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1 **DEVELOPMENT OF A MULTIPLEXED BEAD-BASED IMMUNOASSAY FOR THE**
2 **SIMULTANEOUS DETECTION OF ANTIBODIES TO 17 PNEUMOCOCCAL**
3 **PROTEINS**

4
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23

24 **Abstract**

25
26 Presently, several pneumococcal proteins are being evaluated as potential vaccine candidates.
27 Here, we gather novel insights in the immunogenicity of PLY, PsaA, PspA, PspC, NanA, Hyl,
28 PmpA, SlrA, Eno, IgA1-protease, PdBD, BVH-3, SP1003, SP1633, SP1651, SP0189 and
29 SP0376. We developed a multiplex bead-based immunoassay (xMAP[®] Technology, Luminex
30 Corporation) to simultaneously quantify antibodies against these 17 pneumococcal proteins in
31 serum. The median fluorescence intensity (MFI) values obtained for human pooled serum with
32 the multiplex assay were between 82% and 111% (median 94%) of those obtained with the
33 singleplex assays. For IgG, the coefficient of variation (CV) in serum ranged from 2-9%, for IgA,
34 CV ranged from 3-14% and for IgM, CV ranged from 11-15%. Using this immunoassay, we
35 showed that anti-pneumococcal antibody levels showed extensive inter-individual variability in
36 young children suffering from invasive pneumococcal disease. All proteins, including the
37 proteins with so far unknown function, were immunogenic. In conclusion, the multiplex *S.*
38 *pneumoniae* immunoassay based on proteins is reproducible. This assay can be used to monitor
39 anti-*S. pneumoniae* antibody responses in a material- and time-saving manner.

40
41
42 **Keywords:** *Streptococcus pneumoniae*, vaccine, proteins, antibody detection, Luminex

43 **Introduction**

44
45 *Streptococcus pneumoniae* (*S. pneumoniae*, pneumococcus) is an important human pathogen that
46 causes life-threatening diseases such as pneumonia and meningitis, as well as less serious but
47 highly prevalent diseases such as otitis media and sinusitis. Between 2002 and 2003, pneumonia
48 accounted for 19% of the 10.6 million deaths per annum among children younger than 5 years
49 [1]. Ninety percent of childhood deaths occurred in developing countries [2], including
50 Bangladesh [3, 4]. The global incidence of pneumococcal meningitis in children is 17 cases per
51 100,000. The case-fatality rate (CFR) for pneumococcal meningitis is high. In 2000, the global
52 pneumococcal meningitis CFR was 59%, ranging from 29% in the Western Pacific to 73% in
53 Africa [5].

54 Fortunately, the availability of the 23-valent capsular polysaccharide and 7-valent
55 pneumococcal conjugate vaccines (PCV23 and PCV7) has resulted in a dramatic reduction in
56 morbidity and mortality of pneumococcal diseases. However, the high costs and still limited
57 vaccine-mediated protection, which is restricted to the included serotypes, have prevented its
58 implementation in large-scale immunization programs in developing countries. For these reasons,
59 there is considerable interest in designing alternative and more cost-effective strategies.
60 Presently, several pneumococcal proteins are being evaluated as potential vaccine candidates.
61 Optimally, these would provide protection against pneumococcal infection regardless of serotype
62 [6]. To date, the most promising protein vaccine candidates include pneumolysin (PLY),
63 pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA) and
64 Pneumococcal surface protein C (PspC) [7]. These proteins are produced by virtually all clinical
65 isolates of the pneumococcus. PLY is a 53-kDa protein that causes cytolysis, induces
66 complement activation and the production of cytokines and nitric oxide [8-12]. In addition, PLY

67 has been assigned several functions with respect to modification of the immune response. PLY
68 has recently been shown to interact with Toll-like receptor 4 (TLR-4) [13]. PsaA is a surface-
69 exposed 37-kDa lipoprotein that plays a major role in pneumococcal attachment to the host cell
70 and virulence [14]. PspA is a choline-binding surface protein, which inhibits complement-
71 mediated phagocytosis, binds to lactoferrin and as such prevents lactoferrin-mediated killing [15].
72 Antibodies to pneumococcal proteins PspA, PsaA and PLY have been shown to develop early in
73 life [16, 17]. PspC (also known as CbpA or SpsA) acts as an adhesin and binds the complement
74 regulatory protein factor H, to provide resistance to complement [13, 18].

