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Development of an Extended-Specificity Multiplex Immunoassay for Detection of *Streptococcus pneumoniae* Serotype-Specific Antigen in Urine by Use of Human Monoclonal Antibodies

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ABSTRACT Current pneumococcal vaccines cover the 10 to 23 most common serotypes of the 92 presently described. However, with the increased usage of pneumococcal-serotype-based vaccines, the risk of serotype replacement and an increase in disease caused by nonvaccine serotypes remains. Serotype surveillance of pneumococcal infections relies heavily on culture techniques, which are known to be insensitive, particularly in cases of noninvasive disease. Pneumococcal-serotype-specific urine assays offer an alternative method of serotyping for both invasive and noninvasive disease. However, the assays described previously cover mainly conjugate vaccine serotypes, give little information about circulating nonvaccine serotypes, and are currently available only in one or two specialist laboratories. Our laboratory has developed a Luminex-based extended-range antigen capture assay to detect pneumococcal-serotype-specific antigens in urine samples. The assay targets 24 distinct serotypes/serogroups plus the cell wall polysaccharide (CWP) and some cross-reactive serotypes. We report that the assay is capable of detecting all the targeted serotypes and the CWP at 0.1 ng/ml, while some serotypes are detected at concentrations as low as 0.3 pg/ml. The analytical serotype specificity was determined to be 98.4% using a panel of polysaccharide-negative urine specimens spiked with nonpneumococcal bacterial antigens. We also report clinical sensitivities of 96.2% and specificities of 89.9% established using a panel of urine specimens from patients diagnosed with community-acquired pneumonia or pneumococcal disease. This assay can be extended for testing other clinical samples and has the potential to greatly improve serotype-specific surveillance in the many cases of pneumococcal disease in which a culture is never obtained.

KEYWORDS diagnostics, immunization, monoclonal antibodies, pneumococcus, surveillance studies

Despite being vaccine preventable, *Streptococcus pneumoniae* (pneumococcus) remains a major cause of morbidity and mortality worldwide. Pneumococcal disease (PD) includes both invasive and noninvasive disease. The former includes bacteremia and meningitis; the latter, nonbacteremic pneumonia, sinusitis, and acute otitis media. To date, 97 "serotypes" of pneumococcus have been reported (1); however, some of these show only genotypic differences and produce the same polysaccharide structures (2). Ninety-two serotypes are currently described by the Danish system using commercial typing sera from SSI Diagnostica AG (Hillerød, Denmark). These serotypes differ in

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virulence, prevalence, and antibiotic resistance (3, 4). In 2000 and 2001, a 7-valent protein conjugate vaccine, Prevnar (PCV7; Wyeth/Pfizer), was licensed for use in the United States and the European Union, respectively. In 2010, a 10-valent conjugate vaccine, Synflorix (PCV10; GSK), covering the same serotypes as PCV7 with the addition of serotypes 1, 5, and 7F, and a 13-valent protein conjugate vaccine, Prevnar 13 (PCV13; Pfizer), covering the PCV7 serotypes plus six additional serotypes, were licensed in the United States and Europe (5). The serotypes included in these vaccines were the most prevalent circulating serotypes associated with PD or antibiotic resistance at the time of their development (6–8). A 23-valent nonconjugate pneumococcal polysaccharide vaccine (PPV23) is also licensed for use in the United Kingdom for adults over the age of 65 years and for individuals aged ≥2 years in at-risk groups. PPV23 covers the PCV13 types except for serotype 6A but includes an additional 11 serotypes. Surveillance studies on different continents have identified a decrease in the prevalence of the conjugate vaccine serotypes and an increase in the prevalence of certain serotypes not presently covered by these vaccines (6–10). It is therefore necessary to continue to monitor the distribution of pneumococcal serotypes in order to determine the effectiveness of available vaccines for the prevention of pneumococcal disease. This is particularly important for assessing vaccine impact in low-income countries where there is a very high burden of pneumococcal disease.

A number of different methods for serotyping pneumococci have been published. Currently the gold-standard method for serotyping isolates is the Quellung reaction (11, 12). While this method is capable of identifying 92 pneumococcal serotypes, it requires the use of many specific pneumococcal antisera and can be costly and laborious. Furthermore, this method of typing requires the recovery of a viable pneumococcal culture and thus precludes cases where an isolate is not obtained—for example, when antimicrobial treatment has been administered prior to specimen collection (13, 14), or in cases of noninvasive disease. The development of pneumococcal capsular typing methods using a number of techniques, such as specific enzyme-linked immunosorbent assay (ELISA), competitive enzyme immunoassay (EIA), multiplex immunoassay (MIA), single and multiplex PCRs, and capsular sequence typing (CST) (15–21), has contributed to serogroup/serotype surveillance of invasive and noninvasive PD, particularly when an isolate is unavailable, by their direct application to clinical specimens.

The polysaccharide components of the pneumococcal cell wall can be detected in the urine of individuals infected with pneumococci and in healthy children carrying the organism in their nasopharynx (22–27). The BinaxNOW pneumococcal test (Alere) is a point-of-care test that can detect pneumococcal cell wall C polysaccharide in urine and cerebrospinal fluid (CSF) samples. The test offers a rapid, noninvasive method to aid in the diagnosis of pneumococcal pneumonia and meningitis. With manufacturer-reported sensitivities and specificities in urine of 86 to 90% and 71 to 94% (28), respectively, and independently published sensitivities of 74% and specificities of 94 to 97% (29, 30), the BinaxNOW test is often used in health care settings as a noninvasive method of identifying infections caused by pneumococci without a requirement for culture. However, this test is not capable of reporting any serotype-specific information.

