Development of a sensitive, multiplexed immunoassay using xMAP beads for detection of serotype-specific *Streptococcus pneumoniae* antigen in urine samples

Carmen L. Sheppard,¹ Timothy G. Harrison,¹ Michael D. Smith² and Robert C. George¹

Correspondence

Carmen L. Sheppard
Carmen.Sheppard@hpa.org.uk

¹Respiratory and Systemic Infection Laboratory, Health Protection Agency – Centre for Infections, 61 Colindale Avenue, London NW9 5HT, UK

²Department of Microbiology, Musgrove Park Hospital, Taunton TA1 5DA, UK

In support of the surveillance of pneumococcal infections in the era of conjugate vaccines, a sensitive and specific multiplex immunoassay using xMAP beads has been developed for direct detection of pneumococcal serotype-specific polysaccharides in clinical samples, particularly urine. The assay was tested on panels of spiked urine specimens, clinical urine specimens and bacterial isolates. Each of the 14 serotypes in the multiplex assay can be detected to 0.1 ng purified polysaccharide ml⁻¹, or less. Testing of a panel of urine specimens from patients with culture-confirmed pneumococcal or non-pneumococcal disease indicated that the multiplex assay is both sensitive and specific. The correct pneumococcal serotype was identified directly from urine in 46/58 (79.3%) patients who had a contemporaneous blood culture isolate of a multiplex assay serotype. Furthermore, the specificity of the assay on this panel of samples was 99.3% (145/146). This multiplex assay could be useful, in conjunction with the pneumococcal screening test Binax NOW, in urine for diagnosis of pneumococcal disease and the identification of the aetiological serotype, and potentially be of benefit in culture-negative patients.

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INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is a major cause of morbidity and mortality worldwide. To date, 92 serotypes of pneumococcus have been described (Henrichsen, 1995; Park *et al.*, 2007) but many of these are rarely found in disease. The currently licensed 23 serotype-valent polysaccharide and 7-, 10- and 13-valent protein-conjugate vaccines cover the most important circulating serotypes. With the uptake of any new pneumococcal serotype based vaccines, surveillance of serotypes causing disease is important.

The diagnosis of pneumococcal disease is hampered by the relative insensitivity of culture techniques (Musher, 1992) and therefore the serotype distribution data available to inform the epidemiology of the disease are based on a fraction of the circulating disease-causing strains, most commonly based on isolates from sterile sites (Hanquet *et al.*, 2010; Trotter *et al.*, 2010).

The Binax NOW *Streptococcus pneumoniae* antigen detection kit for use on urine samples offers a non-invasive

method of detecting *S. pneumoniae* infection and has improved the rate of detection in adult patients (Smith *et al.*, 2003); however, this assay does not yield any serotype data to inform epidemiological study and vaccine development. Determination of the serotype of an infecting *S. pneumoniae* strain by non-culture methods would be of benefit for accurate surveillance of pneumococcal infections.

An ELISA test for detection of serotype-specific antigens for 13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) in urine samples was developed by Leeming *et al.* (2005). This is a useful assay for determining serotype in culture-negative cases of pneumococcal infection but has significant limitations. Due to the need for one microtitre well per serotype, it has high sample volume and reagent requirements and is limited in its use for small volume samples such as cerebrospinal fluid (CSF). Furthermore, it is a laborious test for examining large numbers of samples.

Luminex xMAP bead technology can allow the running of multiplex immunoassays that can be read from a small sample volume using a principle based on flow cytometry (Kellar & Iannone, 2002). The beads can be coupled to antibodies or other proteins, and then used for various

Abbreviations: C-PS, C-polysaccharide; CSF, cerebrospinal fluid; %CV, percentage co-efficient of variation; mFI, median fluorescence intensity; T/N, Test to Negative.

assay formats including immunoassay. The beads are differentiated on the Luminex instrument on the basis of the dye labels integral to them. In this way, many different analytes can be evaluated from a single sample. The use of Luminex xMAP bead technology has proven an effective method for detection of pathogens (Dunbar *et al.*, 2003). This technology should enable pneumococcal serotype determination from a single sample well via a multiplex immunoassay comprising pneumococcal serotype-specific capture antibodies, each conjugated to spectrally distinct beads.