75 We developed a multiplex bead-based immunoassay using Luminex xMAP[®] Technology
76 to gather novel insights into the immunogenicity of PLY, PsaA, PspA and PspC and 13 other
77 pneumococcal proteins. With this assay, antibodies to these 17 pneumococcal proteins can be
78 quantified simultaneously. Thus far, for the pneumococcus, this technology was only used for
79 measurement of antibodies directed to different pneumococcal capsular polysaccharides [19-21].

80

81

82 2. Materials and methods

83

84 *Antigens.* The pneumococcal proteins PLY, PsaA, PspA, PspC, neuraminidase A (NanA),
85 Hyaluronidase (Hyl), putative proteinase maturation protein A (PmpA), streptococcal lipoprotein
86 rotamase A (SlrA), α -enolase (Eno), immunoglobulin A1 protease (IgA1-protease), PdBD and
87 BVH-3, SP1003, SP1633, SP1651, SP0189, SP0376 were used. NanA plays an important role in
88 biofilm formation and promotes pneumococcal brain endothelial cell invasion [22, 23]. Hyl is
89 present on the majority of strains. The enzyme degrades essential components of the host's
90 extracellular matrix and as such it may facilitate bacterial spread in host tissues [13, 24]. PpmA
91 and SlrA, two surface-exposed lipoproteins have been shown to play a role in virulence and
92 colonization [25, 26]. Eno has been identified both in the cytoplasm and on the surface of
93 pneumococci and is involved in virulence by the activation of plasmin into plasminogen, a host
94 collagen-degrading enzyme [27]. IgA1-protease plays an important role in pneumococcal
95 colonization. IgA1-protease modifies IgA1 antibodies so that it promotes rather than inhibits
96 pneumococcal adherence to epithelial cells in a model of colonization [28]. PdBD is a mutant
97 version of PLY with less cytolytic activity and reduced ability to activate complement [29].
98 BVH-3 is capable of conferring protection against lethal experimental *S. pneumoniae* infection.
99 This protein family was also designated Pht (for pneumococcal histidine triad) [30, 31]. SP1003,
100 SP1633, SP1651, SP0189, SP0376 are pneumococcal proteins with currently unknown functions.
101 Recently, a comprehensive review summarizing the surface-exposed virulence factors and their
102 functions was published [13].

103 The genes encoding the proteins NanA, PsaA, PspA, PspC, SP1633, SP1651, SP0189,
104 SP0376, Hyl, PLY and PpmA were isolated from *S. pneumoniae* strain TIGR4 chromosomal

105 DNA and cloned in the vector pOPINF using In-Fusion Technology. Cell extracts were made
106 from the recombinant *Escherichia coli* (Rosetta) strains and the recombinant proteins purified by
107 immobilized metal affinity chromatography using the poly-His tag added to the N-terminal end
108 of the protein during the cloning procedure. The genes used for the production of the recombinant
109 antigens Eno, SlrA and PpmA were amplified by PCR from *S. pneumoniae* D39. IgA1-protease
110 was amplified from *S. pneumoniae* TIGR4. The amplified DNA was cloned into a pET11a
111 expression vector (Stratagene) and electrotransformed into *E. coli* BL21(DE3). The expression of
112 recombinant protein was induced by the addition of IPTG and the recombinant proteins were
113 purified by Ni⁺ affinity chromatography, as described before [16, 25, 32]. The genes encoding
114 BVH-3 and PdBD were cloned into plasmid pPA195 and pPA180, respectively, and transformed
115 into *Lactococcus lactis* PA1001. Overexpression of BVH-3 and PdBD was induced by nisin,
116 essentially as described previously [33]. The purity of the recombinant proteins was determined
117 by SDS-PAGE gel electrophoresis. The amino acid sequence of the proteins was confirmed with
118 mass spectrometry (Ultraflex MALDI-ToF, Bruker Daltonics).