Luminex x-MAP technology involves the use of magnetic, spectrally distinct carboxylated polystyrene microspheres, or "beads," that can be coated with a broad range of molecules, including nucleic acids or proteins, and used in various assay formats, such as PCR-based assays and immunoassays (31, 32). Coating such beads with antibodies specific to pneumococcal serotypes allows for the simultaneous detection of different pneumococcal serotype antigens in a single sample, thus reducing the amount of sample required. To date, Luminex microsphere technologies have been used to aid in the detection of pneumococcal serotypes using antibody detection of polysaccharide (33, 34), competitive inhibition assays (21, 32, 35), PCR-based multiplex assays (32), and serological assays to detect antibody responses to pneumococcal serotypes (21, 36).

In 2011, our laboratory developed a sensitive and specific multiplex immunoassay using Luminex x-MAP technologies for the detection of serotype-specific pneumococcal antigen in urine (34). The assay was capable of identifying 14 serotypes (PCV13

TABLE 1 Calculated cutoff values for each serotype, based on results from negative-control urine in repeatability assays[a]

Stated serotype specificity (bead region)	Human MAb designation	Mean normalized negative-control ratio[b]	SD	Calculated cutoff value
1 (77)	pn082p5B06	1.3	0.092	1.59
2 (19)	pn201p3C02	1.4	0.067	1.58
3 (41)	pn201p6E03	1.3	0.091	1.55
4 (65)	pn082p3C05	1.4	0.132	1.77
5 (28)	pn225p1A02	1.4	0.151	1.88
6A (13)	pn134p1D04	1.3	0.076	1.55
6B (25)	pn201p4G01	1.4	0.090	1.66
7F (42)	pn132p8C04	1.2	0.063	1.39
8 (46)	pn201p4C01	1.4	0.070	1.61
9N (34)	pn201p2D02	1.3	0.075	1.48
9V (38)	pn082p4D06	1.3	0.059	1.49
10A (29)	pn219p4B04	1.2	0.040	1.34
11A (57)	pn082p8F05	1.3	0.111	1.66
12F (54)	pn134p3A05	1.3	0.118	1.65
14 (43)	pn225p1A03	1.0	0.021	1.08
15B (61)	pn225p1G05	1.3	0.096	1.63
17F (66)	pn219p1D03	1.2	0.067	1.37
18C (27)	pn082p3G05	1.4	0.114	1.77
19A (35)	pn219p4A05	1.4	0.161	1.89
19F (59)	pn132p7B06	1.4	0.114	1.78
20 (52)	pn219p2D06	1.1	0.262	1.86
22F (26)	pn201p3C03	1.1	0.071	1.27
23F (18)	pn132p2C05	1.4	0.096	1.64
33F (63)	pn219p1C01	0.9	0.038	0.99
CWP[c] (73)	pn082p3C04	1.8	0.119	2.19

[a]Calculated as 3 standard deviations greater than the mean of the normalized negative-control ratios.
[b]Mean *t*/*n* ratio, from six repeatability runs, of negative urine mFI/negative urine mFI (initially 1.0) following the plate normalization procedure.
[c]CWP, cell wall polysaccharide antigen target.

serotypes plus serotype 8) in urine and body fluids based on the detection of serotype-specific capsular polysaccharides using mouse-derived monoclonal antibodies (MAbs). The ability of this assay to provide serotype data in the absence of isolates has enabled its successful application in important, published studies of PD and in outbreaks, facilitating the understanding of serotype distribution in both invasive and noninvasive disease (37–42).

In this paper, we describe the development of an extended-spectrum pneumococcal-serotype-specific multiplex urine antigen detection (UAD) assay that is capable of detecting 24 distinct serotypes/serogroups plus the cell wall polysaccharide (CWP) antigen by use of fully human, full-length pneumococcal polysaccharide monoclonal antibodies, produced by Pamlico Biopharma and the Oklahoma Medical Research Foundation, coupled to Luminex beads.

RESULTS

Determination of the positivity cutoff. Analysis of the fluorescence intensity (FI) data produced from the repeatability runs in the novel 25-plex *S. pneumoniae* polysaccharide assay revealed that a normalized signal-to-noise ratio (test sample/negative control [*t*/*n*] ratio) of 2 is >3 standard deviations above the mean FI of the negative controls in all 25 assays and, therefore, based on these data, is >99% accurate for calling positivity compared to the negative-control urine standard. Furthermore, a normalized ratio of 2.5 is >3 standard deviations above the mean of the normalized ratios for the negative-control samples in all 25 assays (Table 1). Therefore, a *t*/*n* ratio of 2.5 was used as the general positivity cutoff for the analysis of the urine results.

