elsewhere (Lal et al., 2005). Briefly, antigens (except Pn 22F) and beads were activated using 5 mg/ml EDC and 5 mg/ml sulpho-NHS (both Sigma) and the conjugation reaction allowed to proceed for 3 hours at room temperature. Antigen concentrations for conjugation were based on values from published studies, together with a titration to ascertain the concentration that gave the maximal range of MFI in the 19plex assay for the corresponding antibody. Following washing with PBS, beads were resuspended in PBS 0.1% BSA, 0.05% sodium azide

and the concentration of beads labeled with individual antigens was determined using a hemocytometer. Labeled beads were stored at 4°C in the dark until required.

2.5. 19-plexed assay

A series of seven 4-fold dilutions of 89-SF as the standard serum starting at 1:20 was prepared using PBS 1% BSA 0.05% Tween 20 containing 5 µg/ml pneumococcal cell wall polysaccharide (CWPS; Statens Serum Institute, Copenhagen, Denmark) as standard diluent buffer. Test serum samples were diluted 1:100 in PBS 1% BSA, 0.05% Tween 20 containing 5 µg/ml CWPS and 5 µg/ml PnPS 22F to absorb non-specific antibodies (Concepcion and Frasc, 2001). 25 µl of bead mix, containing either 2500 conjugated beads for each of the 19 antigens or 2500 beads for just one antigen (for testing monoplex against 19-plex assay performance), was added to each well of a 96 well filter plate (Millipore).

Beads were washed with PBS 0.05% Tween 20 and washes removed using a vacuum pump. Beads were resuspended in 25 µl diluted standard serum or test serum and incubated for 1 hour in the dark at room temperature at 500 rpm on an orbital shaker. Beads were then washed and incubated with 1:200 mouse anti-human IgG or IgM phycoerythrin (PE)-conjugate (Southern Biotech) for a further 30 minutes prior to washing, resuspending in 125 µl PBS 0.05% Tween 20 and acquisition of data using a Luminex-100 instrument (Bio-plex Systems, BioRad Laboratories, California, USA) using low 'RP1' (low PMT – photomultiplier tube) target setting. Data analysis was performed using Bio-plex Manager 4.1.1 software (BioRad). This created a standard curve of median fluorescence intensity (MFI) against antibody concentration for the 89-SF standard serum which the software used to assign specific antibody concentrations to the test sera.

2. 6. Specificity

Assay specificity was determined by preincubating aliquots of 89-SF serum diluted 1:100 with standard diluent buffer with each of the 19 antigens at 2.5 µg/ml for 1 hour at room temperature before performing the 19-plexed assay.

2.7. Reproducibility

Intra-assay variation was assessed by assaying a control serum for specific antibody levels five times on a single plate in one assay and inter-assay variability by assessing the same serum six times on different plates in different assays and calculating the coefficients of variation.

2.8. Measurement of specific antibody levels in normal adult sera

193 NHSBT sera from healthy adults were tested using the assay. Geometric means, medians and 10th and 90th centile values were determined from log-transformed data for each specific antibody using the SPSS 18 software package. Samples with specific antibody concentrations above the measurable range, were retested at a 1:500 dilution.

3. Results

3.1. Assay dynamics

For the majority of specific antibodies, the 19-plexed assay created a standard curve with a dynamic range over six or seven of the seven dilution steps used (Fig. 1), spanning 2.5 to 3.5 log₁₀ units of MFI. For some IgG specific antibodies, such as Pn 14, Pn 19A, Men C and Hib, there was a loss of dynamic range at the highest concentrations of the standard serum. This probably represents saturation of signal intensity (MFI > 10,000) due to high concentrations of specific antibody in the standard and Pn14 and Pn19A have the highest specific IgG concentrations in 89-SF among the different pneumococcal polysaccharides tested. In contrast, for some IgM specific antibodies, such as Pn 3, Pn 6B, Pn 14 and Pn 23F, there was a loss of dynamic range at the lowest concentrations of the 89-SF standard serum. This is probably the result of a lack of IgM in 89-SF targeting these specific antigens. This observation was particularly marked for the tetanus and diphtheria IgM standard curves, for which peak MFIs barely reached 100.