119
120 ***Coupling methods.*** To quantify antibodies directed against the 17 *S. pneumoniae* proteins
121 simultaneously, the bead-based flow cytometry technique (xMAP[®]; Luminex Corporation) was
122 applied. The purified proteins were coupled to fluorescent SeroMAP beads. The coupling
123 procedure was performed as described elsewhere [34, 35]. In each experiment, control beads
124 were included to determine nonspecific binding. For control beads the coupling procedure was
125 followed, only no *S. pneumoniae* protein was added. In case of nonspecific binding, the median
126 fluorescence intensity (MFI) values were subtracted from the antigen-specific results. As a
127 negative control, PBS-BN was included. Human pooled serum was used as a standard.

128

129 **Multiplex *S. pneumoniae* antibody assay.** The multiplex assay (serum incubated with the
130 different fluorescence-colored antigen coupled beads mixed in 1 well) was validated by
131 comparing the MFI values for HPS obtained with this multiplex assay with the results for HPS
132 obtained with singleplex assays (serum incubated with individual single-color antigen-coupled
133 beads in separate wells). After validation, the different antigen-coupled microspheres were mixed
134 to a working concentration of 4000 beads per color per well. The procedure used was the same as
135 described elsewhere [34-36]. To optimize dilutions, serum samples of children were diluted 1:25,
136 1:50 and 1:100 in PBS-BN. The secondary antibodies were diluted 1:50, 1:100 and 1:200 in PBS-
137 BN. Checkerboard titrations were performed. Considering results in MFI values and amounts of
138 serum and secondary antibody needed, optimal serum dilutions were 1:100 for measurement of
139 IgG and 1:50 for measurement of antigen-specific IgA and IgM. The optimal secondary antibody
140 dilutions were 1:200 for IgG and 1:100 for IgA and IgM. Measurements were performed on the
141 Luminex 100 instrument (BMD) using Luminex IS software (version 2.2). Tests were performed
142 in duplicate, and the MFI values, reflecting semi-quantitative antibody levels, were averaged.

143
144 **Anti-pneumococcal antibodies.** The multiplexed immunoassay was used to compare differences
145 in anti-pneumococcal antibodies in serum samples obtained from 54 children under the age of 5
146 years with clinical suspicion of pneumonia (n=26) or meningitis (n=28). Half of these children
147 suffered from pneumonia/meningitis caused by *S. pneumoniae*, the other half suffered from
148 pneumonia/meningitis caused by a bacterial species other than *S. pneumoniae*. The serum
149 samples were stored at -80°C until use. The research protocol was approved by the Research
150 Review and Research Ethical Committee of ICDDR,B and written informed consent from the
151 parents was obtained.

152

153 **Statistical analysis.** The Mann-Whitney U test was used to compare differences in anti-
154 pneumococcal antibody levels. Differences were considered to be statistically significant when 2-
155 sided *P*-values were <0.05.

156

157 **Results and Discussion**

158

159 **Validation of the multiplex assay and reproducibility.** The MFI values obtained for HPS with
160 the multiplex assay were between 82% and 111% (median 94%) of those obtained with the
161 singleplex assays. Therefore, it was considered legitimate to use the multiplex assay. Serum
162 incubated with control beads resulted in median MFI values for IgG, IgA and IgM of 77 (range,
163 21-7210), 92 (range, 15–11549) and 1282 (range, 171-7966), respectively. This indicates that
164 there was a quite variable level of non-specific binding that was consistently subtracted from the
165 antigen-specific results. The negative control (PBS-BN) incubated with protein-coupled beads
166 resulted in low MFI values (<10). Inter-assay variation was calculated from MFI values obtained
167 from serum samples (n=54) run in 2 separate assays and averaged per protein and antibody
168 isotype. For IgG, the median CV was 5%, range 2% (PsaA) to 9% (SlrA). For IgA, the median
169 CV was 7%, range 3% (PspC and PsaA) to 14% (SlrA). For IgM, the median CV was 13%, range
170 11% (SP1633) to 15% (PspC, PpmA and PspA). Earlier studies found equal CVs for inter-assay
171 variation using other types of antigens [20, 34, 37]. Therefore, this new multiplexed anti-
172 pneumococcal immunoassay is considered reproducible from experiment to experiment.