Analytical sensitivity. The limit of detection for the purified capsular polysaccharides for each assay was estimated as 3 standard deviations above the mean FI of the negative controls in the repeatability runs. Based on these values, it was estimated that serotypes 1, 8, 14, 19A, and 22F can be detected at or below a concentration of 0.0003

TABLE 2 Modal minimum concentration for detection of each of the purified capsular polysaccharides[a] used in the standard curves in the repeatability runs when a normalized *t*/*n* ratio of 2.5 is used as the positivity cutoff

P/S[b] serotype	Concn (ng/ml)
1	0.001
2	0.01
3	0.001
4	0.003
5	0.003
6	0.003
6B	0.03
7F	0.1
8	0.001
9N	0.1
9V	0.001
10A	0.003
11A	0.003
12F	0.01
14	0.001
15B	0.003
17F	0.1
18C	0.3
19A	0.001
19F	1
20	0.03
22F	0.003
23F	0.03
33F	0.03

[a]Excluding the purified pneumococcal cell wall polysaccharide.
[b]P/S, polysaccharide.

ng purified polysaccharide/ml, serotypes 2, 3, 4, 5, 6A, 9V, 10A, 11A, 12F, 15B, 20, and 33F at concentrations as low as 0.001 ng/ml, serotypes 6B, 9N, 17F, and 23F at concentrations as low as 0.01 ng/ml, serotype 7F at 0.03 ng/ml, and serotypes 18C and 19F at 0.1 ng/ml. Table 2 lists the minimum concentration for reporting the positive detection of each of the purified polysaccharides in the standard curves used in the repeatability runs when a normalized *t*/*n* ratio cutoff of 2.5 is applied; the mode value is shown to avoid artificially high or low mean values caused by outlier results.

Analytical specificity. The pneumococcal-serotype specificity of the assay was tested using a panel of purified polysaccharides and a panel of 92 crude pneumococcal serotype reference strain antigens. Pneumococcal specificity tests indicate that 16 of 25 human MAbs (h-MAbs) exhibit cross-reactivity for other nontargeted pneumococcal serotypes, many of which are in the same serogroup (Table 3). Taking the cross-reactions into account, assay results positive for serotype 6A, 6B, 7F, 9V, 10A, 11A, 12F, 15B, 18C, 23F, or 33F are reported as serotype 6A/C, 6B/D, 7A/F, 9A/V, group 10/33C/39, 11A/C/E/45/group 16, 12F/44, group 15, group 18/35C/42, 23F/group 32, or 33A/B/D/F, respectively. However, when an h-MAb cross-reacts with a polysaccharide also targeted by another h-MAb in our UAD assay, this can be taken into consideration in interpreting the results. For example, since the 9N polysaccharide cross-reacts with the h-MAb targeting serotype 14, a sample producing a positive result with both the serotype 9N and serotype 14 MAbs could be interpreted as actually being positive for the serotype 9N polysaccharide antigen. This combination could also be positive for the 9L antigen, but in this case, the 9L antigen also cross-reacts with the h-MAbs targeting 9V and 19A, and therefore, for a sample containing the 9L antigen, a positive result for the 9V and 19A assays as well as for the serotype 9N and 14 assays would be observed. In this manner, results can be interpreted using a checkerboard system similar to that used for the serotyping of isolates by the Quellung reaction and by slide agglutination with the SSI antisera.

Analytical specificity with regard to nonpneumococcal antigens was tested using antigens prepared from 42 nonpneumococcal streptococci, comprising 13 different

TABLE 3 h-MAbs that cross-react with nontargeted pneumococcal serotypes

h-MAb target (bead region)	Serotype(s) with which the h-MAb cross-reacts[a]
6A (13)	6B,[b] 6C, 6D
6B (25)	6A,[b] 6D
7F (42)	7A
9N (34)	9L, **18A**, **43**, **47A**
9V (38)	9A, 9L
10A (29)	10B, 10C, 10F, **33C**, **39**
11A (57)	11C, 11D, 11E, **16A**, **16F**, **18A**, **18B**, **18C**, **45**
12F (54)	12B, **22A**, **44**
14 (43)	**9L**, **9N**[b]
15B (61)	15A, 15C, 15F, **11D**, **12B**, **18A**
18C (27)	18A, 18B, 18F, **35C**, **42**
19A (35)	19F,[b] **9A**, **9L**, **9V**[b]
19F (59)	19A[b]
22F (26)	22A, **43**
23F (18)	**32A**, **32F**
33F (63)	33A, 33B, 33D

[a]Serotypes in boldface indicate a cross-reaction with a different serogroup.
[b]Cross-reaction with a polysaccharide antigen detected in the 25-plex assay.

species, and 111 pure bacterial cultures associated with respiratory infections or the urogenital tract, comprising 21 genera and 59 different species (see Table S1 in the supplemental material). When tested at $>1.2 \times 10^6$ CFU/ml, eight of the nine Lancefield group B streptococcus (GBS) serotype II isolates gave *t*/*n* results of >2.0 for the pneumococcal serotype 14 assay, and six of these had *t*/*n* ratios of >2.5. However, the CWP assay was negative for all eight of these serotype 14-positive GBS serotype II samples. Among the other streptococci, all the *Streptococcus pseudopneumoniae*, *Streptococcus mitis*, and *Streptococcus peroris* (a member of the *S. mitis* group) isolates (two per species) tested positive for the pneumococcal CWP. None of the other bacterial isolates in the panel generated a positive result in this UAD assay, leading to analytical specificities of 98.4% (95% confidence interval [CI], 91.5%, 99.7%) and 95.2% (95% CI, 86.7%, 99.0%) for the serotype and CWP assays, respectively, against nonpneumococcal bacteria. None of the bacteria tested gave positive results for both the serotype and CWP assays.

