

## **Development and Evaluation of a Microarray Platform for Detection of Serum Antibodies Against *Streptococcus pneumoniae* Capsular Polysaccharides**

María A. Campanero-Rhodes<sup>†,‡</sup>, Alicia Lacoma<sup>§,‡</sup>, Cristina Prat<sup>§,‡</sup>, Ernesto García<sup>||,‡</sup>, and Dolores Solís<sup>\*,†,‡</sup>

<sup>†</sup>Instituto de Química Física Rocasolano, CSIC, Serrano 119, 28006 Madrid, Spain

<sup>‡</sup>CIBER de Enfermedades Respiratorias (CIBERES), Avda. Monforte de Lemos 3–5, 28029 Madrid, Spain

<sup>§</sup>Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol. Hospital Germans Trias i Pujol, Carretera de Canyet s/n, 08916 Badalona, Spain

<sup>||</sup>Centro de Investigaciones Biológicas, Ramiro de Maeztu 9, 28040 Madrid, Spain

\*Fax number: 34 91 5642431; email: [d.solis@iqfr.csic.es](mailto:d.solis@iqfr.csic.es)

## ABSTRACT

*Streptococcus pneumoniae* is responsible for severe infections, causing millions of deaths yearly. Immunoglobulin G (IgG) antibodies against the capsular polysaccharide (CPS) offer *S. pneumoniae* serotype-specific protection. In this work, we examined the applicability of the microarray technology to detect CPS type-specific IgGs in serum, using a collection of 22 microarray-printed *S. pneumoniae* CPSs. First, printing of five CPSs onto nitrocellulose-coated glass slides was tested. Successful printing was only achieved for certain CPS types and concentrations. This behavior was tentatively related with diverse viscosities of the CPS solutions. Measurement of dynamic viscosities fully supported this assumption and helped to establish suitable CPS type- and concentration-dependent printing conditions. Next, the potential of CPS microarrays for detecting recognition by anti-CPS IgGs was examined using well-defined rabbit pneumococcal antisera. In all cases, the expected antiserum–CPS binding signals were detected, prompting a proof-of-concept analysis of human serum samples. Clearly distinct serum- and CPS-specific binding patterns and intensities were observed, evidencing selective detection of CPS type-specific IgGs. Compared to the ELISA assay commonly used to quantitate CPS type-specific IgGs in serum, the newly developed *S. pneumoniae* CPS microarrays offer the advantage of enabling the simultaneous analysis of multiple CPS–serum interactions using minute CPS amounts and significantly reduced serum volumes. Therefore, the approach could be particularly valuable for gauging the presence of CPS type-specific IgGs in human serum when sample volumes are limited and/or numerous serum samples are being examined.

*Streptococcus pneumoniae*, a Gram-positive bacterium that asymptotically colonizes the human upper respiratory tract, can also cause severe infectious diseases, like acute otitis media, pneumonia, meningitis, and septicemia,<sup>1</sup> being responsible for millions of deaths each year with special incidence among children < 2 years old, adults  $\geq$  65 years of age, and patients with high-risk conditions. As an example, a systematic analysis covering 195 countries during the period 1990–2016 revealed that *S. pneumoniae* was the leading cause of lower respiratory infection morbidity and mortality worldwide, accounting in 2016 for more deaths than all other aetiologies combined.<sup>2</sup> Moreover, despite vaccination programs, the incidence of invasive pneumococcal disease remains high.<sup>3</sup> An important contributor to *S. pneumoniae* virulence is its polysaccharidic capsule. To date, at least 99 different serotypes, divided into 25 individual types and 21 serogroups, each composed of two to eight serotypes with related capsular antigenic determinants, have been identified.<sup>4,5</sup> The capsule prevents complement activation and phagocytosis by shielding inner bacterial surface structures, and thereby plays a key role in the bacterial defense against the host innate immune system.<sup>4</sup> On the other hand, binding to *S. pneumoniae* cells of serum immunoglobulin G (IgG) antibodies against the capsular polysaccharide (CPS) leads to opsonization and rapid clearance of the bacterium. However, this protection is strictly CPS type-specific. Therefore, current pneumococcal vaccines use as immunogens those CPSs most frequently associated with severe disease.

In 2000, the World Health Organization (WHO) selected a well-characterized enzyme-linked immunosorbent assay (ELISA) protocol for quantitation in human serum of IgG antibodies specific for *S. pneumoniae* CPSs, particularly in response to pneumococcal vaccines.<sup>6</sup> The protocol involved coating microtiter plate wells with individual *S. pneumoniae* CPSs and detection of serum anti-CPS IgGs bound to each well by incubation with anti-human IgG antibody conjugated with alkaline phosphatase, followed by addition of *p*-nitrophenyl phosphate and measurement of the yellow end product, *p*-nitrophenol. A main disadvantage of this and other ELISA plate-binding assays is that they require relatively large amounts of CPS/serum samples and only one CPS–serum pair is examined per well. As an alternative, the microarray technology enables the simultaneous analysis of a large number of interactions using very small amounts of sample. This technology has been successfully used to explore bacterial surface glycans and their interactions with diverse glycan-binding proteins.<sup>7–10</sup> Moreover, microarrays incorporating bacterial carbohydrate structures have proved to be useful for serodiagnosis of bacterial infections, identification of antigenic determinants for vaccine development, and mapping of epitopes recognized by bacteria-specific anti-carbohydrate antibodies.<sup>10</sup> Although non-covalent printing was originally proposed for preventing alteration of carbohydrate epitopes,<sup>11</sup> the strategy typically used for immobilization of bacterial carbohydrates involved chemical conjugation to derivatized slide surfaces.<sup>10</sup> The panel of structures printed in the arrays ranged from large collections of diverse bacterial glycans, including CPSs from some bacterial species and strains, to more focused libraries of a specific glycan type or bacterial origin. In the case of *S. pneumoniae*, a series of

