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Evaluating patient immunocompetence through antibody response to pneumococcal polysaccharide vaccine using a newly developed 23 serotype multiplexed assay

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ABSTRACT

Assessing T-cell independent antibody response to polysaccharide vaccines is crucial for diagnosing humoral immune deficiencies. However, immunocompetence criteria based on *S. pneumoniae* vaccination remain unclear. We evaluated IgG antibody vaccine response in healthy individuals to establish interpretive criteria. Pre- and 4-week post-vaccination sera were collected from 79 adults. Antibody concentrations to PNEUMOVAX 23 serotypes were measured using a multiplexed platform. Immunocompetence was determined by fold increase in post-vaccination response, percentage of serotypes achieving 4- or 2-fold antibody ratio, and post-vaccination concentration $\geq$ 1.3 μg/mL. Immunogenicity varied widely across the 23 serotypes (26.6% to 94.9% for $\geq$4-fold increase, 51.9% to 98.7% for $\geq$2-fold increase). Immunocompetence based on historic criteria of $\geq$4-fold increase in antibody ratio to $\geq$70% of serotypes was low (72.2%), but increased to 98.7% with criteria of at least a 2-fold increase and/or post-vaccination concentration $\geq$ 1.3 μg/mL. Current criteria for assessing immunocompetence may be overly stringent and require updating.

1. Introduction

Streptococcus pneumoniae is a major human pathogen with much of its virulence due to its polysaccharide capsule. Pneumococci possess approximately 90 antigenically specific capsular polysaccharides that confer type-specific immunity. Different serotypes have distinct epidemiological properties with 23 serotypes accounting for most of pneumococcal bacteremia, pneumonia, and meningitis infections worldwide [1,2]. *S. pneumoniae* is also the most common cause of certain bacterial infections (otitis media, pneumonia and meningitis) in children <2 years of age [3]. Geriatric patients and those at high risk (cardiopulmonary and liver disease, recent splenectomy) are also susceptible to pneumococcal infections [4]. Type-specific IgG antibodies to the capsular polysaccharides of *S. pneumoniae* protect against invasive diseases by opsonizing the organism [5,6] and against infection by preventing the acquisition and carriage of the pneumococci [7,8]. Individuals with certain B cell immunodeficiency disorders do not produce antibodies to polysaccharide antigens and, therefore, may experience chronic or recurring respiratory infections caused by *S. pneumoniae* and other encapsulated bacteria [9–11]. In addition, some people may not produce antibodies to polysaccharides even though their serum immunoglobulin levels are normal and may have what is commonly referred to as specific polysaccharide antibody deficiency syndrome [12].

The antibody response to vaccination with the 23-valent pneumococcal polysaccharide (PnPs) vaccine is widely considered the gold standard for assessing a patient's ability to produce a T-cell independent response to polysaccharides [11,13]. The 23-valent PnPs vaccine (PNEUMOVAX 23, Merck, West Point, NY) (PNX) is widely used for this assessment and contains serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9 N, 9 V, 10 A, 11 A, 12F, 14, 15B, 17F, 18C, 19 A, 19F, 20, 22F, 23F, and 33F. Antibody response to the 23 serotypes in the polysaccharide vaccine varies considerably among healthy individuals [14,15]. Assessment of immunocompetence is commonly performed by determining fold increase of post- to pre-vaccination antibody response, the percentage of serotypes for which a defined fold increase occurred and/or post-vaccination antibody concentrations achieving "protective" levels. Guidelines published by the American Academy of Allergy, Asthma & Immunology define an adequate antibody response in healthy adults to pneumococcal

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serotypes as a post-immunization IgG concentration ≥ 1.3 μg/mL or a 4-fold or greater rise between pre-immunization and post-immunization titers [16] and have been applied to several studies for interpretation of diagnostic pneumococcal vaccination for evaluating primary immunodificiency [17–20]. Age dependent responses were also proposed as children 2 to 5 years of age should respond to at least 50% of the serotypes tested, and adults and children older than 6 years should respond to 70% of the serotypes tested [16]. For single time point measurements, studies have indicated that an antibody concentration >1.0–1.3 μg/mL generally confers long-term protection against that serotype [21].

The aim of this study was to establish immunocompetence criteria in normal healthy adult individuals based on their pre- and post-PNEUMOVAX 23 antibody concentrations utilizing a newly developed and validated 23 serotype multiplexed assay.

2. Materials and methods

2.1. Vaccinated normal participants

Seventy-nine (79) self-proclaimed healthy participants between the ages of 18 to 64 years were recruited for this study. Exclusion criteria included taking any prescription medication intended to reduce inflammation or suppress the immune system and having received a PNEUMOVAX 23, or other pneumococcal vaccination within the past five years. An initial pre-vaccination serum specimen was obtained, with a post-vaccination specimen collected 4-weeks after receiving the PNEUMOVAX 23 vaccine, for a total of 158 specimens.