Repeatability. All the results obtained for the clinical urine test samples and those spiked with purified polysaccharide antigen were repeatable over the 6 days, supporting the qualitative repeatability of the assay. Table 4 lists the UAD assay results for the urine samples used in the repeatability runs. The interassay coefficient of variation (CV) for each of the negative controls, except those in the 19A assay, was below 20%. The 19A assay produced high FI values for all three negative controls on day 4, and when these three results were omitted as outliers, the standard deviations, and therefore the CV, were reduced (CV, 11.8%). With the exception of the 7A/F, 18C, 19F, and 20 assays, the intra-assay and interassay *t*/*n* ratio CV for the standard curves were below 25%. As noted above (Table 2), the 7A/F, 18C, and 19F assays are currently among the least analytically sensitive of the 25 assays. Despite this variability, these assays accurately and repeatedly detected the target serotype when it was present at a concentration exceeding the limit of detection.

Clinical testing. The assay was used to test 616 urine samples. Of these, 520 were also tested for *S. pneumoniae* urinary antigen by the BinaxNOW test. All 95 (100%) of the BinaxNOW-positive urine samples tested positive for a pneumococcal serotype and/or the cell wall polysaccharide antigen by our assay. Seventy-four of the urine samples were from patients who had pneumococci isolated from their blood cultures or bronchoalveolar lavage (BAL) specimens, and the UAD assay was able to detect pneumococcal antigen in 69 (93.2%) of these 74 urine samples. Serotype information was available for 65 of these pneumococcal cultures. Six of the culture-identified serotypes are not currently typeable with our multiplex immunoassay; however, the CWP was detected in all six of these matched nontypeable urine samples. For the other

TABLE 4 Bioplex assay results for urine samples tested in the repeatability experiments

Sample	BinaxNOW result	Result for isolate[a] or spiked antigen[b]	Observed result[c]
RP001	Positive	No isolate	9A/V
RP002	Negative	No isolate	Not detected
RP003	Negative	No isolate	Nontypeable[d]
RP004	Spiked urine	19F (1 ng/ml)	19A/F
RP005	Negative	No isolate	Not detected
RP006	Positive	9N	9N
RP007	Spiked urine	33F (0.04)	33A/B/D/F
RP008	Positive	No isolate	Group 15
RP009	Spiked urine	7F (0.4)	7A/F
RP010	Positive	19F	19F
RP011	Positive	No isolate	Group 10 or 33C or 39, 11A/C/E or 16A/F or 45, and 14
RP012	Spiked urine	6B (0.1)	6B/D
RP013	Positive	3	3
RP014	Positive	6A	6A/C
RP015	Positive	8	8
RP016	Positive	7F	7A/F
RP017	Positive	No isolate	14
RP018	Positive	No isolate	9N
RP019	Positive	No isolate	6A/C
RP020	Spiked urine	18C (0.4)	18B/C/F or 35C or 42
RP021	Negative	No isolate	Not detected

[a]Serotype result for pneumococcal culture of blood or a bronchoalveolar lavage specimen from the same patient as the tested urine sample.
[b]For urine samples spiked with antigen, the concentration (in nanograms per milliliter) at which the serotype was detected is given in parentheses.
[c]Taking into account any potential cross-reactions as listed in Table 3.
[d]Sample with a positive CWP assay result but negative for all other assay serotypes.

59 cultures, our assay identified and reported the same serotype in 54 (91.5%) of the corresponding urine samples but detected no serotype or CWP in 4 of the 5 remaining matched urine samples. In the case of the single sample for which a different serotype was reported, the culture isolate was serotyped as 9V, but our UAD assay reported the matched urine sample as positive for 19A. The 9V antigen reacts with the h-MAbs targeting both the 9V and 19A polysaccharides, and therefore, the UAD result for the 9V antigen would be expected to be 9A/V and 19A positive. In this instance, while the Bioplex assay reported a positive result for 19A, the 9V result was below our standard *t*/*n* ratio cutoff of 2.5, though >3 standard deviations above the median value of all the 9V *t*/*n* ratio results for that run. Consequently, the sample was reported as 19A based on the fact that the 9V result fell below the normalized *t*/*n* ratio cutoff of 2.5.

In total, of the 616 urine specimens tested, 132 (21.4%) were obtained from patients with pneumococcal disease as defined by a positive culture isolate from blood or BAL fluid and/or a positive BinaxNOW test result. Our assay identified 127 of the 132 (96.2%) urine samples as positive for pneumococcal antigen, and serotype information was available for 110 of these. Of the 484 urine samples that were negative for pneumococci by the BinaxNOW test and had no associated pneumococcal blood or BAL culture, our assay was able to identify a further 144 as positive for pneumococcal antigen, and 115 of these were serotyped. Multiple (more than one) serotypes were detected in 27 (4.4%) of the 616 urine samples.

A method described by Huijts et al. in 2013 (43) calculated the specificity of a pneumococcal-serotype-specific urine antigen test (UAT) using urine samples from community-acquired pneumonia (CAP) patients by comparing the number of negative UAT samples with the number of "true negatives," defined as CAP cases with bacteremia caused by another pathogen or CAP cases with only a positive *Legionella* urinary antigen test. Using this method, 69 of the urine samples tested by our UAD assay could be described as true negatives, and of these, 62 samples produced a negative result when tested in our assay, resulting in a clinical specificity of 89.9%. Thirty-six of the urine samples tested were from CAP patients defined as "true negative" due to

TABLE 5 Results of Bioplex 7F/A and 22F assays using human or mouse monoclonal antibody-coupled beads on selected urine samples

Sample ID[a]	Result from beads coupled with:	
	Human MAb	Mouse MAb
RR16000141	22F	Not detected
RR16000145	7A/F and CWP	7A/F
RR16000163	7A/F	Not detected
RR16000177	22F and CWP	22F
RR16000199	7A/F and 22F	Not detected
RR16000216	7A/F and 22F	Not detected
RR16000230	7A/F	Not detected
RR16000284	7A/F and 22F	Not detected

[a]ID, identification code.

bacteremia caused by a pathogen other than *S. pneumoniae*; of these, 29 were negative when tested by our UAD assay, with a resulting specificity of 80.6%.

