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MAJOR ARTICLE

Qualification and Clinical Validation of an Immunodiagnostic Assay for Detecting 11 Additional *Streptococcus pneumoniae* Serotype–specific Polysaccharides in Human Urine

Warren V. Kalina,[1] Victor Souza,[1] Kangjian Wu,[1] Peter Giardina,[1] Andrew McKeen,[2] Qin Jiang,[3] Charles Tan,[2] Roger French,[2] Yanhua Ren,[1] Kelly Belanger,[1] Susan McElhiney,[1] Manu Unnithan,[1] Huiming Cheng,[1] Terri Mininni,[1] Donna Giordano-Schmidt,[1] Bradford D. Gessner,[4] Kathrin U. Jansen,[1] and Michael W. Pride[1]

[1]Vaccine Research and Development,Pfizer Research, Pearl River, New York, USA, [2]Early Clinical Development, Pfizer Research, Pearl River, New York, USA, [3]Global Clinical Affairs, Pfizer, Collegeville, Pennsylvania, USA, and [4]Medical and Scientific Affairs, Pfizer, Collegeville, Pennsylvania, USA

Background. Identifying *Streptococcus pneumoniae* serotypes by urinary antigen detection (UAD) assay is the most sensitive way to evaluate the epidemiology of nonbacteremic community-acquired pneumonia (CAP). We first described a UAD assay to detect the *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F, covered by the licensed 13-valent *S. pneumoniae* conjugate vaccine. To assess the substantial remaining pneumococcal disease burden after introduction of several pneumococcal vaccines, a UAD-2 assay was developed to detect 11 additional serotypes (2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F) in individuals with radiographically confirmed CAP.

Methods. The specificity of the UAD-2 assay was achieved by capturing pneumococcal polysaccharides with serotype-specific monoclonal antibodies, using Luminex technology. Assay qualification was used to assess accuracy, precision, and sample linearity. Serotype positivity was based on cutoffs determined by nonparametric statistical evaluation of urine samples from individuals without pneumococcal disease. The sensitivity and specificity of the positivity cutoffs were assessed in a clinical validation, using urine samples obtained from a large study that measured the proportion of radiographically confirmed CAP caused by *S. pneumoniae* serotypes in hospitalized US adults.

Results. The UAD-2 assay was shown to be specific and reproducible. Clinical validation demonstrated assay sensitivity and specificity of 92.2% and 95.9% against a reference standard of bacteremic pneumonia. In addition, the UAD-2 assay identified a *S. pneumoniae* serotype in 3.72% of nonbacteremic CAP cases obtained from hospitalized US adults. When combined with bacteremic CAP cases, the proportion of pneumonias with a UAD-2 serotype was 4.33%.

Conclusions. The qualified/clinically validated UAD-2 method has applicability in understanding the epidemiology of nonbacteremic *S. pneumoniae* CAP and for assessing the efficacy of future pneumococcal conjugate vaccines that are under development.

Keywords. Urine antigen detection; *Streptococcus pneumonia*; polysaccharide.

Community-acquired pneumonia (CAP) is a leading cause of disease and death worldwide [1, 2]. Although the incidence of CAP varies by age, prospective studies conducted in the United Kingdom, Finland, and the United States estimate annual incidences in community-dwelling adults to be between 5 and 11 cases per thousand [3–5]. According to the Global Burden of Disease Study, lower respiratory tract infection (including CAP) was the second leading cause of death and years of life lost in 2013 [6]. CAP is caused by many pathogens, but in approximately 50% of cases [2] an etiological agent cannot be identified with current diagnostic assays [7]. Historically, *Streptococcus pneumoniae* has been the most commonly identified bacterial cause of CAP in adults ≥65 years of age (identified in approximately 50% of patients) [2, 8, 9]. The introduction of routine pediatric pneumococcal conjugate vaccine (PCV) programs has substantially altered pneumococcal epidemiology, including serotype distribution, emphasizing the need for increasingly robust serotype-specific diagnostic tools to help guide future vaccine development.

The standard method of pneumococcal serotyping involves using the Quellung reaction on organisms grown from cultured blood or sputum [10]. Although specific, blood culture (BC) methods are insensitive [11]. Several non–culture-based methods also detect and serotype pneumococcal antigens

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Correspondence: M. W. Pride, 401 N Middletown Road, Pearl River, NY 10965 (michael.pride@pfizer.com).

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in urine, serum, and sputum samples [12, 13]. In 1999, a rapid non–culture-based screening method for evaluating *S. pneumoniae* infection was introduced and marketed as the BinaxNOW *S. pneumoniae* assay. This assay detects pneumococcal C-polysaccharide in urine samples from patients with pneumonia but is not serotype specific. However, both techniques have either low specificity or sensitivity, or require large sample volumes or expensive technology.