Comparing the 89-SF standard curves generated using the 19-plexed assay with equivalent single bead-specificity monoplex assays, there was little difference suggesting an absence of interference between the different specific antibody bead assays. The only specific antibody for which there was a clear separation of the monoplex and multiplexed standard curves was tetanus IgM.

3.2. Assay specificity

To further investigate the presence of individual bead assay interference within the multiplexed assay, we performed the assay using 89-SF standard serum following pre-incubation with each antigen (Fig. 2). For specific IgGs, there was little evidence of the presence of antibody cross-reactivity among the 19 antigens. Specific IgG MFIs were reduced \leq 12% of the unabsorbed level following absorption with homologous antigen for all antibodies (mean 4%, range 1% to 12%). In contrast, when preabsorbed with each of the heterologous antigens, specific IgG MFIs remained above 60% of the unabsorbed level except for Pn 7F following preabsorption with Pn 6B antigen (33%).

Except for tetanus and diphtheria, specific IgM MFIs were reduced to 17% or less than the unabsorbed level following absorption with homologous antigen (mean 8%, range 2% to 17%). There was evidence for cross-reactive antibodies for a small number of pneumococcal polysaccharide antigens when specificity of IgM antibodies was assessed. Non-specific reduction in IgM concentrations was a particular problem for Pn 6B, Pn 19A and Pn 19F IgM MFIs where preabsorption with eight or nine heterologous Pn polysaccharides reduced these values to <50% of that in unabsorbed serum. This non-specific absorption was not observed between antigens from different bacteria. At the 1:100 dilution of 89-SF used to assess specificity, minimal quantities of IgM to tetanus and diphtheria were present that no conclusions could be drawn concerning these IgM specificities.

3.3. Assignment of IgG antibody concentrations to *Haemophilus influenzae* b, tetanus and diphtheria toxoid in 89-SF standard serum

This was achieved by performing the 19plex assay in the same 96-well plate with four-fold serial dilution series of 89-SF, and international reference sera to Hib

polysaccharide, tetanus and diphtheria toxoid. Standard curves were plotted of the MFIs for 89-SF and the reference serum for Hib, tetanus and diphtheria. Concentrations for each specific IgG were assigned to 89SF using the portions of each graph where the standard curve for 89SF and the relevant international reference serum were parallel (Fig. 3), together with the known concentrations in each international reference serum. 89SF concentrations were determined to be 64 µg/ml anti-Hib polysaccharide IgG, 5.13 IU/ml anti-tetanus toxoid antibody and 0.81 IU/ml anti-diphtheria toxoid antibody. No reference sera were available with assigned IgM values for the non-pneumococcal antigens tested, so absolute IgM concentrations could not be determined for the four meningococcal polysaccharides, Hib polysaccharide, tetanus and diphtheria toxoid.

3.4. Clinical range of assay

Protective thresholds of absolute IgG/total antibody concentrations have previously been determined for most of the 19 specificities measured by the assay: 0.35 µg/ml for the pneumococcal serotypes (Siber et al., 2007), 2.0 µg/ml for meningococcal serogroup A (Makela et al., 1975; Peltola et al., 1977) (threshold adopted for other meningococcal serogroups), 1.0 µg/ml conferring long-term protection for Hib (Kayhty et al., 1983), 0.1 IU/ml for tetanus (Plotkin, 2001) and 0.1 IU/ml for diphtheria (Plotkin, 2001). While 0.35 µg/ml is the protective level for pneumococcal serotype IgGs used by WHO, the American Association of Asthma, Allergy and Immunology recommends the use of 1.3 µg/ml for the prevention of colonization and infection (Bonilla et al., 2005). With the assay dynamic range findings and known concentrations of specific antibodies in 89-SF, we were able to use our assay, with 89SF as the standard serum, to measure all 19 IgG and the 12 pneumococcal IgM levels over a 3 log₁₀ range encompassing their protective thresholds: pneumococcal serotypes: 0.01 to 10 µg/ml; meningococcal serogroups: 0.01 to 15 µg/ml; Hib: 0.02 to 20 µg/ml; tetanus and diphtheria: 0.01 to 10 IU/ml.