Preliminary analysis of urine samples from patients with community-acquired pneumonia identified a large number of positive results for the serotype 7F/A, 22F, and 20 assays, leading to the suggestion that these assays were producing false-positive results with some urine samples, possibly due to nonspecific binding. This suspicion was supported when a small number of the urine samples that had tested positive for 7F/A ($n = 3$), 22F ($n = 2$), or both 7F and 22F ($n = 3$) were tested with the mouse monoclonal antibody (m-MAb)-coupled beads used in a 13-valent pneumococcal UAD assay developed by Sheppard et al. in 2011 (34). When tested with m-MAb-coupled beads, only one of six previously positive samples tested positive for 7F/A and one of five tested positive for 22F (Table 5). It was observed that most of the positive serotype 20 and 22F results observed from testing the urine samples by the current 25-valent UAD assay had normalized *t*/*n* ratios below 5, while the m-MAb-confirmed 22F-positive urine sample generated a mean *t*/*n* ratio of 34 with the 25-valent UAD assay. As a result, the serotype 20 and 22F results with *t*/*n* ratios of <20 or the serotype 7F/A, 20, and 22F assay results that were not confirmed by culture serotype were classified as negative.

DISCUSSION

Previous studies have suggested that bacteria are isolated from blood culture in $<25\%$ of adult pneumococcal pneumonia cases (13, 14). Therefore, the ability of our UAD assay to directly test clinical samples for evidence of pneumococcal infection in the absence of cultures offers the potential to detect and serotype many PD infections that would have been missed by relying on culture techniques alone. Our assay is a highly sensitive, extended-range pneumococcal-serotype-specific urine antigen detection assay capable of identifying the PCV13 serotypes plus an additional 11 pneumococcal serotypes and the cell wall polysaccharide. This is of benefit to the surveillance of circulating pneumococcal serotypes, since recent studies have identified a decrease in the numbers and prevalence of PCV13 serotypes and a gradual increase in the numbers of nonconjugate vaccine types. Indeed, the 2011–2015 UK national surveillance figures for the serotype distribution of pneumococci causing invasive disease show that the 10 most prevalent serotypes isolated and referred to Public Health England (PHE) for serotyping were serotypes 8, 12F, 3, 22F, 19A, 9N, 15A, 7F, 33F, and 10A (44). Of these 10 serotypes, only serotypes 3, 7F, and 19A are covered by PCV13. Furthermore, only serotypes 3, 7F, 8, and 19A would have been identified using the previously published urine antigen detection assays (33, 34).

Analytical sensitivity tests indicate that this UAD assay is more sensitive than our previous assay (34) and is capable of detecting some polysaccharide antigens at concentrations as low as 0.3 pg/ml with $>99\%$ accuracy for positivity calling compared to the negative control. For some serotypes, analytical sensitivity and specificity were less than ideal. For example, serotype 19F gave a modal minimum concentration of 1 ng/ml for positive detection, which is much higher than other assays. Also, there are

many cross-reactions with the serotype 11A antibody, which will reduce its usefulness for detecting this serogroup.

Determining positivity as a ratio of the signal to noise (*t*/*n* ratio) relative to a negative-control urine sample helps to overcome any run-to-run variations that may occur, while the inclusion of a standard curve in each run also enables the monitoring of any changes in the sensitivity of each individual serotype assay between runs. Normalization of the *t/n* ratios adjusts for natural variation between test specimens, which may occur when they are run in a highly sensitive immunoassay such as this, as well as helping to overcome any anomalies in the results that may occur due to the antigen-negative urine sample used as the negative control. The normalization of each serotype assay described here can be performed only when there are enough negative samples for the median value to be representative of a negative result. With the analysis of more samples for each assay serotype, it may be possible to specify a tailored standard positivity cutoff *t/n* ratio for the normalized results of each individual serotype assay.

The clinical sensitivity of the UAD assay was assessed based on the existence of matched culture isolates from a normally sterile site or a positive pneumococcal BinaxNOW test. In both instances, our assay showed very good sensitivities of 93.2% (95% CI, 84.9%, 97.7%) and 100% (95% CI, 96.2%, 100%), respectively, with a combined sensitivity of 96.2% (95% CI, 91.3%, 98.7%). The higher sensitivity of our assay than of culture methods and the BinaxNOW test enabled the identification of a further 144 pneumococcus-positive urine samples out of 484 (30%) matched patient urine samples considered negative by either method, suggesting a valuable role for our assay in identifying noncultured PD, particularly noninvasive diseases such as community-acquired pneumonia. Furthermore, with serotyping techniques based on cultured isolates, it is more difficult to detect the presence of multiple serotypes per patient sample. Previous studies using multiplex serotype-specific antigen detection assays show that multiple serotypes can be isolated from single patients with CAP (37, 43). We identified more than one serotype in 27 (4%) of the 616 urine samples tested.