2.2. Quansys pneumococcal 23 serotype multiplexed assay

This assay was developed as a collaboration with Quansys Biosciences, Logan, UT. All kit manufacturing and assembly was performed at Quansys, with validation testing performed at ARUP Laboratories, Salt Lake City, UT. The Quansys Q-Plex technology is based on printing antigens in 350- to 500-μm spots on the surface of polypropylene 96-well plate wells. The assay utilizes the microplate indirect immunoassay technique for the measurement of human IgG antibodies reactive to pneumococcal polysaccharide antigens printed in distinct regions on the bottom of each well. Two separate assays/microplates were used to detect antibodies against the 23 serotypes. One arrayed with 14 pneumococcal polysaccharides (1, 3, 4, 5, 6B, 7F, 8, 9 N, 9 V, 12F, 14, 18C, 19F and 23F) with the other assay arrayed with 9 pneumococcal polysaccharides (2, 10 A, 11 A, 15B, 17F, 19 A, 20, 22F and 33F). Each well also contains two control spots. One control spot is printed with cell wall polysaccharide (CWPS) and acts as a sample specific control for detecting non-specific CWPS antibody binding. An anti-human IgG antibody control spot is also printed in each well for assuring sample addition and aligning each well for proper imaging. A common specimen diluent was formulated for both assays using CWPS and CWPS2, the purified non-antigenic/protective capsule of 22F [22], to allow for adsorption of non-specific binding. A lyophilized stock calibrator, calibrated to the current WHO International pneumococcal capsule 007sp reference material (National Institute for Biological Standards and Control, Hertfordshire, England) for all 23 serotypes [23–25] was diluted to make a 7-Point calibration curve. Patient specimens and three tri-level assay controls pooled from vaccinated individuals were diluted 1:50 in specimen diluent. Fifty uL of each calibrator, a sample blank, diluted patient samples and controls were pipetted into wells of the 96-well plates and allowed to incubate for 30 min at room temperature. The plate was then washed 3 times and 50 uL of HRP-conjugated anti-human IgG antibody was added, followed by a second 30-min incubation at room temperature. Following a final 6 washes, 50 uL of chemiluminescent substrate was added and the plate was then analyzed using a Q-View Imager (Quansys Biosciences). The amount of HRP-conjugated antibody bound to each location of the array is proportional to the amount of serotype-specific antibody initially captured. Quantitative μg/mL results are calculated based on comparison with a standard curve for each serotype using Q-View Software (Quansys Biosciences). The calibrators and high, medium and low controls are included with each run, and along with patient specimens tested in singlicate. For the assay run to be considered valid, the values of the calibrator must decrease linearly for each serotype. Up to one calibration point per serotype may be masked. Additionally, two of the three control results must fall within their defined ranges for each of the 23 serotypes. If the result for one of the controls does not fall within range, then borderline results around the 1.3 μg/mL cut-off are repeated. Performance parameters including linearity; precision; detection capability; cross-reactivity; interference due to hemolysis, triglycerides and bilirubin; and specimen stability were evaluated following Clinical and Laboratory Standards Institute (CLSI) guidelines as described in the results section. The assay was validated to be performed either manually or on an automated QUANTA-Lyser 3000 liquid handler (Werfen).

2.3. Luminex bead-based pneumococcal 23 serotype multiplexed assay

A pneumococcal 23 serotype multiplexed laboratory developed assay previously validated and utilized by our laboratory was included for comparison studies. Similar to the Quansys assay, two separate bead-based panels were used to detect antibodies against the 23 serotypes. The serotypes contained in each panel were identical to the Quansys assay. This assay has been previously described [26,27].

3. Results

3.1. Performance characteristics of the multiplexed assay

Linearity was assessed in accordance with CLSI guidelines [28]. Four unique post-vaccination specimens were proportionally mixed with a pre-vaccination specimen which had low pneumococcal antibody levels and tested using a minimum of 9 concentrations covering the analytical measurement range (AMR) for the 23 different serotypes. Linearity was assessed using Analyze-it statistical software for Microsoft Excel (Analyse-it Software, Ltd. Leeds, UK) allowing for 25% non-linearity. All serotypes demonstrated a linear fit throughout the AMR of the assay which varied by serotype depending on the assigned value of the stock calibrator.