microarrays containing the CPS of a particular serotype and small synthetic substructures thereof have been used for deciphering antigenic determinants in the search of vaccine candidates.<sup>12-16</sup> However, to the best of our knowledge, a focused array including a collection of CPSs from different *S. pneumoniae* serotypes has not been developed so far.

In this work, we evaluated the potential applicability of the microarray technology to detect *S. pneumoniae* type-specific antibodies in human serum using a repertoire of microarray-printed *S. pneumoniae* CPSs. The collection of CPSs in the arrays comprised 21 different polysaccharides included in the pneumococcal polysaccharide vaccine PPSV23 (Pneumovax<sup>®</sup>), which is currently licensed worldwide for those aged  $\geq 2$  years, plus one polysaccharide (serotype 6A) not included in this vaccine but found to be responsible for causing pneumococcal disease in USA and/or EU in the last ten years. First, the challenge of generating microarrays displaying uniform non-covalent printing of these CPSs was addressed. The diverse viscosities of the CPS solutions made necessary the establishment of printing conditions in a CPS type- and concentration-dependent basis. Next, binding and competition assays using well-defined rabbit pneumococcal antisera against particular CPS serotypes printed in the array served to prove the specificity of the assay. Finally, serum samples from pneumonia patients were examined. The results supported the utility of *S. pneumoniae* CPS microarrays for selectively detecting CPS type-specific IgGs in human sera.

## EXPERIMENTAL SECTION

**Materials.** Pneumococcal CPSs of serotypes (Danish nomenclature) 1, 2, 3, 4, 5, 6B, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 19A, 19E, 20, 22F, 23F, and 33F, all of them included in the 23-valent vaccine, as well as serotype 6A CPS, were purchased from the American Type Culture Collection (ATCC). CPSs that are underlined are also included in the currently licensed 13-valent pneumococcal conjugate vaccine (PCV13). The candidate 15-valent PCV contains those serotypes included in PCV13 plus serotypes 22F and 33F, and is currently under clinical evaluation.<sup>17,18</sup> Rabbit pool sera against *S. pneumoniae* CPSs types 3, 4, and groups 9, 12 (pool R), against CPSs types 5, 8, and groups 10, 15, 17 (pool S), and against CPSs types 2, 20, and groups 11, 22, 33 (pool T) were from Statens Serum Institut (SSI Diagnostica). Group serum against *S. pneumoniae* CPSs 19F, 19A, 19B, and 19C (designated group serum 19) and type serum against *S. pneumoniae* type 1 (type serum 1) were also from SSI Diagnostica. Sera from patients with pneumonia of diverse bacterial aetiology were collected at the Hospital Universitari Germans Trias I Pujol following the ethical principles of the Declaration of Helsinki (Fortaleza 2013), the Standards of Good Clinical Practice, the legislation on biomedical research (Law14/2007) and biobanks (RD1716/2011). Samples and databases were coded to keep patients' anonymity and privacy following the Spanish legal normative (LOPD 15/1999 and RD 1720/2007) and the European General Data Protection Regulation.

**Microarray Preparation.** Dynamic viscosities of 1 mg/mL CPS solutions in milli-Q water and/or printing solution (47% glycerol and 0.06% (v/v) Triton X-100) were measured at 25 °C with a

Kinexus Ultra+ rheometer (Malvern Panalytical), using parallel plate geometry of diameter 40 mm (PU40), 0.1 mm gap and 0.5 Pa shear stress. CPS solutions at 3 mg/ml in milli-Q water were used to prepare serial dilutions, from 0.1 to 0.01 mg/mL, in printing solution, diluted printing solution, or milli-Q water, as required to keep for each CPS and concentration the viscosity in a range suitable for printing. The Cy3 fluorophore (GE Healthcare) was added to these dilutions at a final concentration of 1 µg/mL. All solutions were printed as triplicates on 16-pad nitrocellulose-coated glass slides (Grace Biolabs ONCYTE NOVA) using a noncontact arrayer (Sprint, Arrayjet Ltd.). Immediately before printing, samples were gently centrifuged (200g for 1 min) to avoid the presence of bubbles, which would cause problems in sample aspiration, and of undesired particles that could block the microarrayer capillaries.<sup>19</sup> Printed slides were scanned to monitor spot location and grid Cy3 signals (excitation at 532 nm, green laser) using a GenePix Autoloader 4200AL (Axon Instruments) microarray scanner. The slides can be stored at room temperature in a dry and dark place, and remain stable for at least 1 year.