This paper describes the use of a Bio-Plex suspension array instrument to run a multiplex immunoassay using Luminex xMAP beads coupled to mAbs for detection of pneumococcal serotype-specific polysaccharide and C-polysaccharide (C-Ps) antigen directly from urine. The assay was developed for use in conjunction with Binax NOW, providing both non-invasive immediate diagnosis of *S. pneumoniae* disease by Binax NOW and subsequent identification of infecting serotype by the Bio-Plex assay.

METHODS

Bacterial strains used for specificity testing of the Bio-Plex assay. Pneumococcal isolates representing 85 different pneumococcal serotypes and 35 other strains including non-typable pneumococci (exhibiting a rough phenotype by conventional serotyping) ($n=9$), *Streptococcus mitis* ($n=13$) and *Streptococcus oralis* ($n=13$) were recovered from the Respiratory and Systemic Infection Laboratory (RSIL) culture collection and cultured on Columbia blood agar (ColBA) for use in specificity tests. RSIL is the national reference laboratory and serotypes all submitted *S. pneumoniae* strains from invasive disease in England and Wales, numbering around 5000 isolates per year (Trotter *et al.*, 2010).

Preparation of crude bacterial antigen for specificity testing. A thin-wall 96-well PCR plate (ABgene) was used and 200 μ l sterile distilled water containing 2% bile (sodium deoxycholate) was added to the number of wells required for the isolates. A 1 μ l loop of overnight culture from a ColBA agar plate was added and gently swirled until the cell clump was removed from the loop into the liquid. The bile solution lysed pneumococcal cells, breaking down any debris that could block wells or cause aggregation of the assay beads. The wells were capped and the plate was vortexed briefly, then left on the bench for 10–15 min until clearing was seen in the wells. The PCR plate was placed into a thermocycler (MJ Research/GRI) and heated to 100 °C for 5 min to kill any remaining bacteria. The sample was allowed to cool and then diluted 1:100 by adding 2 μ l to 198 μ l PBS in a second 96-well plate. For antigen detection, 40 μ l of the killed, diluted suspension was added to 60 μ l PBS on the Bio-Plex assay plate.

Preparation of spiked urine standards for sensitivity analysis. As the assay is intended for use on urine samples, urine from a healthy staff member was spiked with various concentrations of a pooled 'pneumococcal polysaccharide mix' to produce spiked urine standards. The polysaccharide mix comprised purified polysaccharides for serotypes 1, 3, 4, 5, 6A, 6B, 7F, 8, 9V, 14, 18C, 19A, 19F and 23F, all polysaccharides from ATCC, except 6A, which was kindly donated by Pfizer (formerly Wyeth Vaccines Research). It had been shown in optimization experiments that mixing of polysaccharides did not affect the sensitivity of detection. The concentrations of each

polysaccharide in the standards were: 10, 3, 1, 0.3, 0.1, 0.03 and 0.01 ng ml⁻¹. Negative urine was also used as a control. HEPES buffer (Fisher Scientific) was added to all standard and negative urine samples at 25 mM final concentration, and samples were centrifuged to remove any sediment.

For reproducibility evaluation, standards plus unspiked negative control urine were run in triplicate in the assay on five separate occasions. Each reproducibility evaluation assay run used the same preparation of xMAP beads and other reagents.

Panel of urine specimens from patients with confirmed invasive pneumococcal and non-pneumococcal disease. Aliquots of urine from 146 patients with confirmed invasive infections (bacteraemia/pneumonia) taken during a large study of the use of the Binax NOW urinary antigen test for diagnosis of *S. pneumoniae* infection (Smith *et al.*, 2003) were obtained for testing. The samples were part of a series of clinical studies on the diagnosis of severe community-acquired infections conducted in South West England. The study was approved by the South and West Multicentre Research Ethics Committee and informed consent was obtained from patients or relatives.

Of these patients, 63 had *S. pneumoniae* cultured from blood and 82 had bacteraemia due to non-pneumococcal species, e.g. *Staphylococcus aureus*, *Escherichia coli*, *Legionella pneumophila*, *Streptococcus constellatus* and *Klebsiella pneumoniae*. The urine samples were all collected between 1998 and 2002 and had been stored at -80 °C prior to testing.