Within run precision studies were performed by testing high, medium and low value pools in replicates of five on three separate runs. Performance criteria were set at ≤20% CV for results ≥1.3 μg/mL cut-off and ≤ 30% CV results <1.3 μg/mL that were greater than the lower limit of quantitation (LOQ). Performance criteria were met for most serotypes (Table 1) with the following exceptions. The high pool for serotypes 3, 6B, 12F and 15B had CVs of 28.3, 21.2, 20.5, and 25.1% respectively. The medium pool for serotypes 3, 9 N, 15B and 19F had CVs of 21.4, 20.5, 24.7 and 20.5% respectively. All values for the replicates of these discrepants were in qualitative agreement of ≥1.3 μg/mL. For the low pool, the percent CV was 31.5% for serotype 1 and 36.2% for 19 A. These values were measured at the low end of the AMR and results were in qualitative agreement of less than the 1.3 μg/mL cut-off.

Between run precision was evaluated using the same three pools tested in singlet on three separate days. Performance criteria were set at ≤30% CV. All CVs for the high, medium and low pools were <30% for all 23 serotypes (Table 1).

The precision acceptance criteria were based on previous experience with the Luminex bead-based version of the assay and other publications on multiplexed assays [29,30]. The CVs >20% for the within run precision studies were most often associated with a single significant outlier for one of the five replicates. Removal of these outliers would decrease the %CV to withing the acceptable range. In addition, the outliers agreed qualitatively with the other replicates with respect to being above or below the 1.3 μg/mL cut-off.

Table 1
Validation performance characteristics of the multiplexed assay.

Parameter		High Pool	Medium Pool	Low Pool
		Mean (Range)	Mean (Range)	Mean (Range)
Precision	Within Run CV	12.9% (2.1–28.3)	11.0% (2.7–24.7)	11.9% (2.0–38.1)
	Between Run CV	11.0% (1.9–22.5)	11.6% (1.9–19.5)	17.8% (3.9–29.9)
Interference	Hemoglobin Δ of Control	5.7% (−25.8–23.3)	0.4% (−13.2–11.1)	2.0% (−22.2–33.0)
	Triglycerides Δ of Control	6.2% (−20.6–28.1)	−9.8% (−27.5–8.0)	5.9% (−4.2–33.0)
	Bilirubin Δ of Control	0.6% (−19.6–20.4)	−4.1% (−28.9–19.3)	−0.2% (−25.0–26.2)
Stability	24 h ambient Δ of Day 0	3.2% (−7.8–16.9)	2.0% (−19.3–26.5)	2.1% (−16.4–17.5)
	14 Days 4 °C Δ of Day 0	−1.5% (−16.5–24.2)	8.2% (−12.1–38.1)	19.5% (−20.5–57.7)
	3× Freeze/ Thaw Δ of Day 0	−0.9% (−23.3–23.2)	−2.3% (−17.7–16.8)	7.7% (−17.2–42.1)
	69 Days -20 °C Δ of Day 0	7.1% (−17.6–27.9)	0.8% (−20.7–26.6)	8.4% (−20.5–64.7)

Specimen interference was assessed in accordance with CLSI guidelines [31]. Three serum pools with high, medium and low pneumococcal serotype concentrations were spiked with hemoglobin (1530 mg/dL), triglycerides (4260 mg/dL) and bilirubin (22.2 mg/dL) and analyzed in parallel with PBS- or NaOH-spiked control samples. Performance criteria were set at ≤30% change from the controls. Percent change was >30% for serotype 1 for the low pool for hemoglobin and triglycerides but were measured at the low end of the AMR and results were in qualitative agreement of <1.3 μg/mL. Results are summarized in Table 1.

Analyte stability was determined using 3 serum pools with high, medium and low pneumococcal serotype concentrations tested after storage for 2 days at room temperature (20–25 °C), 14 days refrigerated (2–8 °C), 69 days frozen (−20 °C) and after 3 freeze/thaw cycles. Performance criteria were set at ≤30% change from the Day 0 baseline values. Percent Change was >30% for some of the low pool storage conditions. All of these values were measured at the low end of the AMR and results were in qualitative agreement of <1.3 μg/mL. (Table 1).

Detection capability for limit of blank (LOB), limit of detection (LOD) and limit of quantitation (LOQ) were assessed in accordance with CLSI guidelines [32] and calculated using Analyze-it statistical software for Microsoft Excel. For LOB and LOD, 4 blank specimens (assay specimen diluent, calf serum, goat serum and fetal bovine serum) and 4 low pre-vaccination specimens were tested in replicates of 4 on 3 separate runs for a total of 96 data points. For LOQ, two specimen pools with medium valued pneumococcal serotype results were tested at 50%, 25%, 10%, 5% and 1% concentrations. Each dilution was tested in triplicate on 4 separate runs for a total of 120 data points. The lowest mean μg/mL concentration which had a CV of <20% was used to determine the LOQ for each serotype. LODs ranged from 0.01 to 0.15 μg/mL and LOQs from 0.02 to 0.17 μg/mL for the 23 serotypes. The AMR for each serotype was based on the upper LOQ determined by linearity and precision studies and the lower LOQ determined by detection capability studies are shown in Table 2.