**Microarray binding and competition assays.** Before the binding/competition assays, 1 µL-samples of rabbit pneumococcal antisera were diluted in 100 µL (400 µL in the case of type serum 1) of an absorption buffer composed of 5 mM sodium phosphate, pH 7.2, 0.2 M NaCl (PBS), 0.1% (v/v) Tween 20, 1% bovine serum albumin (BSA), and 10 µg/mL of a 1:1 mixture of pneumococcal cell wall polysaccharides CWPS and CWPS2 (CWPS Multi, SSI Diagnostica), and incubated for 1 h at 20 °C. In competition experiments, 25 µg/mL of the appropriate CPS was added to this absorption buffer. Human sera were processed similarly, 1 µL-samples being diluted in 100 µL of absorption buffer. Pre-absorption of CWPSs-specific antibodies (step also included in the WHO ELISA protocol) is essential to minimize non-CPS-specific binding signals, as CWPSs are commonly expressed by pneumococci and expected to be present in purified CPSs, and most people have antibodies targeting CWPS, possibly in response to pneumococcal carriage.<sup>6</sup>

To test the binding of sera to the microarrays, these were first blocked for 1 h with 0.25% Tween 20 and 1% BSA in PBS. Then, slides were washed once with PBS and incubated for 90 min with 100 µL per pad of pre-absorbed serum samples. To exclude a direct binding to the CPSs of the secondary antibodies and/or streptavidin, subsequently used for detection, blank assays by incubation with 100 µL of absorption buffer, instead of sera, were run in parallel. After four short rinses with PBS, slides were incubated for 1 h with a 1:1000 dilution in absorption buffer of biotin-labeled goat anti-rabbit (Dako) or anti-human IgGs (Vector Laboratories), as appropriate. After four further washes with PBS, slides were incubated for 35 min in the dark with AlexaFluor-647 (AF647)-labeled streptavidin (Invitrogen) at 1 µg/mL in PBS containing 0.1% Tween 20. All incubation steps took place at 20 °C and protected from dust. Finally, slides were thoroughly washed with PBS and milli-Q water, drained, let dry, and scanned for AF647 fluorescence signals red (excitation at 635 nm, red laser), using a GenePix Autoloader 4200AL microarray scanner. The signals were quantified with the GenePix Pro 6.1 software (Molecular Devices). For spot segmentation, a fixed 100-µm diameter circle and the grid

of Cy3 signals as reference for spot coordinates were employed. Median spot intensities minus median local background were used for final quantitation.

## RESULTS AND DISCUSSION

**Preparation of CPS microarrays.** Different microarray surfaces can be used for printing samples of interest (the probes) to be evaluated for recognition by relevant targets. Among these surfaces, nitrocellulose-coated slides have proved to be very useful for straightforward adsorption of a variety of probes, spanning from purified biomolecules to entire cells. The ELISA protocol designated by the WHO as reference assay for quantitation in human serum of anti-*S. pneumoniae* CPS IgGs is based on adsorption of CPSs on microtiter plate wells. Therefore, nitrocellulose-coated slides were selected for the preparation of microarrays of purified *S. pneumoniae* CPSs. Printing on this surface should avoid damaging the nitrocellulose membrane with the microarrayer dispensing capillaries, what could lead to errors in the quantitation of spot intensities. Therefore, the use of a non-contact microarray printer is indicated. The successful performance of this type of printers crucially depends on the viscosity of probe solutions, as sample droplets are ejected from printing nozzles over the slide surface, traveling a short distance through the air. For printing using the Arrayjet Sprint non-contact arrayer, used in this work, probe solutions are routinely prepared in the recommended printing solution that contains 47% glycerol, what in principle should ensure an appropriate viscosity for uniform printing. Triton X-100 is also included in the printing solution to ensure the cleanliness of the system. To examine the applicability of this set up for printing CPS solutions, we first tested the behaviour of five CPSs, namely 1, 3, 6A, 6B, and 19A at 1 to 0.03 mg/mL in printing solution. Disappointingly, successful printing was only achieved for CPS 19A at 0.1 mg/mL and below, and for CPSs 1 and 6A at 0.03 mg/mL, while for CPSs 3 and 6B printing failed even at the lowest concentration. Moreover, several sample loading capillaries of the microarrayer were clogged up with the solutions, pointing at an exceedingly high viscosity (please note that, before printing, samples were centrifuged to avoid the presence of undesired particles that could obstruct the capillaries). Therefore, CPS solutions in different dilutions of printing solution were next tested. Again, successful printing was only observed for certain combinations of CPS concentration and printing solution dilution, evidencing that printing conditions should be established in a CPS type- and concentration-dependent basis. A possible explanation for this behavior was that CPS solutions themselves could exhibit very diverse viscosities, due to their different chemical structure and size. Therefore, dynamic viscosities of solutions in printing solution of all CPSs to be included in the arrays were determined.