Antibodies used for assay. Purified mAbs (mouse derived) for serotypes (1, 3, 4, 5, 6A/C, 6B, 7F/A, 8, 9V, 14, 18, 19A, 19F and 23F) were kindly provided by Pfizer (formerly Wyeth Vaccines Research). An IgM mAb for pneumococcal C-Ps (HASP-8) was purchased from the Statens Serum Institut (SSI), Copenhagen, Denmark.

The secondary detection antibodies used in the assay were SSI polyclonal sera (rabbit serum) to pneumococcal serogroups or types (where no subtypes exist) 1, 3, 4, 5, 6, 7, 8, 9, 14, 18, 19, 23 and C-Ps. Optimization experiments determined that some sera performed best when purified for IgG, and others performed equally well when desalted only. Where appropriate, a Pierce Melon Gel Spin Purification kit (Pierce/Perbio) was used to purify the IgG from SSI polyclonal sera, following the manufacturer's instructions. If full purification was not necessary, the serum was filtered with a 0.2 μ m syringe filter (Minisart) and desalted using PD-10 columns (Amersham). A list of all the antibodies used in the assay is shown in Table 1.

Coupling of mAb to Luminex xMAP beads. Each mAb preparation was coupled to spectrally distinct carboxylated Luminex xMAP beads. The beads used and the antibody coupled to them are shown in Table 1.

For all assays, the protein coupling method as available on the Luminex website ([http://www.luminexcorp.com/uploads/data/Protein Protocols FAQs/Protein Coupling Protocol 0407 10207.pdf](http://www.luminexcorp.com/uploads/data/Protein%20Protocols%20FAQs/Protein%20Coupling%20Protocol%200407%2010207.pdf)) was followed. MES buffer (pH 5.0) was used for all serotype mAbs except serotype 3 and 19F, which were found to be more effectively coupled if PBS buffer (pH 7.0–7.4) was substituted for MES buffer in the coupling reaction. A concentration of 3.2 μ g antibody per million beads coupled was used, except for 19A, for which 3.6 μ g antibody per million beads was used to ensure efficient coupling. The coupled beads were stored in PBS + 1% BSA (Sigma-Aldrich).

The beads were enumerated after coupling by use of a disposable counting chamber (ISL Laboratories) and light microscope (Zeiss) at $\times 10$ magnification.

Multiplex serotype-specific xMAP bead assay. Single-use aliquots of mixed antibody-coupled beads containing 4×10^5 beads of each assay type ml⁻¹ ($4 \times$ working concentration) were prepared

Table 1. mAbs and secondary polyclonal antibodies used in the assay

Stated specificity (serotype)	Mouse mAb*	Rabbit polyclonal antibody type	Antibody preparation
1	Pn 26-2	SSI type 1 serum	Filtered and desalted
3	Pn 459-1	SSI type 3 serum	Filtered and desalted
4	Pn 31-1	SSI type 4 serum	Purified IgG
5	Pn 55-1	SSI type 5 serum	Filtered and desalted
6A	Pn 10-2.1	SSI group 6 serum	Purified IgG
6B	Pn 36-1	SSI group 6 serum	Purified IgG
7F	Pn 503-1	SSI group 7 serum	Filtered and desalted
8	Pn 814-1	SSI type 8 serum	Purified IgG
9V	Pn 45-1	SSI group 9 serum	Filtered and desalted
14	Pn 42-1	SSI type 14 serum	Filtered and desalted
18C	Pn 56-1	SSI group 18 serum	Purified IgG
19A	Pn 177-7	SSI group 19 serum	Purified IgG
19F	Pn 63-1	SSI group 19 serum	Purified IgG
23F	Pn 53-2	SSI group 23 serum	Filtered and desalted
C-Ps	HASP-8	SSI C-Ps serum	Filtered and desalted

*All mAbs were from Pfizer (formerly Wyeth Vaccines Research), except HASP-8, which was obtained from Statens Serum Institut (SSI), Copenhagen.

and transferred to light-protective amber microcentrifuge tubes (Alpha Laboratories), which were stored at 2–8 °C until use.