173

174 **Anti-pneumococcal antibodies.** Anti-pneumococcal antibody levels showed extensive inter-
175 individual variability (Figure 1), probably due to the variable number of previous encounters with
176 different *S. pneumoniae* strains (colonization, subclinical infection) as well as inter-individual

177 differences in the ability to mount a humoral immune response. The levels of IgM were not
178 significantly different between children suffering from a pneumonia/meningitis caused by *S.*
179 *pneumoniae* or by a different bacterial species. The levels of IgG directed against NanA and the
180 levels of IgA directed against Hyl and PpmA were higher in children suffering from a pneumonia
181 caused by *S. pneumoniae* than in children suffering from a pneumonia caused by other bacterial
182 pathogens ($P<0.05$, Figure 1a). In contrast, significantly higher levels of antibodies directed
183 against IgA1-protease were detected in serum samples of patients suffering from a non-*S.*
184 *pneumoniae* pneumonia. This might be explained by the fact that IgA1-protease is also present in
185 *Haemophilus influenzae* [38], another frequent cause of pneumonia in non-vaccinated children. In
186 meningitis patients, the levels of IgG directed against PspC, PdBD and Ply were significantly
187 higher in children suffering from meningitis caused by a bacterial pathogen other than *S.*
188 *pneumoniae* ($P<0.05$, Figure 1b). Furthermore, the levels of IgA directed to PdBD, PLY, SP0189
189 and SP1003 were higher in this group ($P<0.05$). We speculate that the lower *S. pneumoniae*
190 antibody levels in the children suffering from *S. pneumoniae* meningitis might be due to
191 consumption of antibodies. However, such data can be obscured by the fact that we do not know
192 the exact patient delay (time between the onset of disease and the arrival in the hospital and the
193 collection of serum). Furthermore, sera from children with different ages were included.

194
195 In conclusion, using our novel non-polysaccharide-based high-throughput immunological
196 test we were able to determine the level of antibodies to 17 pneumococcal proteins
197 simultaneously in small volume serum samples. The requirement for small sample volumes only
198 is a great advantage as compared to ELISA, especially when limited amounts of samples are
199 available, which is usually the case in young children. Novel antigens can be easily included and
200 combinations of protein and polysaccharide antigens may help profiling the complete immune

201 response of both patients and healthy individuals to a common opportunistic pathogen, especially
202 relevant in children in developing countries.

203

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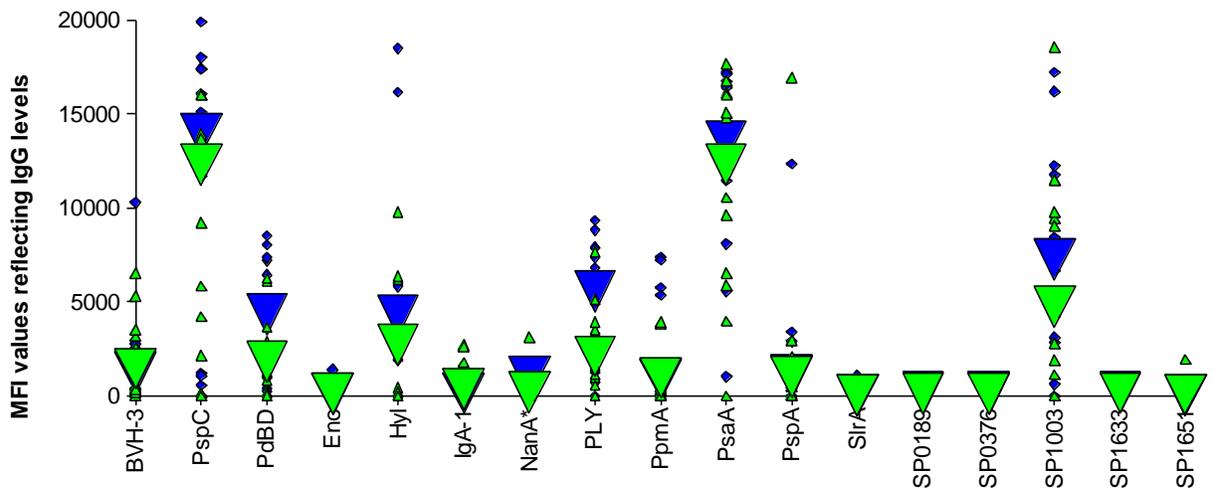
320 **Figure caption**