As compiled in Table 1, there were obvious differences in viscosity among 1 mg/mL CPS solutions in printing solution, ranging from 3.3 to 46.8 cP. Of note, the printing performance observed for the five CPSs used in the initial tests entirely correlated with their viscosities, as CPS 19A showed the lowest viscosity (5.4 cP), followed by CPSs 6A and 1 (6.6 and 9.45 cP, respectively), while for CPSs 3 and 6B, whose printing failed even at a concentration of 0.03 mg/mL, measured viscosities were

significantly higher (27–28 cP). To help in the establishment of appropriate printing conditions, measurements in milli-Q water for those CPSs exhibiting the highest viscosities were also performed (Table 1). Based on the values obtained, different percentages of printing solution were tested for each CPS type and concentration. The best conditions are given in Table 1. Please note that although most CPSs could be successfully printed at a concentration of 0.3 mg/mL when the appropriate percentage of printing solution was used, only 0.1 to 0.01 mg/mL dilutions were finally used for assembling the CPS microarrays. The aim was to facilitate a straightforward comparison of the signals observed for the different array-printed CPSs in binding assays with rabbit pneumococcal antisera and human serum samples.

**Table 1. Dynamic viscosity of pneumococcal CPS solutions and percentage of printing solution used for microarray printing of CPS dilutions**

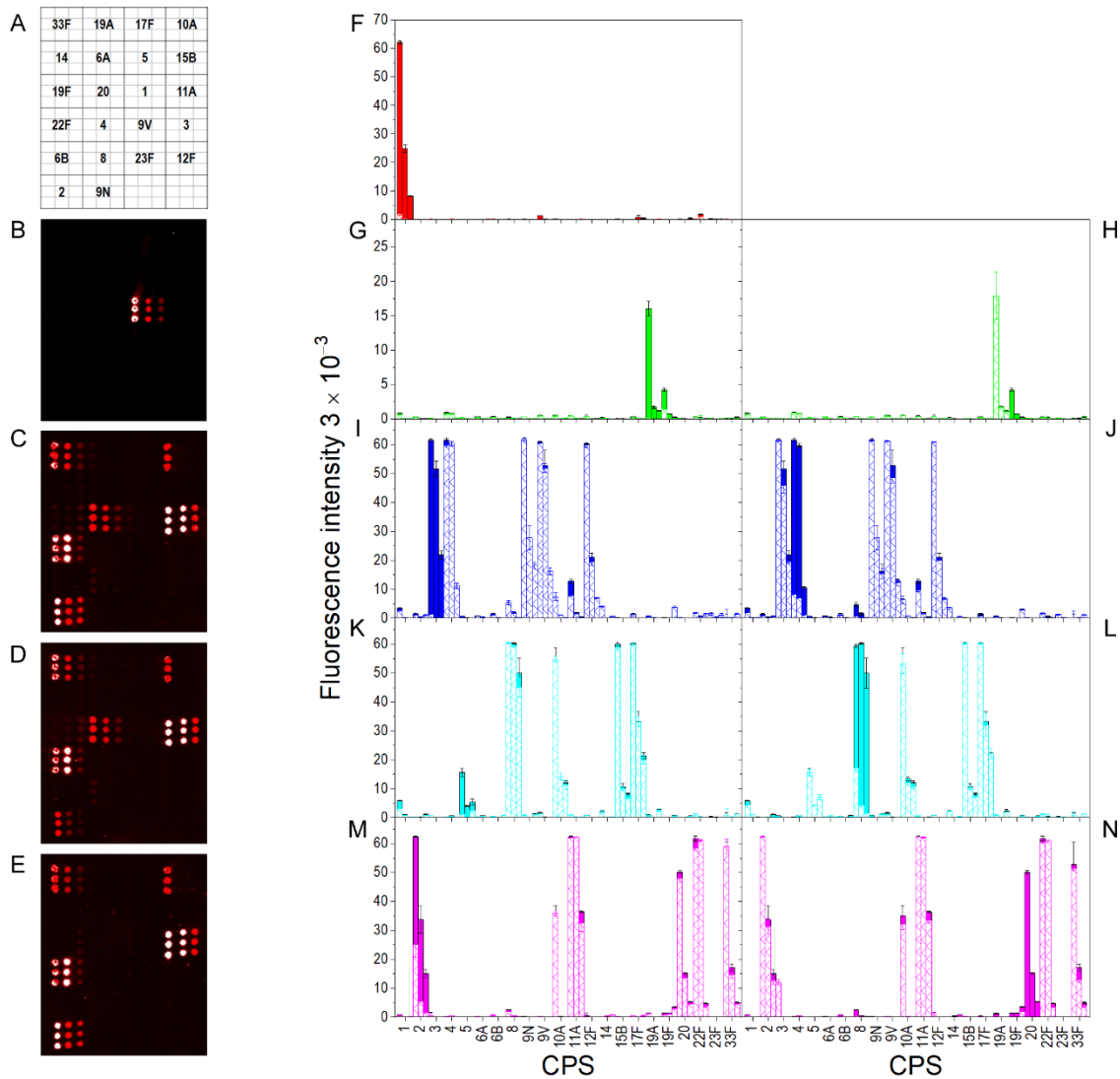
CPS (1 mg/mL)	Solvent	Viscosity (cP)	Percentage of printing solution used for 0.1–0.01 mg/mL CPS dilutions		
			0.1	0.03	0.01
	PS <sup>a</sup>	3.5			
	water	0.8			
1	PS	9.45	70%	100%	100%
2	PS	3.5	70%	100%	100%
3	PS water	27 8.4	50%	70%	70%
4	PS	11.1	70%	70%	100%
5	PS	7.2	70%	100%	100%
6A	PS water	6.6 1.4	100%	100%	100%
6B	PS water	28 5	50%	70%	70%
8	PS water	34.5 4.2	30%	50%	70%
9N	PS	15	70%	70%	100%
9V	PS water	22.1 4.4	30%	50%	70%
10A	PS	6	100%	100%	100%
11A	PS	9.7	70%	100%	100%
12F	PS	4.4	100%	100%	100%
14	PS	6.3	100%	100%	100%
15B	PS	7.2	70%	100%	100%
17F	PS	5.6	100%	100%	100%
19A	PS	5.4	100%	100%	100%
19F	PS	8.3	70%	100%	100%
20	PS	9.3	70%	100%	100%
22F	PS	10.4	50%	70%	100%
23F	PS water	46.8 9.6	30%	50%	70%
33F	PS	3.3	100%	100%	100%