PBS (100 µl) was used to pre-wet the wells of an opaque filter plate (Millipore multiscreen MSBVN1B) and the wells were emptied by vacuum using a BioTek eLX 50 microplate washer with vacuum manifold accessory (Fisher Scientific). The bottom of the plate was blot dried and 100 µl test urine sample or control sample was added to the assay plate. At least one 'no antigen' control well, consisting of PBS or negative control urine from a healthy donor, was added to each plate tested and a standard titration containing purified capsular polysaccharides (ATCC, LGC Standards) of each serotype at concentrations of 10, 3, 1, 0.3, 0.1, 0.03 and 0.01 ng ml⁻¹ was included on every plate.

For each assay plate, an aliquot of pre-prepared multi-bead mix was removed from the refrigerator, made up to the final working concentration with PBS, vortexed and sonicated using an ultrasonic bath (Grant) for 30 s. Then, 25 µl of the multi-bead mixture (containing 2500 beads of each coupled bead type) was added to each of the assay wells containing sample or control. The plate was covered with a plastic microtitre plate lid with anti-contamination rings (Costar corner notch lid; Corning). To protect the beads from photobleaching, the lid was made opaque by the application of a foil plate-sealer (ABgene) to the outside. The plate was incubated, shaking at low speed on a Jencons titre plate shaker (VWR International) at speed level 2, overnight at room temperature.

The plate was washed four times with 200 µl per well PBS plus 0.5 % Tween 20 (Media services, Centre for Infections, London, UK), followed by vacuum filtration as before. After the first wash step, a plate shake step was incorporated to resuspend the beads. The polyclonal secondary antibodies were pre-mixed and stored in single-use aliquots at -80 °C. The final concentration of polyclonal antibody in the pre-mixed vials was 1 µg ml⁻¹ for the purified IgGs and a final dilution of 1/21 000 (compared to neat serum) for the desalted sera. For each assay, an aliquot of the stored mixed polyclonal antibodies was thawed and the volume was made up using Starting Block buffer (SBB; Pierce/Perbio) to form a multi-antibody mixture as detailed in Table 1.

One hundred microlitres of the multi-antibody mixture was added to each well and the dark lid was placed on the plate. The plate was

incubated shaking for 1 h at room temperature then washed as stated before. After washing, 100 µl per well goat anti-rabbit IgG R-phycoerythrin conjugate (Pierce/Perbio) at 1 : 200 dilution in SBB was added. The plate was incubated shaking for 30 min at room temperature, then washed as previously described. The beads were resuspended in 125 µl PBS, the dark lid was applied and plate was then placed on a plate shaker while the Bio-Plex multi-analyte suspension array instrument (Bio-Rad) was calibrated using the Bio-Plex calibration kit (Bio-Rad) and prepared for reading. For each well, a minimum of 100 beads per region were counted and the median fluorescence intensity (mFI) value was given as the result for that bead region.

Statistical methods. The Bio-Plex assay was tested for sensitivity and reproducibility using the standard preparations of negative urine spiked with various concentrations of pneumococcal polysaccharide antigen, as described above.

To determine the reproducibility of the triplicates within a run, the intra-assay mFI percentage co-efficient of variation (%CV) values from all five runs were used to calculate the overall mean intra-assay %CV for each standard. The intra-assay %CV for the mFI of all the serotype assays was below 15 % for all the standards in all the assays (Table 2).

The inter-assay reproducibility was assessed by calculation of the %CV of the five run mean of the mFI readings for each point on the standard curve for each serotype assay [%CV=(SD/mean) × 100].

Interpretation of results. To easily compare the results of different serotype assays when their overall signal strengths were different, a Test to Negative (T/N) signal ratio was used. The use of this ratio enabled direct comparisons of the different serotype assays. A cut-off was assigned as a T/N ratio of 2.0 (i.e. the mFI of a positive sample was at least twice that of the signal from the negative control). The T/N value of 2.0 was chosen because for all serotypes the negative mean + 3 standard deviations (intra-assay SDs) was always far less than twice the negative mean.