3.5. Assay reproducibility

The mean intra-assay coefficient of variation for the specific IgG concentrations was determined as 8% (range 4% to 25%) and the mean inter-assay variability as 31% (range 17% to 57%). For IgM concentrations, these values were 14% (range 8 to 24%) and 33% (range 14% to 39%) respectively.

3.6. Healthy adult range

A range of specific antibody concentrations were determined for sera from 193 healthy adult donors with differences between 10th and 90th centiles greater for IgG (often 1.5 to 2 log₁₀) compared with IgM levels (1 to 1.5 log₁₀) (Table 1). All 10th centile values were in the assigned clinical range for the assay, along with all 90th centile values except those for Pn 14, Pn 18C, Pn 19A and Men A IgG. Median IgG levels against the Pn polysaccharides in the assay were generally higher than the corresponding IgM levels. High median concentrations (>1 µg/ml) were detected for Pn 14, Pn18C, 19A, 19F and Men A, and Pn 6B IgM.

For 10/12 specific Pn polysaccharide IgGs, over 50% of sera assessed contained greater than the WHO protective threshold (> 0.35 µg/ml) with protective levels against Pn 14 and Pn 19A in ≥80% of sera and against Pn 1 and Pn 4 in <50% of sera. While protective IgG concentrations were present in around half of the sera to Hib (41%) and

Men A (56%), less than 20% had protective concentrations against Men C (10%), Men W-135 (4%) and Men Y (18%). Most sera had protective IgG concentrations against tetanus (95%), though only a third against diphtheria (34%).

4. Discussion

The present study extends previous work (Borgers et al., 2010; Lal et al., 2004; Lal et al., 2005) in demonstrating the utility of a multiplexed bead-based flow cytometric assay for the simultaneous measurement of antibody against 19 antigens found in five different vaccines. Previous reports have measured specific antibody concentrations against different serogroup-specific antigens of the same vaccine or pathogen, in particular pneumococcus (Borgers et al., 2010; Lal et al., 2005) and meningococcus (Lal et al., 2004). There have been limited reports of these assays being used to investigate IgM as well as IgG levels. The availability of isotype-specific secondary antibodies makes such studies straightforward. The assay was established first to investigate the *in vivo* response to vaccines containing polysaccharide, protein and polysaccharide-protein conjugate antigens, and second to determine antibody concentrations which may be related to previously assigned protective levels.

This 19-plex assay measures a wide dynamic range of antibody levels, typically covering 3 log₁₀. Despite the large number of specific antibody concentrations assessed, comparison of data from the multiplexed assay with each constituent monoplex assay indicates very little interference (Fig. 1). The IgG specificity is further supported by the antibody pre-absorption studies (Fig. 2). Some cross-reactivity was apparent for IgM antibodies to three target pneumococcal polysaccharide antigens and as has been previously reported (Baxendale et al., 2008). However, there was a lack of such cross-reactivity between IgM antibodies to vaccine antigens from different bacterial species. As multiplexed assays continue to expand, in relation to the number of antibody specificities measured at one time, the potential for cross-reactivity between components of the assay will increase. Therefore, it will be important to carefully evaluate this in each new assay.

The assay requires the availability of a standard serum containing sufficient quantities of antibodies to all 19 antigens measured. 89-SF was produced by the pooling 17 high-titered sera from a group of 68 adults who had been immunized with a 23-valent pneumococcal polysaccharide vaccine, a quadrivalent meningococcal polysaccharide vaccine and a *Haemophilus* conjugate vaccine (Quataert et al., 1995). Given the universal use of tetanus and diphtheria toxoid vaccines, it is unsurprising that 89-SF contains sufficient specific IgG antibodies to adequately determine IgG antibody concentrations against all 19 antigens. The range of specific antibodies required in the standard serum makes it difficult to substitute 89-SF with a secondary serum.