Specificity was assessed by homologous inhibition of binding studies performed by Quansys Biosciences. Twenty μg/mL of each serotype was spiked individually into a 1:100 dilution of the 007sp reference standard along with a buffer only control and allowed to incubate. Each sample was then tested by both the 9-plex and 14-plex assays. The greatest percent inhibition for each of the 23 serotypes was observed for the corresponding serotype and ranged from 57 to 99% for the 9-plex assay and 80–99% for the 14-plex assay (Table 3). In the 9-plex assay, serotypes 11 A and 23F demonstrated >40% non-homologous inhibition for serotypes 10 A and 23F, respectively (Table 3). Six serotypes showed >40% non-homologous inhibition in the 14-plex assay, 9 V to 4, 11 A to 5, 9 N to 6B, 1 to 7F, 4 to 12F and 33F to 18C. (Table 3)

Table 2
Detection capability of the 9 and 14 valent pneumococcal valent assays.

Pneumo 9 μg/mL			Pneumo 14 μg/mL		
Serotype	LLOQ	ULOQ	Serotype	LLOQ	ULOQ
2	0.09	27.30	1	0.05	60.19
10 A	0.02	29.06	3	0.09	14.01
11 A	0.02	3.45	4	0.02	11.94
15B	0.10	27.72	5	0.03	21.21
17F	0.04	11.68	6B	0.02	18.71
19 A	0.15	48.18	7F	0.04	27.96
20	0.04	19.06	8	0.03	19.77
22F	0.03	38.00	9 N	0.05	11.04
33F	0.07	13.39	9 V	0.03	11.73
			12F	0.02	20.50
			14	0.03	35.84
			18C	0.05	11.15
			19F	0.17	112.96
			23F	0.02	9.46

Cross-reactivity of the pneumococcal multiplexed assay was evaluated by testing pre- and post-vaccination specimens from healthy participants between the ages of 19 and 65 who had been enrolled in an IRB approved study for assessment of antibody response to other vaccinations. Seventeen Diphtheria, Tetanus, and *H. influenzae* b pre- and post-vaccination pairs (34 total) and 18 *Neisseria meningitidis* (NMEN) tetravalent pre- and post-vaccination pairs (36 total) were included. All 35 of the individuals receiving these two polyvalent vaccines would have been considered non-responders by the pneumococcal multiplexed assay indicating minimal cross-reactivity of these similar vaccines on the *Streptococcus pneumoniae* IgG antibodies assay. As these were healthy adult individuals it is unlikely that they would have received a pneumococcal vaccine within the past 5 years, but clinical information regarding recent pneumococcal vaccination was unavailable.

3.2. Assessment of immunocompetence

3.2.1. Immunogenicity of the 23 serotypes

Immunogenicity to each of the 23 serotypes contained in the vaccine was determined by calculating the percent of the 79 individual healthy participants having at least a 4-fold or 2-fold post- to pre-vaccination response (Table 4). Immunogenicity based on a ≥ 4-fold response was greatest for serotype 1 with 75 of the 79 (94.9%) individuals mounting a 4-fold or greater response (Table 4). Serotype 19 A was the lowest, with only 26.6% of participants generating a ≥ 4-fold response. Based on a ≥ 2-fold response, immunogenicity was greater for all 23 serotypes compared to ≥4-fold response, with Serotype 20 having the most responders (98.7%) and serotype 19 A again the fewest with 51.9% of individuals responding. There did not appear to be a consistent correlation for reduced immunogenicity based on pre-vaccination responses of ≥1.3 μg/mL. While serotype 19 A did have the greatest percentage of pre-vaccination individual responses ≥1.3 μg/mL (81.0%, Table 4) and the lowest immunogenicity, serotype 12F only had 6.3% of pre-vaccination responses ≥1.3 μg/mL but still demonstrated poor immunogenicity of 49.4% for a 4-fold or greater response, and 73.4% for a 2-fold or greater response. Other serotypes (1,2,7 F and 33 F) had relatively high percentages (22.8 to 35.4%) of individuals with pre-vaccination responses >1.3 μg/mL, but still demonstrated immunogenicity of >90% (Table 4.) Upon further analysis, when using a threshold of a ≥ 4-fold response, only 2 of the 23 serotypes (1 and 5) would have had >90% response compared to a ≥ 2-fold threshold where 13 of the 23 serotypes had a > 90% response (Table 4).