This cut-off was used for all testing of spiked samples. Preliminary work showed that when testing clinical specimens (including CSF,

Table 2. Mean %CV values, with maximum and minimum %CV, at each point on the standard curve and negative control, determined from five assay runs

Standard	Intra-assay variation			Inter-assay variation		
	Mean %CV from all assays	Max %CV	Min %CV	Mean %CV from all assays	Max %CV	Min %CV
10 ng	3.9	5.9	1.7	4.9	10.0	1.8
3 ng	4.2	6.9	3.1	5.0	8.3	1.2
1 ng	4.2	9.5	1.8	5.2	10.6	2.2
0.3 ng	5.5	12.4	1.8	5.4	9.1	2.2
0.1 ng	5.7	8.7	3.0	11.4	20.0	6.9
0.03 ng	5.6	9.0	4.2	14.6	31.3	8.8
0.01 ng	6.5	13.9	3.8	8.0	17.2	2.2
Negative	6.2	9.1	3.6	9.1	16.6	2.8

urine, pleural fluid; results not shown), some specimens gave an elevated background in all serotype assays compared to the negative control, which led to multiple and erroneous positives if a single T/N ratio cut-off was used on the raw mFI values.

To address this, a normalization procedure was developed which assumes that most cases should not have more than one serotype positive (and certainly not more than two). For each specimen, the 85th percentile of the T/N results of the 14 assays (not including C-Ps) was calculated, which gives approximately the 11th highest serotype result. The T/N results for all assays were then divided by this figure. This enabled the cut-off T/N of 2 to be applied to all the normalized data. Note that the reason for choosing the 85th percentile rather than the median was because high background results tended to affect a few, rather than all, assays results for a specimen. This normalization method was applied to all clinical specimens tested.

RESULTS

Bio-Plex assay specificity testing

The multiplex assay was tested for serotype specificity by testing isolates of 85 serotypes of *S. pneumoniae* plus a panel including 13 *S. mitis* strains, 13 *S. oralis* strains and nine non-typable pneumococcal strains.

On testing these strains, it was found that the serotype 18C assay cross-reacted with all serotypes within serogroup 18. Similarly, the serotype 7F assay cross-reacted with serotype 7A though with a weaker signal than that obtained from the serotype 7F isolate. These results were confirmed by repeat testing and these two assays were designated serogroup 18- and serotypes 7F/A-specific, respectively, from this point on.

Once a strain of the newly designated serotype 6C became available (SSI), the assay was retested using this strain and it was noted that the 6A assay also cross-reacted with serotype 6C and therefore from this time the assay was designated 6A/C-specific.

There were no other cross-reactions noted between the serotype-specific assays and any other serotype or the non-pneumococcal bacterial species tested. One of the pneumococcal isolates that was recorded on the database as 'non-typable' gave a clear signal for serotype 9V during the

specificity testing. This isolate was rechecked by conventional serotyping and found to indeed be serotype 9V.

All of the pneumococcal isolates tested gave a C-Ps-positive result and, interestingly, 18 of the 26 non-pneumococcal species tested also gave a C-Ps-positive result. Of these, nine were *S. mitis* and nine were *S. oralis* isolates. It is known that structures similar to the pneumococcal C-Ps (phosphorylcholine) can be found on the oral streptococci (Gillespie *et al.*, 1993).

The inter-assay %CV was <20% at most points on the standard curve in all assays (Table 2), the highest %CV at any point on the curve for any assay being serotype 3, with a figure of 31.3% for the inter-assay %CV at point 0.03 ng ml⁻¹. In general, there was an increase in inter-assay %CV as the concentration of polysaccharide decreased; this was expected due to the greater chance of distribution errors associated with testing small quantities of polysaccharide.

Assessing assay limits of detection

Using the data from the standard curves run in triplicate over five assay runs, the assay sensitivity was determined as the lowest standard on the standard curve giving a mean mFI at or above twice that of the mean negative control mFI (≥ 2.0 T/N ratio). The lower limits of detection (LLOD) found using this method were 0.1 ng ml⁻¹ for serotype 3, 9V and 19A assays, 0.03 ng ml⁻¹ for serotype 1, 6A/C, 6B, 8, 14 and 18 assays, 0.01 ng ml⁻¹ for serotype 5, 19F and 23F assays and <0.01 ng ml⁻¹ for serotype 4 and 7F/A assays. Each of the mean mFI readings giving the T/N values used to determine these LLODs was at least 10 standard deviations greater than the mean negative control mFI (range 11–27 SD), suggesting that the T/N=2.0 cut-off was very specific. Each of the purified polysaccharides for the serotypes contained some contamination with C-Ps, therefore it was not possible to get an accurate LLOD for the C-Ps assay using these standard curves. However, a separate single-estimate standard curve was run containing only purified C-Ps and a LLOD of <0.1 ng ml⁻¹ was obtained.