This assay was extended to measure specific IgM antibodies. Although the clinical utility of measuring IgM antibodies is currently uncertain and these antibodies are usually of lower affinity than IgG, IgM antibodies appear early in infections and are important in the protection against some infections including pneumococcus. This antibody class can represent natural antibody, but also forms the early primary response to vaccination and is particularly important for responses to T-independent antigens. Therefore, measurement of IgM in addition to the IgG antibody isotype, could be useful in distinguishing acute from past or chronic infections, in determining the driver of the immune response in relation to T-dependent and T-independent antigens, and the presence or absence of normal antibody class switching which is absent in hyper-IgM syndrome. Specific IgM was not abundantly present in 89-SF for all antigens examined,

particularly tetanus and diphtheria toxoids. These elicit strong T-dependent antibody responses with significant class-switching from IgM. By obtaining the relevant standard sera from NIBSC, we were able to assign to 89-SF concentrations for Hib, tetanus and diphtheria IgG antibodies. No standard sera are currently available with specific IgM concentrations to the meningococcal and Hib polysaccharides, tetanus or diphtheria. Should such concentrations become available, the dynamics of the assay (Fig. 1) would permit measurement of IgM concentrations to the meningococcal and Hib polysaccharides using 89-SF, but not tetanus or diphtheria toxoids owing to the lack of IgM to these antigens in 89-SF.

A wide range of IgG and pneumococcal IgM concentrations around the protective threshold was measurable for each specific antibody using a single 1:100 serum sample dilution. This indicates that the assay is suitable for the clinical purpose of determining response to vaccination and presence of protective antibody against a group of different pathogens. Despite reasonable mean intra- and inter-assay coefficients of variation for both specific IgG and IgM concentrations (8% and 31%, respectively for IgG and 14% and 33% for IgM), the lower intra-assay value indicates that when measuring responses to vaccination, it is advisable to measure concentrations in pre- and post-vaccination samples at the same time. To provide external quality assurance, the assay has been enrolled in the NEQAS (National External Quality Assurance Scheme, UK) Specific Microbial Antibodies Scheme, although this is only available for the IgG class of antibodies.

The determination of antibody concentrations in 193 NHSBT sera served two main purposes. First, this demonstrated that all specific antibody IgG 10th centile values, and 15 out of 19 specific antibody IgG 90th centile values, are within the range we established for clinical use. Adequate response to univalent vaccines is typically assessed as exceeding the relevant protective antibody concentration or a 4-fold increase from the pre-vaccination concentration (Hare et al., 2009) and so this range will be adequate for standard clinical use without requiring repeat sample runs at higher dilutions. For multivalent vaccines, assessment is more complex and is based on the proportion of specific antibody concentrations measured that exceed designated protective levels. Proposed criteria for multivalent pneumococcal vaccines are 50% of specific antibody concentrations exceeding the protective level in children and 70% in adults (Hare et al., 2009). For all antibody specificities tested, the dynamic range of the assay ensures that the defined levels of protective antibodies will be detectable with each sample diluted once at 1:100. In instances where clinicians request peak absolute antibody concentrations, repeat assay runs at higher dilutions may be required.

Second, the ranges determined for the NHSBT samples give some indication of which vaccinations will provide helpful data in relation to the investigation of immunodeficiency, particularly antibody deficiency. Among apparently healthy adult donors, such as NHSBT donors, only a few will lack protective antibody levels to tetanus, potentially limiting the usefulness of test immunization with this vaccine. This likely reflects the widespread use and frequent boosting with tetanus vaccine, together with the T-dependent nature of the protein antigens in the vaccine. By contrast, only a third of the adult sera tested had protective concentrations against diphtheria toxoid which may reflect the lack of booster vaccinations given to adults against diphtheria

compared with tetanus. Variable proportions of individuals had protective IgG concentrations against the different pneumococcal serotypes, most likely reflecting variation in natural exposure to the different serotypes, since it is unlikely that many of these donors will have previously received a pneumococcal vaccine.