321

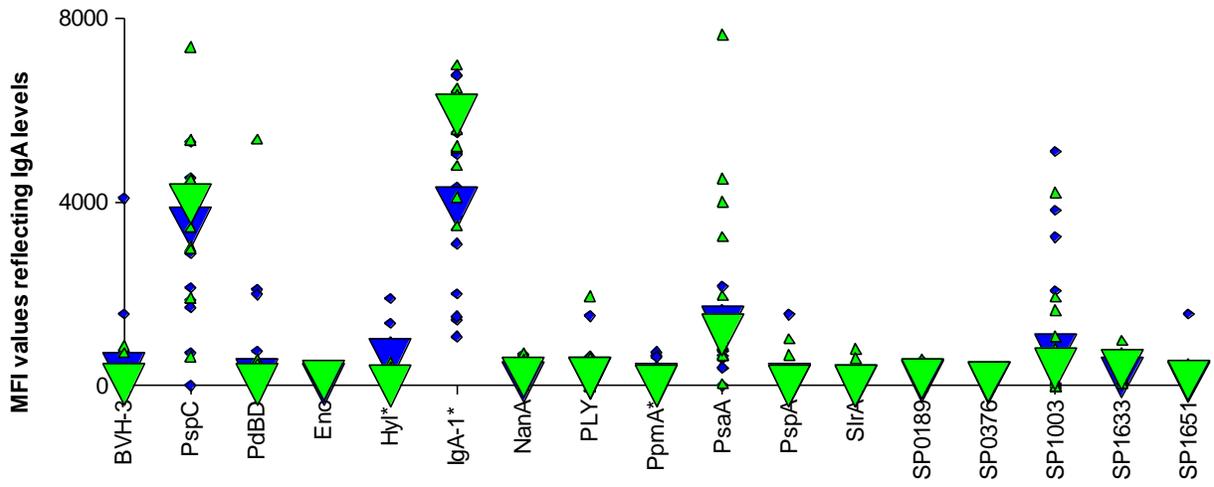
322 **Fig. 1** Median fluorescence intensity (MFI) values reflecting levels of antigen-specific IgG and
323 IgA for 17 *Streptococcus pneumoniae* antigens in serum samples of pneumonia [a] and
324 meningitis patients [b]. Blue diamonds represent pneumonia/meningitis patients caused by *S.*
325 *pneumoniae*, green triangles represent pneumonia/meningitis patients that are positive for
326 bacterial species other than *S. pneumoniae*. Horizontal lines indicate median levels of anti-
327 pneumococcal antibodies for these two groups. Asterisks indicate significant differences.