3.2.2. Responder status based on individual responses to at least 50 or 70% of the 23 serotypes

Responder status was calculated by the number of individual

Table 3
Homologous inhibition of binding studies for the 9-plex and 14-plex assays.

	9-Plex Assay								
Serotype	2	10 A	11 A	15B	17F	19 A	20	22F	33F
Homologous Inhibition (HI)	99%	57%	98%	97%	92%	78%	98%	96%	99%
Non-HI Mean	4%	1%	8%	-2%	7%	−12%	−1%	−19%	−19%
Non-HI Range	−8-20%	−10-49%	−10-37%	−16 − 9%	-9-19%	−58-17%	−11-8%	−36-44%	−31-1%
Serotype with >40% Non-HI		11 A						23F	

	14 -Plex Assay													
Serotype	1	3	4	5	6B	7F	8	9 N	9 V	12F	14	18C	19F	23F
Homologous Inhibition (HI)	80%	96%	95%	97%	91%	94%	95%	91%	95%	89%	99%	99%	93%	98%
Non-HI Mean	5%	8%	6%	8%	9%	5%	3%	5%	7%	5%	4%	6%	2%	3%
Non-HI Range	−3-35%	−5-19%	−6-69%	−3-65%	−15-45%	−9-47%	−9-47%	−7-36%	−5-32%	−14-75%	−5-11%	−7-53%	−16-27%	−10-17%
Serotype with >40% Non-HI			9 V	11 A	9 N	1				4		33F		

Table 4
Immunogenicity of individual serotypes by response ratio of 79 vaccinated healthy controls.

Serotype	≥ 4 Fold	≥ 2 Fold	% individuals with Pre-vaccination >1.3 μg/mL
1	94.9%	97.5%	29.1%
5	91.1%	97.5%	16.5%
10 A	87.3%	94.9%	16.5%
17F	87.3%	92.4%	15.2%
2	86.1%	93.7%	26.6%
9 V	84.8%	94.9%	16.5%
20	83.5%	98.7%	8.9%
8	83.5%	96.2%	6.3%
9 N	83.5%	996.2%	15.2%
15B	82.3%	87.3%	35.4%
33F	79.7%	93.7%	22.8%
4	79.7%	94.4%	17.7%
7F	75.9%	91.1%	35.4%
19F	75.9%	88.6%	64.6%
11 A	73.4%	89.9%	13.9%
6B	72.2%	91.1%	6.3%
23F	70.9%	87.3%	8.9%
22F	70.9%	87.3%	17.7%
3	68.4%	84.8%	19.0%
14	67.1%	81.0%	29.6%
18C	65.8%	86.1%	26.6%
12F	49.4%	73.4%	6.3%
19 A	26.6%	51.9%	81.0%

participants that had either a ≥ 2-fold or ≥ 4-fold response to at least 50% or 70% of the 23 serotypes. The number and percentage of individuals meeting those responses is shown in Table 5. Only 57 of our 79 (72.2%) healthy vaccinated participants generated a ≥ 4-fold response to a minimum of 70% of the serotypes. When the criteria were changed to a ≥ 4-fold response to ≥50% of the serotypes, 91.1% of individuals would have been considered responders. Using the same percentage criteria but looking at ≥2-fold response, 97.5% of individuals responded to at least 70% of the serotypes, and 98.7% to at least 50% of the serotypes.

Table 5
Responder status based on individual responses to at least 50 or 70% of the 23 serotypes.

Post- to Pre-Vaccination Ratio	≥4-fold	≥2-fold
# individuals with ≥50% of Serotypes	72	78
% individuals with ≥50% of Serotypes	91.1%	98.7%
# individuals with ≥70% of Serotypes	57	77
% individuals with ≥70% of Serotypes	72.2%	97.5%

3.2.3. Responder status based on 1.3 μg/mL post-vaccination antibody concentration

We next examined the number and percent of individuals that had post-vaccination antibody concentrations of at least 1.3 μg/mL to at least 50% or 70% of the of the 23 serotypes. Sixty-two of the 79 (78.5%) healthy vaccinated participants demonstrated 1.3 μg/mL or greater concentrations to at least 70% of the serotypes. This increased to a 91.1% response to a minimum of 50% of the serotypes (Table 6).

3.2.4. Responder status based on either post-vaccination concentrations of 1.3 μg/mL and/or response ratios of either 2- or 4-fold increase

For the final assessment of immunocompetence, we calculated the number and percent of individuals that had either a post-vaccination concentration of at least 1.3 μg/mL or at least a ≥ 2-fold or ≥ 4-fold post- to pre-vaccination response to at least 50% or 70% of the of the 23 serotypes.