Very low proportions of sera had protective antibody levels against Men C. This would not be expected in a pediatric population, due to the incorporation of the Men C conjugate vaccines into the Extended Programme of Immunization in an increasing number of countries, but this vaccination is not administered routinely to adults. By contrast, for 10/12 pneumococcal polysaccharides, over half of the NHSBT sera had protective antibody concentrations with 41% having protective levels against Hib in the absence of an adult program of pneumococcal and Hib vaccination. These findings are consistent with exposure of the adult population in Britain to pneumococcus and Hib, but not to Men C. Limited data are available on specific antibody concentrations in adult populations. The average pneumococcal serotype concentrations in the 193 NHSBT sera from 2007 were generally lower than those published using sera collected in the UK between 2000 and 2004 (Balmer et al., 2007), and in both sets of sera average antibody concentration to MenA was higher than that for serogroup C, W-135 and Y (Trotter et al., 2008). The average tetanus and Hib antibody concentrations were comparable to those found in healthy adults in Germany (Schauer et al., 2003).

A choice of vaccines are available to use in combination with the assay, particularly with reference to pneumococcus and meningococcus for which there are both pure polysaccharide and polysaccharide-protein conjugate vaccines. The former kind of vaccines, including the 23-valent pneumococcal polysaccharide, Pneumovax 2 (Sanofi Pasteur), and quadrivalent A-C-W135-Y meningococcal polysaccharide, ACWYvax (GSK), are suitable for assessing the immune response to T-independent antigens. An increasing number of glycoconjugate vaccines are available including the pneumococcal Prevenar-7, Prevenar-13 (both Wyeth Vaccines) and Synflorix (GSK) vaccines (Levine et al., 2010), and meningococcal quadrivalent Menveo (Novartis Vaccines) and Menactra (Sanofi Pasteur) vaccines. Protein carriers, such as CRM₁₉₇, in these vaccines effectively convert the capsular polysaccharides into T-dependent antigens and so they assess a T cell-dependent immune response (Wuorimaa et al., 2001). Use of the assay to assess T-independent and T-dependent immune responses to vaccination is currently underway in several different clinical settings.

In summary, we have developed and demonstrated the use of a 19-plexed specific antibody assay capable of measuring antibody responses to a combination of five bacterial subunit vaccines. The performance characteristics of the assay are compatible with its adoption into standard use in clinical laboratories.

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Figure legends

1. Specific antibody levels in a standard serum as assessed by a 19-plexed assay compared with monoplex assays. Specific IgG (A) and IgM (B) levels shown as mean fluorescent intensities (MFI) for standard serum 89-SF through a series of seven four-fold dilutions starting at 1:20. Red lines are data acquired using the 19-plexed assay, while blue lines are data from monoplex assays.

2. Effect of preabsorption with specific antigens on antibody measurements by a 19-plexed assay. IgG (A) and IgM (B) measurements for each specific antibody in 89-SF at a 1:100 dilution following preabsorption with each antigen (shown on x-axis) at 2.5 µg/ml for 1 hour at room temperature.

3. Assigning of antibody concentrations to reference serum 89SF against Hib polysaccharide, tetanus and diphtheria toxoid The 19-plex assay was performed with a four-fold dilution series of 89SF and international reference sera 96/536 (Hib), TE-3 (tetanus) and 00/496 (diphtheria). Standard curves were plotted for **A.** Hib, **B.** tetanus and **C.** diphtheria beads and specific antibody concentrations determined for 89SF for each antigen using the known concentrations in the international reference sera.

Table 1. Specific IgG and IgM antibody levels to 19 bacterial antigens in sera from 193 healthy adult Blood Transfusion Service donors measured by a 19-plexed antibody assay. Concentrations are $\mu\text{g/ml}$, except for tetanus and diphtheria which are IU/ml. Protective IgG concentrations: 0.35 $\mu\text{g/ml}$ for pneumococcal (Pn) polysaccharide, 2.0 $\mu\text{g/ml}$ for meningococcal (Men) polysaccharide, 1.0 $\mu\text{g/ml}$ for *Haemophilus influenzae* b (Hib), 0.1 IU/ml for tetanus and diphtheria antigens.