<sup>a</sup> PS: printing solution



**Binding of rabbit pneumococcal antisera to microarray-printed CPSs.** The potential of the established CPS microarrays for detecting recognition by anti-pneumococcal CPS IgGs was first examined by testing the binding of five well-defined rabbit pneumococcal antisera against particular CPS serotypes printed in the array, namely, pool serum R (expected to recognize serotypes 3, 4, 9N, 9N, and 12F), pool serum S (expected to recognize serotypes 5, 8, 10A, 15B, and 17F), pool serum T (expected to recognize serotypes 2, 11A, 20, 22F, and 33F), group serum 19 (expected to recognize serotypes 19A and 19F), and type serum 1. As shown in Figure 1, serum-specific binding patterns were observed, and the fluorescence intensity of the binding signals correlated with the array-printed concentration of the recognized CPS(s), evidencing CPS- and dose-dependent responses. Worth mentioning, the observation of dose-dependent responses is crucial to discern between meaningful and irrelevant fluorescent signals. Thus, detection of serum binding to only one particular CPS dilution is indicative of an artefact, as e.g., contamination. An example is the spurious binding of pool serum T to one dilution of CPS 10A (Figure 1C–D and M, N), although this serum does not contain anti-CPS 10 IgGs.

To further examine the specificity of antisera binding signals, competition assays were carried out in the presence of selected CPSs in solution. In detail, CPSs used in these experiments were: *i*) CPS 1 for competition assays with type serum 1, *ii*) CPSs 19A and 19F for group serum 19, *iii*) CPSs 3 and 4 for pool serum R, *iv*) CPSs 5 and 8 for pool S, and *v*) CPSs 2 and 20 for pool T. The presence of these CPSs significantly reduced or even abolished antisera binding to the corresponding array-printed CPS (Figure 1), confirming the serotype specificity of the respective IgG antibodies. Altogether, these assays proved the efficiency of the established CPS-based microarray set-up for detecting binding of serotype-specific anti-pneumococcal antibodies in rabbit antisera. Therefore, its usefulness for detecting anti-pneumococcal antibodies in human sera was next examined.



**Figure 1.** Binding of rabbit pneumococcal antisera to microarray-printed CPSs. CPSs were printed as triplicates at three different concentrations (0.1, 0.03, and 0.01 mg/mL), and the binding of antisera in the absence or presence of selected CPSs in solution was detected using AF647-labelled streptavidin as final step, as described in the Experimental Section. (A) CPS printing layout. (B–E) Representative images of fluorescent signals observed for the binding of type serum 1 (B) and of pool serum T in the absence (C) or presence of CPS 2 (D) or CPS 20 (E). (F–N) Plot of fluorescence intensities for the binding of type serum 1  $-/+$  CPS 1 (F), group serum 19  $-/+$  CPS 19A (G) or 19F (H), pool serum R  $-/+$  CPS 3 (I) or 4 (J), pool S  $-/+$  CPS 5 (K) or 8 (L), and pool T  $-/+$  CPS 2 (M) or 20 (N). Fluorescence intensities in the absence of CPSs in solution are represented by filled columns, while those from competition assays are represented by crosshatched columns. Data shown correspond to the mean of triplicate spots and error bars indicate the standard deviation of the mean.