This assay possesses good pneumococcal-serotype analytical specificity. Some cross-reactivity between serotypes within closely related serogroups is common when one uses an antibody-based assay and is difficult to avoid. The interpretation for these cross-reactions should be handled with care, and it is not always possible to determine the serotype to subtype level. Nevertheless, the data gained are useful for the surveillance of circulating serotypes in nonculture situations. The checkerboard approach helps with data interpretation and the determination of the serotype detected. Although multiple serotype positivity might be confused with cross-reactivity, in our experience, the multiple serotypes tended to be common serotypes associated with unexpected checkerboard results rather than serotypes that could be misinterpreted.

Testing of the extensive nonpneumococcal specificity panel showed good specificity when pneumococcal polysaccharide antigen-negative urine was spiked with these antigens: only the GBS serotype II antigen showed any cross-reactivity with our UAD assay, producing positive results for serotype 14. Interestingly, structural analyses of the GBS capsular polysaccharide have shown that when the sialic acid is removed from the GBS serotype III polysaccharide, it becomes identical to the pneumococcal serotype 14 capsular polysaccharide, with resulting serological and immunological cross-reactivity (45–48). However, the GBS serotype III isolates did not show any cross-reactivity with our assay. None of the GBS antigens appear to cross-react with the CWP, and GBS cases of adult pneumonia are uncommon and are usually diagnosed by the isolation of GBS from blood or other normally sterile sites; therefore, it should be possible to distinguish a false-positive GBS result from a true pneumococcal serotype 14 result. Nevertheless, care should be taken in interpreting results for serotype 14 in the absence of a positive CWP result. Seven of the urine samples tested were from a suspected outbreak of pneumococcal serotype 6C and influenza on a hospital ward. Of these, three were from patients who had tested positive for influenza virus. None of these samples showed any unexpected cross-reactions with the UAD assay.

The cell wall C polysaccharide antigen common to all pneumococci is detected by the BinaxNOW test so as to identify and diagnose adult pneumococcal pneumonia from urine samples. Testing of urine samples identified the potential of our assay for detecting CWP from nontypeable pneumococcal serotypes. Thus, our assay could be used as an indicator for determining the proportion of noninvasive pneumococcal disease caused by non-PPV types. Our UAD assay reported a positive result for 100% (95/95) of the BinaxNOW-positive urine samples tested. Furthermore, as well as being able to provide serotype information for the serotypes that are most commonly isolated currently, this assay is more sensitive than the BinaxNOW test. Tests show that our assay could identify pneumococcal antigen in 32% of the BinaxNOW-negative samples. (BinaxNOW has reported a sensitivity and specificity of 74 to 90% and 71 to 94%, respectively [28, 29, 30]).

Streptococcus mitis, *S. pseudopneumoniae*, and *S. peroris* are all members of the *S. mitis* group of streptococci, to which the pneumococci also belong. A C polysaccharide similar to that found in the cell wall of *S. pneumoniae* is present in other members of the *S. mitis* group (49–51), and therefore, it is not surprising to find positive CWP results for these organisms. Indeed, the manufacturer's instructions for the BinaxNOW test state that the test may also report positive results for *S. mitis* (28), and positive BinaxNOW results for *S. pseudopneumoniae* have also been reported (52). *Streptococcus mitis* is a commensal organism typically found in the oropharynx and sometimes associated with invasive disease, particularly in immunocompromised individuals. Although *S. pseudopneumoniae* is occasionally isolated from respiratory and blood specimens, its clinical significance is not yet well defined (52–55). The first strain of *S. peroris* to be described was isolated from the pharynx of a child (56), and so far, isolates from both healthy and unhealthy individuals appear to be extremely rare. For all three organisms, isolation from patients' samples and from CAP patients is uncommon (53, 54, 57–60). Therefore, in the testing of urine samples from adult pneumonia patients, this cross-reactivity is unlikely to be of clinical significance, although its possible presence should be considered when one is reviewing any nontypeable CWP-positive results.

The specificity of any diagnostic test should be tested in the patient population in which the test will be applied. In the case of respiratory tract infections, there is increasing recognition of the presence of coinfection, and *S. pneumoniae* is implicated as the commonest co-occurring pathogen, in association with both viruses and bacteria (14, 61–63). For this reason, even in cases of nonpneumococcal bacteremia, the possibility of coinfection with *S. pneumoniae* cannot be ruled out. Furthermore, this situation, together with the lack of a microbiological gold standard, poses a challenge for assessment of the true specificity of modern, highly sensitive nonculture microbiological tests such as the assay described here (64). Other factors that influence the assessment of specificity in the clinical context include issues of carriage or colonization. The high sensitivity of this UAD assay allows for the possibility that it will also detect small amounts of polysaccharide antigen in urine as a result of carriage as well as disease. Pneumococcal carriage rates in children range from 30% to 86%, with higher rates in lower-income countries (65–72), and studies have found positive BinaxNOW results in healthy children carrying pneumococci in their nasal pharynges (24–27). Adult carriage rates are much lower than those of children; carriage in the United Kingdom is predicted to be approximately 3.4% (66). Carriage density may also be a contributing factor that determines whether the UAD assay would detect the pneumococcal antigen carried. Taking into account the high sensitivity of our UAD assay, the ability of the assay to detect small amounts of pneumococcal polysaccharide antigen that may be present in the urine of adults as a result of carriage cannot be ruled out. For these reasons, the UAD serotype results should always be interpreted in the context of the clinical presentation and other test results, as appropriate. We obtained serotype specificity results of 98% (95% CI, 91.5%, 99.7%) and 90% (95% CI, 80.2%, 95.8%) for cultured nonpneumococcal bacterial antigens and clinical samples, respectively.