Antigen	IgG		% with Protective concentration	IgM	
	Geometric Mean (95% CI)	Median (10 th - 90 th centile)		Geometric Mean (95% CI)	Median (10 th - 90 th Centile)
Pn 1	0.36 (0.28-0.46)	0.22 (0.05-4.76)	40	0.18 (0.16-0.21)	0.20 (0.09-1.00)
Pn 3	0.81 (0.62-1.04)	0.56 (0.15-8.1)	59	0.30 (0.26-0.35)	0.30 (0.15-1.31)
Pn 4	0.30 (0.26-0.35)	0.30 (0.09-1.03)	45	0.38 (0.33-0.44)	0.40 (0.24-1.81)
Pn 5	0.47 (0.37-0.59)	0.47 (0.06-3.50)	58	0.60 (0.52-0.70)	0.59 (0.29-3.64)
Pn 6B	0.75 (0.59-0.95)	0.87 (0.14-6.56)	69	1.29 (1.07-1.55)	1.35 (0.60-10.60)
Pn 7F	0.91 (0.74-1.13)	0.87 (0.13-5.52)	76	0.80 (0.69-0.94)	0.87 (0.43-4.20)
Pn 9V	0.58 (0.47-0.72)	0.60 (0.07-3.87)	61	0.63 (0.55-0.74)	0.65 (0.33-3.04)
Pn 14	1.39 (1.07-1.81)	1.93 (0.12-11.25)	81	0.38 (0.31-0.47)	0.35 (0.16-2.91)
Pn 18C	1.12 (0.87-1.45)	1.11 (0.10-11.10)	76	0.32 (0.27-0.38)	0.34 (0.14-1.86)
Pn 19A	2.05 (1.68-2.50)	2.20 (0.31-10.59)	89	0.83 (0.73-0.94)	0.82 (0.46-3.74)
Pn 19F	1.12 (0.90-1.39)	1.07 (0.15-7.13)	75	0.96 (0.81-1.13)	0.91 (0.46-8.14)
Pn 23F	0.73 (0.57-0.94)	0.66 (0.10-8.07)	65	0.22 (0.17-0.27)	0.18 (0.09-2.81)
Men A	3.06 (2.48-3.78)	2.63 (0.56-22.92)	56	-	-
Men C	0.14 (0.10-0.19)	0.12 (0.01-1.88)	10	-	-
MenW-135	0.142 (0.12-0.18)	0.16 (0.02-0.77)	4	-	-
Men Y	0.36 (0.27-0.47)	0.23 (0.04-4.75)	18	-	-
Hib	0.61 (0.49-0.78)	0.73 (0.08-4.9)	41	-	-
Tetanus	1.4 (1.12-1.73)	1.77 (0.18-7.24)	95	-	-
Diphtheria	0.07 (0.06-0.09)	0.06 (0.02-0.54)	34	-	-