**Detection of CPS-specific IgGs in human serum samples.** Randomly selected serum samples from patients with pneumonia of diverse bacterial aetiology, i.e., *S. pneumoniae* (samples *Sp*1–3), *Haemophilus influenzae* (sample *Hi*), *Legionella pneumophila* (sample *Lp*), or suspected (because the bacterium was only isolated from a respiratory sample) *S. pneumoniae* aetiology (sample *sSp*) were used for a proof-of-concept study. Binding signals were obtained for all the samples, although the binding patterns and fluorescent intensities were clearly distinct (Table 2). As an example, strong dose-dependent binding of serum *Sp*1 to many microarray-printed CPSs was observed, whereas the binding of serum *Sp*4 was much more selective. Anti-pneumococcal CPS IgGs were also detected in samples from patients with *H. influenzae*, *L. pneumophila*, or suspected *S. pneumoniae* pneumonia, although again different binding profiles and intensities were evident (Table 2). A thorough analysis of the results clearly goes beyond the scope of this work. Still, it is interesting to mention the detection in all the samples tested of antibodies against CPS 14, one of the most common *S. pneumoniae* serotypes worldwide before the introduction of pneumococcal conjugate vaccines. Competition assays carried out with sample *sSp* in the presence of CPS 14 in solution confirmed the specificity in the recognition, as the binding signals to printed CPS 14 were completely abolished. In striking contrast, significant binding to CPSs 4, 12F, and 22F was only detected for serum *Sp*1, and the same was found for CPS 19F and *Sp*3. Furthermore, no significant binding signals were detected for CPSs 5 and 6A for any of the serum samples tested (please note that CPS 5 was found to be recognized by the appropriate rabbit antiserum, proving its availability in the array for IgG recognition). Therefore, the results supported the utility of *S. pneumoniae* CPS microarrays for selectively detecting CPS type-specific IgGs in human serum.

**Table 2. Binding intensities of human serum samples to microarray-printed *S. pneumoniae* CPSs**

CPS	Serum samples						
	<i>Sp1</i>	<i>Sp2</i>	<i>Sp3</i>	<i>Sp4</i>	<i>sSp</i>	<i>Hi</i>	<i>Lp</i>
<i>Maximum fluorescence intensity<sup>a</sup> (rfu × 10<sup>-3</sup>)</i>							
	57.5 ± 0.1	36 ± 1	6.8 ± 0.6	50 ± 6	43 ± 3	6.7 ± 0.4	31 ± 6
<i>Relative fluorescence intensity<sup>b</sup></i>							
<b>1</b>	+++	·	++	·	·	++	·
<b>2</b>	+++	+	+	+	+	·	++
<b>3</b>	+	+	+	+++	·	·	·
<b>4</b>	+	·	·	·	·	·	·
<b>5</b>	·	·	·	·	·	·	·
<b>6A</b>	·	·	·	·	·	·	·
<b>6B</b>	++	·	·	·	·	+++	·
<b>8</b>	++	·	+	·	·	·	++
<b>9N</b>	+	+	+	·	+	·	+
<b>9V</b>	+	++	+	·	·	·	+
<b>10A</b>	++	+	++	·	+	·	+
<b>11A</b>	++	·	++	·	+	·	++
<b>12F</b>	+	·	·	·	·	·	·
<b>14</b>	++	+	+++	+	+++	+++	++
<b>15B</b>	·	+	·	·	++	·	+++
<b>17F</b>	+	+	·	·	·	·	+
<b>19A</b>	++	++	·	·	·	·	·
<b>19F</b>	·	·	+	·	·	·	·
<b>20</b>	++	·	·	·	+	+	++
<b>22F</b>	+	·	·	·	·	·	·
<b>23F</b>	++	+	·	·	++	·	·
<b>33F</b>	+++	+++	+	·	+++	·	+++

<sup>a</sup> Maximum fluorescence intensity (in relative fluorescent units, rfu) observed for the binding to a 0.1 mg/mL CPS dilution printed in the array. <sup>b</sup> Percent binding intensities taking as 100% the maximum fluorescence intensity: +++ ≥ 60%; ++ > 30%; + > 10%; · < 10%.

## CONCLUSIONS

The microarray technology is a versatile tool for high-throughput analysis of molecular interactions and identification of binding partners. Consistent and uniform printing of relevant samples in the arrays at comparable concentrations is critical for obtaining reliable information. In this work, we developed a strategy for non-covalent printing of a collection of *S. pneumoniae* CPSs. It involves measurement of the viscosity of CPS solutions and adjustment of printing conditions, based on the measured viscosities, in a CPS type- and concentration-dependent manner. This strategy, which could also be exploited for non-contact printing of other samples with high viscosity, enabled the establishment of a microarray set-up for detecting binding of serotype-specific anti-pneumococcal antibodies.