By these criteria, 74 of our 79 (93.7%) normal donors would have had ≥1.3 μg/mL and/or ≥ 4-fold response to at least 70% of the serotypes, and 97.5% to at least 50% of the serotypes (Table 7). Moving to ≥1.3 μg/mL and/or ≥ 2-fold response increased immunocompetence to 98.7% response to at least 70% of serotypes, and 100% to at least 50% of the 23 vaccine serotypes (Table 7). Based on these data, the following immunocompetence interpretive criteria were established: 1) a "non-responder was defined as having less than a 2-fold pre-to post-vaccination increase and post-vaccination concentration less than 1.3 μg/mL and 2) a "good responder" was defined as having at least a 2-fold increase and/or post-vaccination concentration greater than or equal to 1.3 μg/mL to 70% of serotypes.

3.3. Comparison of responder status between Quansys and Luminex bead-based multiplexed platforms

We next compared the performance of the Luminex assay using the same immunocompetence interpretive criteria. By Luminex, 75 of our 79 (94.9%) normal donors would have had ≥1.3 μg/mL and/or ≥ 4-fold response to at least 70% of the serotypes, and 100% to at least 50% of the

Table 6
Response status based on ≥1.3 μg/mL post-vaccination antibody concentration.

Post-vaccination Concentration	≥1.3 μg/mL
# individuals with ≥50% of Serotypes	72
% individuals with ≥50% of Serotypes	91.1%
# individuals with ≥70% of Serotypes	62
% individuals with ≥70% of Serotypes	78.5%

Table 7
Responder status based on either post-vaccination concentrations of ≥1.3 μg/mL and/or response ratios of either 2- or 4-fold increase.

	≥1.3 μg/mL or ≥ 4 Fold		≥1.3 μg/mL or ≥ 2 Fold	
≥1.3 μg/mL or Fold Increase	Quansys	Luminex	Quansys	Luminex
# individuals with ≥50% of Serotypes	77	79	79	79
% individuals with ≥50% of Serotypes	97.5%	100.0%	100.0%	100.0%
# individuals with ≥70% of Serotypes	74	75	78	76
% individuals with ≥70% of Serotypes	93.7%	94.9%	98.7%	96.2%

serotypes (Table 7). A ≥ 1.3 μg/mL and/or ≥ 2-fold response increased immunocompetence to 96.2% to at least 70% of serotypes, and 100% to at least 50% of the 23 vaccine serotypes (Table 7).

3.4. *Retrospective analysis of patient samples submitted for pneumococcal testing*

In addition to our normal healthy vaccinated participants, we performed a retrospective analysis of results obtained from patients for which both pre- and post-vaccination samples were submitted to our laboratory and tested using the newly developed multiplexed pneumococcal assay. We identified 18 who would have been classified as non-responders. Of these 18 patients, 8 had testing performed at ARUP Laboratories that provided additional information on the status of their immune system such as total immunoglobulin levels and/or absolute number and frequency of B cells. All the patients for which total IgG was ordered (7/8) had very low levels of total IgG. Total IgA and IgM were measured in 6 of the 7 patients with low IgG, and one or both were also low in all 6 of the patients. Three of the patients for which IgG testing had been performed also had lymphocyte phenotyping performed which showed a low frequency as well as absolute number of B cells. These results are consistent with a diagnosis of B cell immunodeficiency and demonstrate that the multiplex method for detecting *Streptococcus pneumoniae* IgG antibodies can identify patients with confirmed disease.

4. Discussion

Assessment of humoral immunity to pneumococcal vaccination has traditionally been performed using individual enzyme-linked immunosorbent assays (ELISA) for each serotype. The "WHO ELISA" [33] is still considered the gold standard and was widely adopted in the 2000's for serotype specific pneumococcal testing [33–36]. ELISA testing, however, has limitations in requiring the development and validation of 23 separate assays for measuring each serotype contained in the pneumococcal polysaccharide vaccine. The dynamic range of the colorimetric signal of ELISA's is also limited compared to fluorescent and chemiluminescent signals of current multiplexed assays, often requiring additional dilutions to obtain results by ELISA. Multiplexed assays, therefore, have gained greater use and acceptance as a cost-effective, time efficient and comprehensive approach for assessing pneumococcal antibody responses in both the clinical laboratory and vaccine development studies [26,27,29,37–40]. Along with the advantages of multiplex testing, there are some drawbacks. A common single standard must be developed, containing an adequate antibody response to all 23 serotypes, which then must be calibrated to the WHO reference material. With antibody/antigen interactions to multiple serotypes occurring simultaneously within the same reaction, parameters such as incubation time and anti-human IgG detection antibody concentration cannot be optimized for each serotype. With certain polysaccharides sharing structural similarities, cross-reactivity is also more evident in a multiplexed assay which was observed for a few serotypes in our homologous inhibition of binding studies.