328

Pneumonia patients



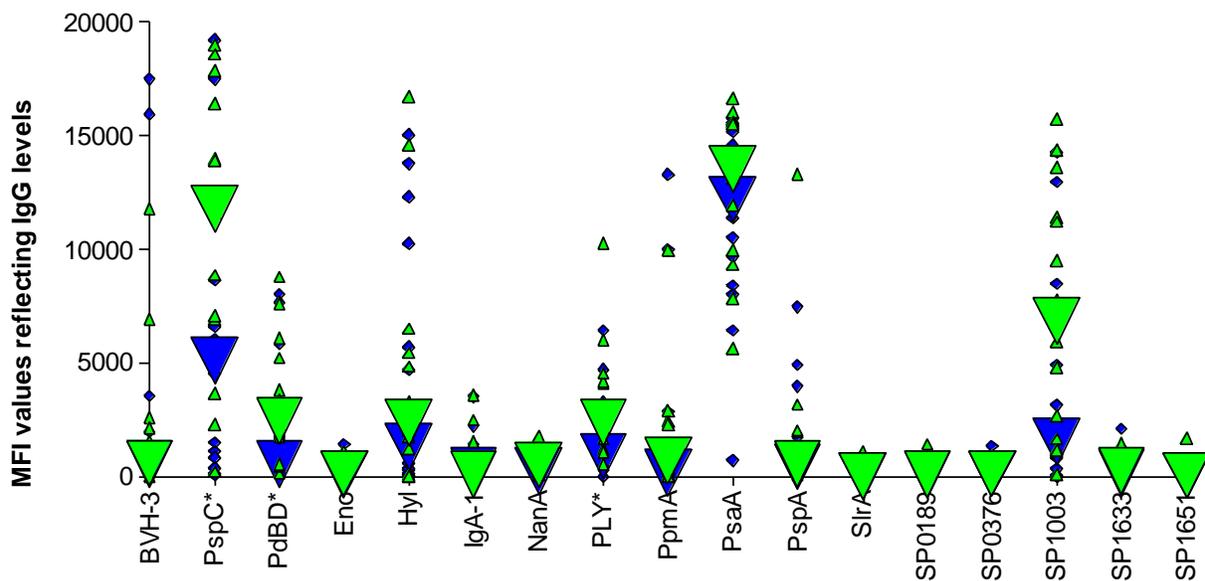
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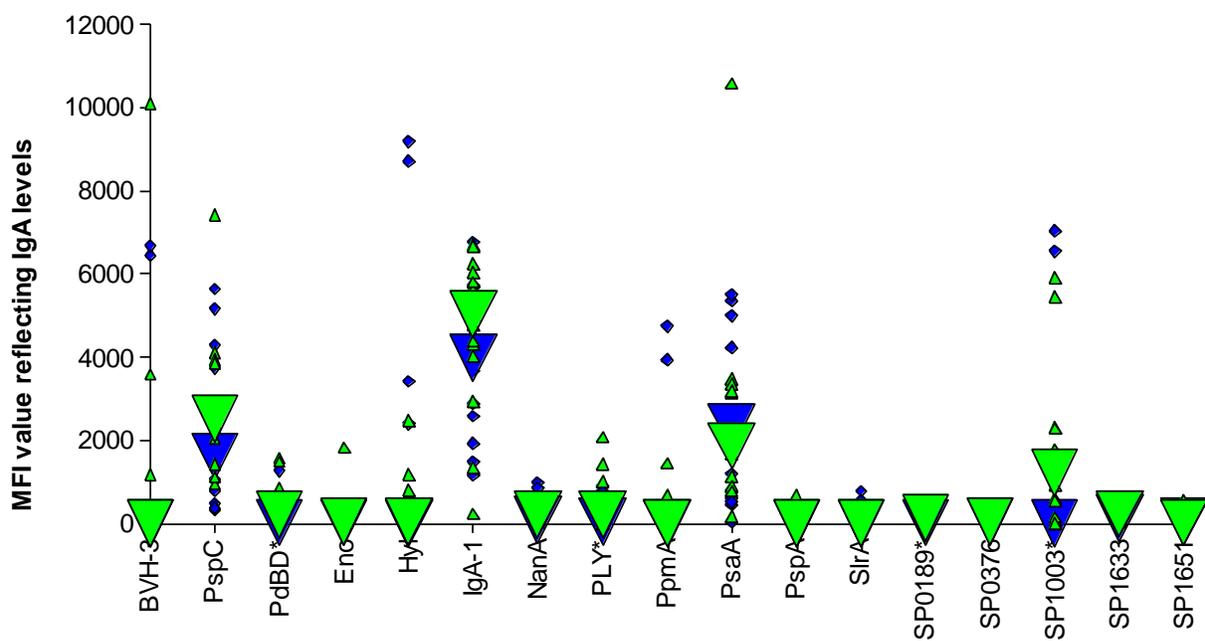
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Meningitis patients



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