The ELISA assay selected by the WHO for quantitation in human serum of IgG antibodies specific for *S. pneumoniae* CPSs involves the adsorption of CPSs onto microtiter plate wells, generally using 100  $\mu\text{L}$  per well of CPS solutions at 1–10  $\mu\text{g}/\text{mL}$  (0.1–1  $\mu\text{g}$  per well), incubation of each well with 50  $\mu\text{L}$  of serially diluted serum, and subsequent detection of bound antibodies by incubation with alkaline phosphatase-conjugated anti-human IgG followed by addition of an alkaline phosphatase substrate and colorimetric quantitation of the enzyme product developed.<sup>6</sup> The pilot study here presented put forward the possibility of using CPS-based microarrays for this purpose. A main advantage is the requirement of much smaller amounts of CPSs for testing a given serum dilution. As the microarrayer used in the present study dispenses  $\sim 100$  pL of CPS solution per spot, the amount of printed CPS at the highest concentration here used was 10 pg, that is, 4 orders of magnitude smaller than the minimum amount recommended for coating each microtiter plate well in the ELISA assay. The volume of serum dilution used was also substantially smaller, as 100  $\mu\text{L}$  were sufficient for examining the presence of antibodies against all the CPS types included in the array, in our case 22 CPSs printed as triplicates at three different concentrations. Of note, the possibility of comparing binding signals to different amounts of printed CPS facilitates the detection of false positives, which are easily spotted when dose-dependent responses are not observed. Thus, multiple CPS–serum interactions are simultaneously examined in a single microarray pad, as opposed to the ELISA assay in which only one CPS–serum pair is examined per well. All things considered, the use of CPS-microarrays for measuring *S. pneumoniae* serotype specific IgGs in human serum could be an alternative (or complement) to the ELISA assay, calling for a detailed quantitative analysis using quality control and reference standard sera. At any rate, the semi-quantitative study reported here demonstrates the utility of the approach for gauging the presence of CPS type-specific IgGs in serum, what could be particularly valuable when sample volumes are limited and/or in studies involving numerous serum samples, e.g. for determination of serotype-specific contributions to current pneumococcal diseases or in studies aimed at evaluating the impact of vaccination strategies.

## AUTHOR INFORMATION

### Corresponding Author

\*E-mail: d.solis@iqfr.csic.es

### ORCID

María A. Campanero-Rhodes: 0000-0002-3373-0064

Alicia Lacoma: 0000-0002-2049-3872

Cristina Prat: 0000-0001-6974-9165

Ernesto García: 0000-0002-1741-5486

Dolores Solís: 0000-0002-8148-1875

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGEMENTS

We gratefully acknowledge financial support from the Spanish Ministry of Economy, Industry and Competitiveness (Grants BFU2015-70052-R and SAF2017-88664-R), the Spanish Ministry of Science, Innovation, and Universities, the Spanish State Research Agency, and the European Regional Development Fund (RTI2018-099985-B-I00, MCIU/AEI/FEDER, UE), and the CIBER of Respiratory Diseases (CIBERES), an initiative from the Spanish Institute of Health Carlos III (ISCIII). We thank Alberto Escobar (IESMAT) for measurements of dynamic viscosity of the pneumococcal capsular polysaccharide solutions.

## REFERENCES

1. Weiser, J. N.; Ferreira, D. M.; Paton, J. C. *Streptococcus pneumoniae*: transmission, colonization and invasion. *Nat. Rev. Microbiol.* **2018**, *16*, 355–367.
2. GBD 2016 Lower Respiratory Infections Collaborators. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory infections in 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Infect. Dis.* **2018**, *18*, 1191–1210.
3. Hanquet, G.; Krizova, P.; Valentiner-Branth, P.; Ladhani, S. N.; Nuorti, J. P.; Lepoutre, A.; Mereckiene, J.; Knol, M.; Winje, B. A.; Ciruela, P.; Ordobas, M.; Guevara, M.; McDonald, E.; Morfeldt, E.; Kozakova, J.; Slotved, H. C.; Fry, N. K.; Rinta-Kokko, H.; Varon, E.; Corcoran, M.; van der Ende, A.; Vestheim, D. F.; Munoz-Almagro, C.; Latasa, P.; Castilla, J.; Smith, A.; Henriques-Normark, B.; Whittaker, R.; Pastore Celentano, L.; Savulescu, C., on behalf of The SpIDnet/I-

MOVE+ Pneumo Group. Effect of childhood pneumococcal conjugate vaccination on invasive disease in older adults of 10 European countries: implications for adult vaccination. *Thorax* **2019**, *74*, 473–482.

4. Paton, J. C.; Trappetti, C. *Streptococcus pneumoniae* capsular polysaccharide. *Microbiol. Spectr.* **2019**, *7*, GPP3-0019-2018.

5. Geno, K. A.; Gilbert, G. L.; Song, J. Y.; Skovsted, I. C.; Klugman, K. P.; Jones, C.; Konradsen, H. B.; Nahm, M. H. Pneumococcal capsules and their types: past, present, and future. *Clin. Microbiol. Rev.* **2015**, *28*, 871–899.

6. Wernette, C. M.; Frasc, C. E.; Madore, D.; Carlone, G.; Goldblatt, D.; Plikaytis, B.; Benjamin, W.; Quataert, S. A.; Hildreth, S.; Sikkema, D. J.; Käyhty, H.; Jonsdottir, I.; Nahm, M. H. Enzyme-linked immunosorbent assay for quantitation of human antibodies to pneumococcal polysaccharides. *Clin. Diagn. Lab. Immunol.* **2003**, *10*, 514–519.