Validation studies performed following current CLSI guidelines support the use of the multiplexed pneumococcal antibody assay for clinical testing. Performance characteristics including precision, linearity, specimen stability, interference and specificity/cross-reactivity met the expected validation criteria. The analytical measurement range was determined independently for each serotype. The average lower limit of quantitation for the 23 serotypes was 0.05 μg/mL and easily allowed quantitation of values below the 1.3 μg/mL cut-off. The average upper limit of quantitation of 26.3 μg/mL allowed adequate range for the calculation of post- to pre-vaccination ratios. Advantages of this method over bead-based multiplexed testing are that the capsular polysaccharides can be spotted directly on the polystyrene microtiter plates in their native form. The bead-based assays require modification of the antigen by either poly-L-lysine [26], sodium periodate oxidation [41] or 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methyl-morpholinium (DMTMM) [42] before conjugation to the carboxyl functional groups of the microspheres. These methods can cause conformational epitope changes to the polysaccharide antigens [42] and may also lead to more lot-to-lot variability due to the double conjugation required. This assay also does not use bovine serum albumin (BSA) as a blocking agent, eliminating the non-pneumococcal specific binding that has been shown to be associated with falsely elevated results particularly in younger patients being evaluated for B cell or humoral deficiencies [27]. Current recommendations for pneumococcal serologic testing advocate for addition of both CWPS and pneumococcal serotype 22F to the specimen diluent to allow for adsorption of non-specific cell wall polysaccharides [43]. However, when the assay contains serotype 22F this would cause adsorption of specific 22F vaccine response requiring a separate diluent containing only CWPS for panels including 22F. The formulation of a common diluent containing both CWPS and CWPS2 [22] allowed for a single specimen dilution for use in both the 14 and 9 valent assays greatly improving workflow and improved the specificity of the assay containing 22F.

In addition to establishing the performance characteristics of the multiplex pneumococcal assay, 79 pre- and 4-week post-vaccination serum specimens obtained from normal healthy individuals vaccinated with the PNEUMOVAX 23 unconjugated polysaccharide vaccine were tested to determine their pneumococcal antibody levels with the goal of establishing criteria for our assay that could be used for assessing immunocompetence. Assessment of a patient's vaccine response is commonly done by either measuring the post-vaccination response of a single sample to determine if it has achieved serotype specific "protective" concentrations or preferably, using paired pre- and post-vaccination specimens. Determining the fold change between the post- and pre-vaccination antibody levels helps to ensure that the response is due to vaccination and not prior exposure to pneumococcal infection and allows the quantitation of response in patients that have residual protective levels of antibody prior to the vaccine challenge. Various recommendations have been proposed regarding what qualifies as a sufficient vaccination response. Historically, the commonly advised criteria for an effective vaccination response in adults included either achieving a "protective" level of 1.3 μg/mL or a 4-fold increase in antibody concentration after vaccination in at least 70% of the tested serotypes [15,16,44]. In cases where the pre-vaccination response exceeded 1.3 μg/mL, more recent studies have suggested that a 4-fold post- to pre-vaccination response may be too stringent and that a 2-fold increase may be more applicable [16,45] and could be applied regardless of pre-vaccination antibody concentrations [11,14,46]. As part of the evaluation of immunocompetence, it has also been observed that the percentage of measured serotypes achieving a specified fold-increase is dependent on age. Children between 2 and 6 years old are expected to respond to at least 50% of the tested serotypes, while adults and children above 6 years old should exhibit a response in 70% of the tested serotypes [16,46]. In our study, we found that only 72.2% of