The assay described in this report represents a solid first iteration of an extended-

specificity UAD assay. This assay could be used to test other clinical samples, such as blood, cerebrospinal fluid, and pleural fluid. The panel of antibodies used here provides the assay with several advantages over those previously reported. Most importantly, the antibodies are fully characterized, are available commercially, and are recombinant, allowing the possibility of further development at a molecular level. We expect further improvements in cross-reactivity to accrue alongside ongoing monoclonal developments. We believe that the assay presented here is of value for clinical diagnosis as well as seroepidemiological surveillance, offering the potential for an increased yield of pneumococcal-serotype-specific data from cases where no isolate is obtained.

MATERIALS AND METHODS

Bacterial strains. Bacterial isolates and control strains, including 13 nonpneumococcal streptococcal species (*n*, 42 strains) and a selection of various bacteria that are associated with respiratory infections or that may be found in the urogenital tract (*n*, 111 strains) were obtained from laboratories in the Bacteriology Reference Department, Public Health England (PHE) National Infection Service, Colindale, United Kingdom. A panel of 91 pneumococcal serotype reference strains obtained from SSI Diagnostica AG, Hillerød, Denmark, and the reference strain for serotype 6D, kindly provided by The National Institute of Health and Welfare, Helsinki, Finland, were also included in order to assess the serotype specificity of the assay.

Preparation of crude bacterial antigen for specificity testing. Pure bacterial cultures were suspended in phosphate-buffered saline (PBS) (PHE-Colindale Media Services), and colony counts were performed on a selection of the bacterial suspensions covering a range from the least to the most optically dense. All samples counted were estimated to have $>1.2 \times 10^9$ CFU/ml. The bacteria were heat killed in a heat block (Grant Instruments) at 100°C. An additional preparation of killed *Legionella pneumophila* was obtained by overnight treatment of a suspended *L. pneumophila* culture in 2% formalin, followed by centrifugation at 13,000 × *g* for 10 min before resuspension of the bacterial pellet in PBS. All of the killed bacterial antigen preparations were stored at −20°C. Prior to testing, the antigen preparations were thawed and were spun in a centrifuge at 13,000 × *g* for 3 min, and an aliquot of the supernatant was diluted 1:100, 1:2,000, and/or 1:1,000 in 25 mM HEPES (Sigma)-buffered polysaccharide antigen-negative urine prior to testing by the UAD assay. Aliquots of the pneumococcal antigen preparations were diluted 1:2,500 in 25 mM HEPES-buffered antigen-negative urine.

Panel of purified polysaccharides. Purified capsular polysaccharides from 25 different pneumococcal serotypes (serotypes 1, 2, 3, 4, 5, 6B, 6C, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15A, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F) and the pneumococcal cell wall polysaccharide (CWP) were obtained from the ATCC, Manassas, VA, USA. The purified capsular polysaccharide for serotype 6A was acquired from SSI Diagnostica. For specificity testing, each polysaccharide was diluted in 25 mM HEPES-buffered antigen-negative urine to a final concentration of 10 ng/ml.

Preparation of controls and standards. A pneumococcal polysaccharide antigen-negative urine sample was kindly supplied by a healthy donor for use as a negative control. The same negative-control urine sample was also used to dilute a standard titration consisting of a mixture of purified capsular polysaccharides for each of the 25 antibody targets. The standards were titrated at concentrations of 10, 3, 1, 0.3, 0.1, 0.03, and 0.01 ng/ml for all assay runs except for the repeatability experiments, where further dilutions of 0.003, 0.001, and 0.0003 ng/ml were also included.

Panel of urine specimens. Urine samples were obtained from individuals presenting to secondary-care centers in the United Kingdom, either as part of a prospective cohort study of patients with community-acquired pneumonia (CAP) or for confirmation testing following a positive BinaxNOW result. All urine samples were anonymized for use in specificity testing.

A panel of 588 urine samples were obtained from patients diagnosed with CAP between 2006 and 2015 from two UK regional NHS hospital trusts. Patients treated by the admitting team with the presence of new or progressive infiltrates on a chest radiograph and at least one symptom of acute lower respiratory tract infection, such as cough, fever, dyspnea, sputum, or pleuritic chest pain, were diagnosed with CAP. These urine samples were frozen prior to transportation to PHE-Colindale for testing by the UAD assay.

A further 28 urine samples from patients with suspected PD based on clinical symptoms, contact links with outbreak cases, and/or a positive BinaxNOW test were obtained from the Respiratory and Vaccine Preventable Bacteria Reference Unit (RVPBRU) at PHE-Colindale. Three of these urine samples were from a family cluster with severe pneumonia, and seven were from a suspected outbreak of pneumococcal serotype 6C and influenza on a hospital ward. In addition to the negative-control urine samples, a further two urine samples were obtained from healthy donors who had tested negative for pneumococcal urinary antigen by the BinaxNOW test. The RVPBRU urine samples were stored at 2 to 8°C. All other urine samples were frozen at ≤−70°C for long-term storage and were then refrigerated at 2 to 8°C prior to testing.

Antibodies used for bead coupling. Purified human monoclonal antibodies (h-MAbs) to the target serotypes and CWP were supplied by Pamlico Biopharma/Oklahoma Medical Research Foundation (73, 74). These antibodies were fully human, full-length monoclonal antibodies produced from single antibody-secreting cells sorted 7 days after vaccination with either Pneumovax 23 (Merck & Co., Inc.) or Prevnar 13 (Wyeth Pharmaceuticals, Inc.).