South West study panel of clinical urine samples

The results in Table 3 were obtained from the samples in the panel that had a matched pneumococcal isolate.

The urine panel contained samples which had matched cultures representing all the serotypes in the assay panel except for serotype 5. The overall sensitivity of the assay against samples with matched culture-confirmed serotypes covered by the assay was 79 % (i.e. 46 of 58 urines were positive for the appropriate pneumococcal serotype-specific capsular polysaccharide). However, of concern is the fact that only one of the five samples with matching serotype 6B cultures gave a 6B-positive in the assay. Excluding serotype 6B, the sensitivities of the individual assays as determined by the urine panel samples ranged from 40 to 100 %, although the number of samples available for each serotype was small (Table 3).

One sample which had a matched blood culture isolate recorded as 22F gave a repeatable serotype 5 in the Bio-Plex assay. This assay was repeated three times on three separate aliquots of this urine specimen but each time gave a clear serotype 5-positive result. Another aliquot of urine from the same patient but taken 2 days after the original aliquot was sourced from the study collection; this sample also tested positive for serotype 5 in the urine assay.

During the testing of the clinical urine specimens, it was observed that only one sample gave a positive signal for

C-Ps in the Bio-Plex assay. This was obtained from a sample with a strong serotype 23F-positive signal.

No positive signals for any serotype-specific assay or C-Ps were obtained from any of the 82 clinical urine specimens from patients with confirmed non-pneumococcal infection.

DISCUSSION

Understanding the serotype distributions in culture-negative samples (i.e. urine, blood, CSF) by use of antigen detection assays and how they relate to the distributions in isolates will provide valuable epidemiological information for vaccine development and disease monitoring and diagnosis. The Bio-Plex serotype-specific pneumococcal assay would be an ideal tool for use on such samples.

The multiplexed serotype-specific antigen detection assay described here is similar to but offers benefits over the ELISA method reported previously (Leeming *et al.*, 2005) for the determination of serotype in culture-negative infections. The use of multiplexing on the Bio-Plex instrument enables determination of all serotypes available in the assay panel from a single well, thus reducing reagent and sample volume requirements. The ELISA method and the multiplex described here use nearly the same reagents in terms of the pneumococcal-specific mAbs and polyclonal antibodies. The ELISA used the polyclonal antibody

Table 3. Testing of South West study urines with matched pneumococcal culture isolates

Culture confirmed serotype	No. of urine samples	Correct assay serotype detected	Assay serotype not detected	Incorrect serotype	Individual assay sensitivity (%)
Assay serotypes					
1	7	7	0	0	100
3	3	2	1	0	67
4	3	3	0	0	100
6A	2	2	0	0	100
6B	5	1	4	0	20
7F	2	2	0	0	100
8	4	4	0	0	100
9V	2	2	0	0	100
14	17	15	2	0	88
18C	2	2	0	0	100
19A	2	2	0	0	100
19F	5	2	3	0	40
23F	4	2	2	0	50
Non-assay serotypes					
9N	1	NA	1	0	
12F	2	NA	2	0	
15A	1	NA	1	0	
22F	1	NA	0	1*	
Totals	63	46	16	1	

NA, Not applicable.

*Serotype 5 antigen detected.

ies as the capture antibody and the mAbs to detect; however, in the multiplex assay, the mAbs were used as the capture antibody in order to distinguish between types within groups (such as 19A and 19F) using the differing Luminex beads.

The multiplex assay detected 0.1 ng purified polysaccharide ml⁻¹ or less of all assayed serotypes, similar to the ELISA method. This level of detection is at least as good as the commercial Binax NOW pneumococcal antigen test in terms of ng polysaccharide ml⁻¹ (Binax NOW analytical sensitivity was determined by our laboratory to be 1 ng purified polysaccharide ml⁻¹ when read at 15 min) and has the advantage of providing serotype information. Some assays within the multiplex can detect levels of polysaccharide below 0.01 ng ml⁻¹.