7. Campanero-Rhodes, M. A.; Llobet, E.; Bengoechea, J. A.; Solís, D. Bacteria microarrays as sensitive tools for exploring pathogen surface epitopes and recognition by host receptors. *RSC Adv.* **2015**, *5*, 7173–7181.

8. Kalograiaki, I.; Euba, B.; Proverbio, D.; Campanero-Rhodes, M. A.; Aastrup, T.; Garmendia, J.; Solís, D. Combined bacteria microarray and quartz crystal microbalance approach for exploring glycosignatures of nontypeable *Haemophilus influenzae* and recognition by host lectins. *Anal. Chem.* **2016**, *88*, 5950–5957.

9. Kalograiaki, I.; Euba, B.; Fernández-Alonso, M. C.; Proverbio, D.; St. Geme III, J. W.; Aastrup, T.; Garmendia, J.; Cañada, F. J.; Solís, D. Differential recognition of *Haemophilus influenzae* whole bacterial cells and isolated lipooligosaccharides by galactose-specific lectins. *Sci. Rep.* **2018**, *8*, 16292.

10. Campanero-Rhodes, M. A.; Palma, A.; Menéndez, M.; Solís, D. Microarray strategies for exploring bacterial surface glycans and their interactions with glycan-binding proteins. *Front. Microbiol.* **2020**, *10*, 2909.

11. Wang, D.; Liu, S.; Trummer, B. J.; Deng, C.; Wang, A. Carbohydrate microarrays for the recognition of cross-reactive molecular markers of microbes and host cells. *Nat. Biotechnol.* **2002**, *20*, 275–281.

12. Geissner, A.; Pereira, C. L.; Leddermann, M.; Anish, C.; Seeberger, P. H. Deciphering antigenic determinants of *Streptococcus pneumoniae* serotype 4 capsular polysaccharide using synthetic oligosaccharides. *ACS Chem. Biol.* **2016**, *11*, 335–344.

13. Parameswarappa, S. G.; Reppe, K.; Geissner, A.; Ménová, P.; Govindan, S.; Calow, A. D. J.; Wahlbrink, A.; Weishaupt, M. W.; Monnanda, B. P.; Bell, R. L.; Pirofski, L.-a.; Suttorp, N.; Sander, L. E.; Witzenrath, M.; Pereira, C. L.; Anish, C.; Seeberger, P. H. A semi-synthetic oligosaccharide

conjugate vaccine candidate confers protection against *Streptococcus pneumoniae* serotype 3 infection. *Cell Chem. Biol.* **2016**, *23*, 1407–1416.

14. Emmadi, M.; Khan, N.; Lykke, L.; Reppe, K.; Parameswarappa, S. G.; Lisboa, M. P.; Wienhold, S.-M.; Witzernath M.; Pereira, C. L.; Seeberger, P. H. A *Streptococcus pneumoniae* type 2 oligosaccharide glycoconjugate elicits opsonic antibodies and is protective in an animal model of invasive pneumococcal disease. *J. Am. Chem. Soc.* **2017**, *139*, 14783–14791.

15. Lisboa, M. P.; Khan, N.; Martin, C.; Xu, F. F.; Reppe, K.; Geissner, A.; Govindan, S.; Witzernath, M.; Pereira, C. L.; Seeberger, P. H. Semisynthetic glycoconjugate vaccine candidate against *Streptococcus pneumoniae* serotype 5. *Proc. Natl. Acad. Sci. U.S.A.* **2017**, *114*, 11063–11068.

16. Ménová, P.; Sella, M.; Sellrie, K.; Pereira, C. L.; Seeberger, P. H. Identification of the minimal glycotope of *Streptococcus pneumoniae* 7F capsular polysaccharide using synthetic oligosaccharides. *Chemistry* **2018**, *24*, 4181–4187.

17. Stacey, H. L.; Rosen, J.; Peterson, J. T.; Williams-Diaz, A.; Gakhar, V.; Sterling, T. M.; Acosta, C. J.; Nolan, K. M.; Li, J.; Pedley, A.; Benner, P.; Abeygunawardana, C.; Kosinski, M.; Smith, W. J.; Pujar, H.; Musey, L. K. Safety and immunogenicity of 15-valent pneumococcal conjugate vaccine (PCV-15) compared to PCV-13 in healthy older adults. *Hum. Vaccin. Immunother.* **2019**, *15*, 530–539.

18. Rupp, R.; Hurley, D.; Grayson, S.; Li, J.; Nolan, K.; McFetridge, R. D.; Hartzel, J.; Abeygunawardana, C.; Winters, M.; Pujar, H.; Benner, P.; Musey, L. A dose ranging study of 2 different formulations of 15-valent pneumococcal conjugate vaccine (PCV15) in healthy infants. *Hum. Vaccin. Immunother.* **2019**, *15*, 549–559.

19. Kalograiaki, I.; Campanero-Rhodes, M. A.; Proverbio, D.; Euba, B.; Garmendia, J.; Aastrup, T.; Solís, D. Bacterial surface glycans: Microarray and QCM strategies for glycophenotyping and exploration of recognition by host receptors. *Methods Enzymol.* **2018**, *598*, 37–70.



# For Table of Contents Only