Bead-coupling method. Spectrally distinct Luminex carboxylated magnetic beads (Luminex) are mapped to unique bead regions. Each h-MAb preparation was coupled to a distinct bead region by use of the method described in the Luminex *xMAP Cookbook*, 3rd edition (75), with the following adaptations: all the postactivation reagents were kept on ice to maintain a cold temperature; a coupling protein concentration of 3.2 μg of antibody per million beads was used; and the beads were blocked overnight in PBS–1% bovine serum albumin (BSA) (Sigma) with mixing by rotation at room temperature. The bead regions used and the antibodies coupled to them are shown in Table 1. The coupled beads were stored in StabilGuard (SurModics) at 2 to 8°C in the dark and were stable for 1 year. Prior to use, the beads were counted using a Bio-Rad TC10 automated cell counter (Bio-Rad), and aliquots of bead mixtures containing approximately 15.25×10^4 beads of each bead type/μl were prepared. Bead mixtures were stored in light-protective amber microcentrifuge tubes (Alpha Laboratories) at 2 to 8°C until required. One batch of each coupled bead preparation was used for testing the 588 CAP urine samples, but different batches were used for sensitivity, specificity, and reproducibility experiments. Each batch of beads was subject to a quality control check before use to ensure equivalence of results.

Multiplex serotype-specific antigen detection assay. Each bead mixture was diluted in PBS prior to use to produce final concentrations of 100 beads of each bead type/μl. Twenty-five microliters of the bead mixture was then added to each test well of a Greiner black 96-well assay plate (Greiner Bio-One), resulting in approximately 2,500 beads of each bead type per well. At least one antigen-negative control urine well and a standard titration were included on each test plate; for sensitivity, specificity; and reproducibility experiments, the samples, controls, and standards were added in the replicates stated in each section. For routine sample testing, samples were added in duplicate, and controls and standards were added in single wells. HEPES buffer (Sigma) was added to all clinical, negative-control, and standard urine samples at a final concentration of 25 mM, and urine samples were spun in a centrifuge at 16,000 $\times$ *g* for 2 min to remove any debris before use. All samples, controls, standards, and reagents were added at 100 μl per well to the appropriate wells of the assay plate, and for all incubations, the plate was sealed with a foil-backed plate lid (Corning) and was placed on a plate shaker (VWR International) set to shake at 500 rpm at room temperature.

The samples, controls, and standards, along with the h-MAb-coupled beads, were allowed to incubate on a plate shaker overnight. After incubation, the assay plate was washed with PBS–0.05% Tween 20 (PHE-Colindale Media Services) three times using a Bioplex Pro 2 magnetic plate washer (Bio-Rad). This washing method was used for all subsequent wash steps. Omni antiserum (SSI Diagnostica), previously purified for IgG using the Melon Gel IgG Spin purification kit (Thermo Fisher Scientific), was diluted 1:1,000 in the assay diluent (PBS–2% BSA and a negative human reference serum diluted to produce a total IgG concentration of 10 μg/ml) and was added to the assay plate, incubated on the plate shaker for 1 h, and then washed. After washing, a polyclonal goat anti-rabbit–R-phycoerythrin (RPE) conjugate solution (Thermo Fisher Scientific) diluted to 1:400 in the assay diluent was added to the assay plate. The plate was covered and was incubated for 30 min. The plate was then washed and the beads resuspended in 150 μl PBS–0.05% Tween 20. The assay plate was then loaded onto the Bioplex 200 suspension array system (Bio-Rad) and the resulting fluorescence intensities read with the system set to read $\geq$100 beads per region with the low photomultiplier tube (PMT) setting.

Interpretation of results. Results were interpreted by observing the signal-to-noise ratio of the median FI, referred to as the *t*/*n* (test sample-to-negative control) ratio. In order to enable results to be comparable between test samples, these ratios were normalized using the method described by Sheppard et al. in 2011 (34). This method assumes that there are no more than 3 positive results for each sample. The normalized data were then normalized further by calculating the median ratio result for each assay (in cases where the majority of samples were negative, and therefore, the median would be representative of a negative result). Each of the individual ratios for that assay were then divided by the median ratio for the same assay. Any results above the normalized *t*/*n* ratio cutoff of 2.5 were considered positive. To establish the cutoff threshold, negative-control ratios (negative/negative $-$ expected to be $t/n = 1$) were normalized.

Repeatability. To test the repeatability of the assay, three standard curves, consisting of a mixture of purified capsular polysaccharides for each of the 25 antibody targets at individual concentrations of 10, 3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001, and 0.0003 ng/ml, were tested over 6 days. Twenty-one urine samples were included in the repeatability runs. Fourteen of these were urine samples obtained between 2008 and 2013 from patients with culture-confirmed or suspected PD based on clinical symptoms, contact links, and/or BinaxNOW results (Table 4), two were donated urine samples from healthy donors who had previously tested negative for pneumococcal cell wall C polysaccharide by the BinaxNOW test, and five were spiked samples in which antigen-negative urine was spiked with purified polysaccharide antigen to produce a final concentration just above the estimated sensitivity cutoff. The spiked urine samples consisted of polysaccharides of serotype 19F (at 1 ng/ml), 18C (at 0.4 ng/ml), 6B (at 0.1 ng/ml), 33F (at 0.04 ng/ml), or 7F (at 0.4 ng/ml). Each urine sample was tested in triplicate over 5 days and was tested once on day 6.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/CVI.00262-17.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

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