The analytical specificity testing of the multiplex assay using culture isolates and spiked urine specimens showed that the overall serotype specificity of the assay is excellent. The only cross-reactions noted were between the serotype 18C-specific mAb and all the serotypes within group 18, the 7F-specific mAb and serotype 7A and the 6A mAb with 6C.

Some of the inter- and intra-assay variation seen may reflect the cumbersome technical process that was necessary to prepare the reagents and standards and run the assay, as well as the underlying assay reproducibility. The multiplex assay method is a complex process requiring many reagents. However, due to the 96-well plate format of the assay, it could be fully automated with the use of robotic plate handlers, reducing manual labour and improving the reproducibility of results. This assay is intended, in its current format, to give qualitative positive/negative results, in which case some of the variation may not matter as long as the cut-off value is reliable. However, it could also be used in a quantitative form to infer concentration of polysaccharide ml⁻¹, which may be related to infection severity, in which case the %CVs of the standards and samples would be much more important.

Testing of the South West evaluation panel of clinical urine samples showed that the Bio-Plex assay gave an overall sensitivity of 79.3 % against specimens from patients who had an *S. pneumoniae* culture of a serotype available within the assay. The specificity of the assay was 99.3 % with only one specimen giving a result for a serotype that was not expected. This specimen had a matched blood culture isolate of serotype 22F but gave a repeatable and clear serotype 5 result in the Bio-Plex assay. Investigation of the matched isolate revealed that it was correctly typed as a 22F. A separate urine aliquot from the same patient but taken 2 days later than the original specimen was also tested; this aliquot also gave a positive result for serotype 5. This would seem good evidence for the validity of the urine result and the possibility of a sample match error affecting the culture isolate cannot be ruled out. One possible alternative explanation for this result is dual-serotype infection, where serotype 5 was not cultured and serotype 22F is not detectable in this assay. Testing of serotype 22F

cultures showed that the type 5 assay does not cross-react with serotype 22F pneumococci.

One unexpected finding of testing real clinical urine specimens was that very few of the pneumococcal-positive samples gave any signal with the C-Ps assay, despite good sensitivity of this assay using culture and purified polysaccharides and most urine samples also being positive by the Binax NOW assay, which also detects C-Ps.

The reason for the consistent negativity of the C-Ps and the low rate of positivity of the 6B, 19F and 23F assays on the clinical urine specimens, when the assays have been proven to detect the polysaccharide in spiked urine, has not yet been explained. It could possibly be due to the specificity of the mAb. If the polysaccharide is somehow degraded or processed during its transport through the kidneys, this could be the reason that the mAb can no longer detect it in the clinical urine specimens. If so, this is a drawback of this assay in its current form, for detecting these polysaccharides in urine. The C-Ps assay was originally intended to indicate whether a sample was likely to be a non-assay type (by the strength of C-Ps signal in the absence of a positive serotype result) and this outcome has rendered this particular component of the assay useless on urine specimens.

Tests using the Bio-Plex assay have shown that C-Ps and 6B polysaccharide are both readily detectable in other clinical specimens such as CSF, pleural fluid and blood (C. Sheppard, unpublished data) from patients with *S. pneumoniae* infection.

This assay should be a useful tool for investigating the serotypes of pneumococci present in pneumococcal infections, particularly where a culture isolate cannot be grown. It has limitations, but has been proven to be capable of detecting pneumococcal serotype-specific polysaccharides directly from urine specimens, which is a widely available, non-invasive sample type. Used in conjunction with the Binax NOW assay for initial diagnosis of pneumococcal infection, the multiplex assay could provide valuable extra serotype information to help inform surveillance activities and in outbreak situations.

Continuing development by sourcing further mAbs to increase the serotype range, automation and further optimization of the method will improve the assay further and increase its usefulness. Finally, it is important to note that this methodology as applied to clinical samples detects phenotypic expression of the specific capsular serotypes *in vivo* rather than inferring this from a DNA/PCR-based method, which may or may not indicate capsule expression during infection.

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