Reference List

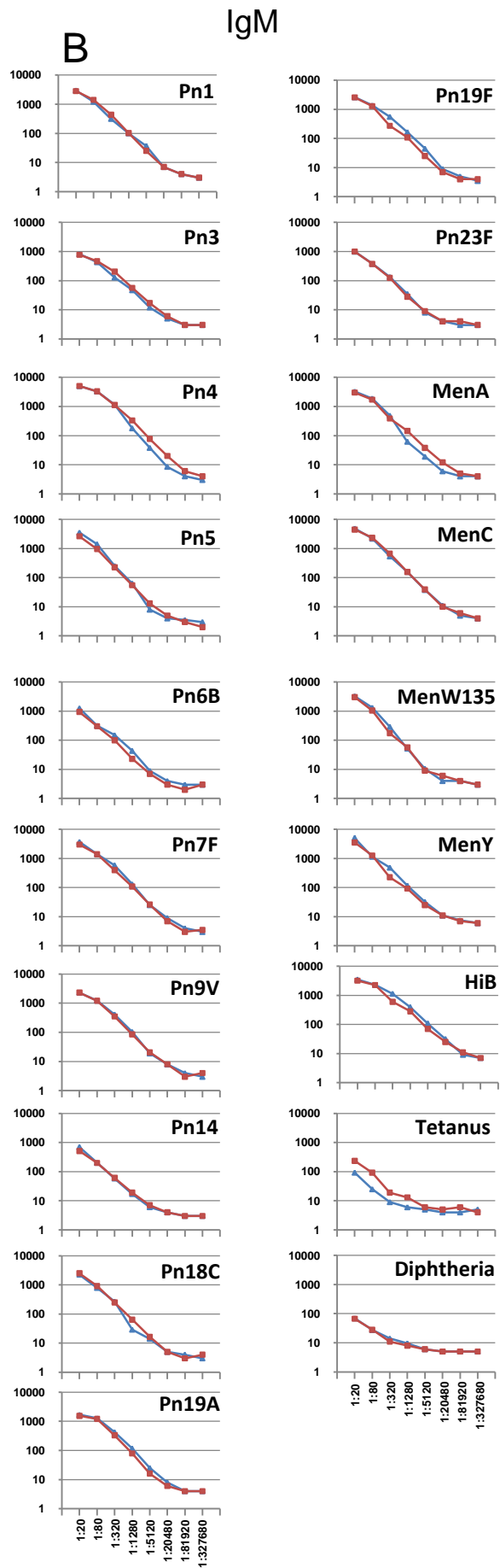
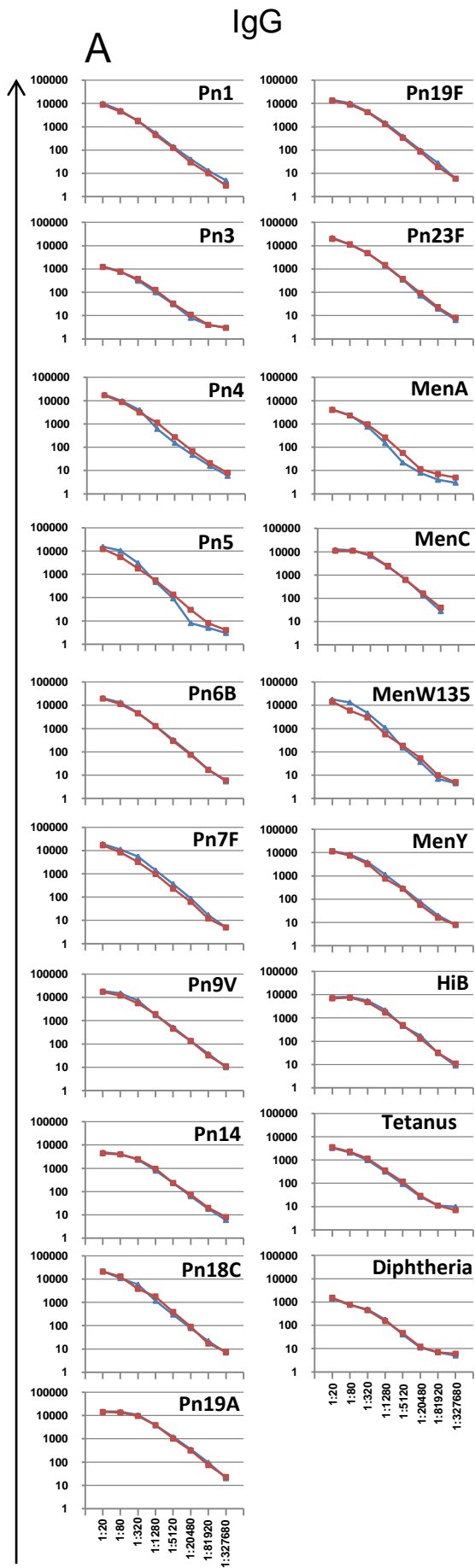
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