normal healthy adult controls would have been classified as immunocompetent based on a 4-fold post- to pre- response to at least 70% of 23 serotypes. Similarly, a study by Borgers et al. found that only 44% of normal individuals mounted a 4-fold increase to 60% of the 14 serotypes included in their study [45]. When reassessing our data using a 2-fold response, we found that 97.5% of our healthy subjects achieved this threshold. In the Borgers study they found that 79% mounted a 2-fold response to 60% of the serotypes tested. The use of a 2-fold response is further supported by a comparison of commercial and laboratory developed pneumococcal assays at three reference laboratories. In this study they observed a 57% agreement between the three assays based on a 4-fold response and 96% agreement for a 2-fold response [20]. A meta-analysis of anti-pneumococcal antibody responses also showed that the majority of normal healthy individuals could mount at least a 2-fold response to most serotypes [47]. Further analysis of our data looking at a combination of either post-vaccination concentrations of $\geq$1.3 μg/mL and/or a response ratio of at least 2-fold classified 78 of our 79 (98.7%) healthy adult donors as immunocompetent. These traditional interpretation criteria have also been questioned by others with alternative criteria evaluated and proposed. Hansen et al. propose the use of a *Z*-score for compiling the standard deviations of multiple individual serotype specific antibody responses into a simple mean [48]. This eliminated variability introduced by the number and serotype specificity of the antibodies measured. In their study the Z-score provided a useful way to interpret and compare individual antibody responses within the context of a larger population, helping to assess the effectiveness of vaccines and identify variations in immune responses. Similarly, fifth-percentile cut-offs have also been proposed to address the variability in serotype specific responses [17,49,50]. Another study by Park et al. suggested using serotype specific cut-offs for pneumococcal antibody response [51]. In this study, using 100 pre- and 4 week post-vaccinated adults, they determined optimal cut-offs for each serotype which best discriminated the number of individuals with negative pre-vaccination responses to positive post vaccination responses.

Our study, as well as others, demonstrate that there is no established consensus for interpretation of pneumococcal antibody responses, but do indicate that the traditional immunocompetence criteria for adults having a 4-fold or greater post- to pre-vaccination ratio to at least 70% of serotypes is too strict. For our laboratory we have adopted the following interpretive criteria for adult immunocompetence. A "good responder" is defined as having at least a 2-fold increase to 70% of the serotypes and/or a post-vaccination concentration $\geq$ 1.3 μg/mL. A "non-responder" is defined as having less than a 2-fold increase and a post-vaccination concentration <1.3 μg/mL. These criteria are further supported when we performed the same analysis using our previously validated Luminex bead-based pneumococcal assay and found very comparable results to the Quansys assay. The strong correlation between the two different assay platforms based on these immunocompetence criteria is impressive considering the differences between the assays. The pneumococcal serotype antigens employed for each assay were sourced from different manufacturers, with the further need of first conjugating the Luminex antigens to poly-L-lysine before coupling to the beads. Additionally, the Quansys assay was calibrated to the WHO 007sp standard, with the Luminex assay calibrated to the original WHO 89 s material.

Our study is unique in that we measured antibody responses to all 23 serotypes contained in the PNEUMOVAX 23 unconjugated polysaccharide vaccine employing a newly developed and validated multiplexed assay. Vaccine immunogenicity is known to vary greatly in individuals between different serotypes [47,52]. Based on a 2-fold response, serotype specific immunogenicity ranged from 51.9% to 98.7% for the 23 serotypes measured in our study, with serotypes demonstrating poor immunogenicity not necessarily correlating with $\geq$1.3 μg/mL pre-vaccination responses. Additional confounding factors in assessing immunocompetence by diagnostic vaccine challenge include previous clinical and subclinical infections or previous vaccination with conjugated pneumococcal vaccines leading to increased pre-vaccination specimen concentrations. Conjugated pneumococcal vaccines illicit a T-cell dependent response generating increased antibody persistence, avidity and memory compared to the B-cell mediated immunity of the pure polysaccharide vaccines. With a 20 valent pneumococcal conjugated vaccine now available, the assessment of primary immunodeficiency disorders based on pure polysaccharide diagnostic vaccination is becoming increasingly difficult. The pneumococcal vaccination status of the individuals included in our study was unknown beyond the 5 year "no previous vaccination" exclusion criteria. Recommendations for performing polysaccharide diagnostic vaccine challenge in individuals previously vaccinated with conjugated Prevnar 13 or 20 vaccines are currently not well defined and present a gap in current knowledge that should be addressed in future studies. When vaccinating adults with specified immunocompromising conditions, it is recommended to administer the polysaccharide vaccine at least 8 weeks after the conjugate vaccine. This interval allows the immune system sufficient time to fully respond to the conjugate vaccine before being challenged with the polysaccharide vaccine [53]. It is therefore important to test for as many serotypes as possible when evaluating vaccine response [54]. The development of the multiplexed assay was a collaborative effort between Quansys Biosciences and ARUP Laboratories with the goal of making a commercially available assay for measuring antibody response to 23 pneumococcal serotypes. Previously, there were no commercial multiplexed methods available, with testing performed in individual laboratories using laboratory developed tests (LDTs). These assays lack standardization between laboratories and demonstrate considerable interlaboratory differences [20]. The multiplexed assay employed in our study would allow for a standardized method between laboratories if more widely adopted.

CRediT authorship contribution statement

Thomas B. Martins: Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Harry R. Hill:** Writing – review & editing, Supervision, Conceptualization. **Lisa K. Peterson:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization.

Data availability

Data will be made available on request.

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