To improve diagnosis of pneumococcus, a 13-valent Luminex multiplex urinary antigen detection (UAD) assay that identifies serotypes covered by the 13-valent PCV (PCV13; Prevnar 13®, Pfizer) was developed and validated [14, 15]. This assay combines multiple spectrally distinct microspheres conjugated to a different serotype-specific monoclonal antibodies (mAbs) in a single test to simultaneously detect 13 pneumococcal polysaccharide (PnPS) serotypes in a small volume of urine. The UAD assay demonstrated 97% sensitivity and 100% specificity when validated against samples from bacteremic patients with CAP of known cause [14] and provided a robust tool for clinical and epidemiological evaluation of pneumococcal disease (PD) in adults [16, 17].

UAD assays offer an alternative to other serotyping methods for both invasive and noninvasive disease. The original UAD assay (UAD-1) identified the PCV13 serotypes. Because new PCVs are under development with increased serotype coverage, we developed the UAD-2 assay, which is capable of detecting additional pneumococcal serotypes. The current article describes the development, qualification, and clinical validation of a UAD-2 assay that identifies 11 additional non-PCV13 pneumococcal serotypes (serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20, 22F, and 33F). Combined with the original UAD-1 assay, 24 *S. pneumoniae* serotypes can be identified with high sensitivity and specificity in patients with invasive PD (IPD) or noninvasive PD.

MATERIALS AND METHODS

Reagents and Assay Specificity

Serotype specific reagents were generated as described elsewhere [14]. After screening for suitable mAbs and polyclonal antibodies, the specificity of the UAD-2 assay was assessed with urine samples from healthy donors spiked with PnPS (Supplementary Material).

Positivity Cutoffs

Like the original UAD-1 assay, the UAD-2 is a limit assay [15]. To determine UAD-2 positivity cutoffs, 48 reference standard curves were generated for each serotype to ensure the suitability of the 11-point reference standard. Positivity cutoffs (antigen concentrations read from a standard curve) were established for each serotype, using 400 control urine specimens obtained from individuals undergoing elective surgery, healthy patients with stable chronic obstructive pulmonary disease (COPD), and healthy donors with no apparent signs of PD. Nonparametric tolerance intervals were computed from these concentrations, giving a range predicted to contain 98% of negative urine samples with 99% confidence, thus achieving ≥97% assay specificity for each serotype [18]. The positivity cutoffs were refined using additional samples (400 control urine samples from healthy adults and 110 urine samples from patients with chest radiographically confirmed CAP and a corresponding BC obtained from a sterile site (BC or pleural fluid)].

Multiplex Serotype-specific UAD-2 Assay

The UAD-2 assay was performed as described elsewhere [14], with some modifications such as use of automated Tecan Fluent-1080 Luminex Systems and FlexMap-3D readers (Luminex). All samples were tested in duplicate and scored as serotype positive if both duplicate values were greater than the serotype-specific positivity cutoff. Statistical analyses and data processing were performed using a validated SAS program version 9.4.

Assay Qualification

Assay robustness was assessed using a fractional factorial design of experiment to study various assay robustness factors (day, operator, primary incubation time and temperature, secondary incubation time and temperature, reporter antibody time and temperature). Qualification consisted of assay runs over multiple days that addressed precision, sample linearity, and accuracy, as described elsewhere, with some modification [15] (Supplementary Material).

Study Participants and Clinical Validation

To clinically validate the diagnostic potential of the UAD-2 assay, we evaluated urine specimens obtained from patients >18 years old with radiographically confirmed CAP, who were enrolled in a multicenter US epidemiological study [19]. This study included 11 087 patients with CAP with corresponding urine samples and BCs obtained from a sterile site (BC or pleural fluid). Of the 11 087 patients, 10 220 had nonbacteremic and 867 had bacteremic CAP diagnosed. The etiological agent in a bacteremic CAP case was supported by positive BC.

For clinical validation of the sensitivity and specificity of the UAD-2 assay, the BC in patients with CAP was considered the "true" result. The 867 positive BC results represented 144 *S. pneumoniae* and 723 non–*S. pneumoniae* organisms. Of the 144 *S. pneumoniae* BCs, 17 were not serotyped and were excluded from the determination of sensitivity and specificity. Of the 723 non–*S. pneumoniae* organisms, 260 were potential contaminating organisms (*Bacillus* sp, coagulase-negative *Staphylococcus* sp, *Corynebacterium* sp, and *Propionibacterium* sp) but were included in the sensitivity and specificity analyses so as not to bias results by excluding potential true cases of bacteremic CAP.

Urine samples were analyzed in the BinaxNOW *S. pneumoniae* urine antigen test (Abbott) according to the manufacturer's instructions. All work was approved by local research ethics committees. Written informed consent was obtained from all enrolled patients.

RESULTS

Assay Specificity

The specificity of the 11-valent UAD-2 assay was assessed using bacterial lysates obtained from 93 *S. pneumoniae* strains and 413 non–*S. pneumoniae* organisms (Table 1) spiked into assay buffer. Reactivity to bacterial lysates was assessed using a signal-to-noise ratio (S/N) >5 (PnPS-specific mAb coated bead median fluorescence intensity compared with assay buffer median fluorescence intensity). Of the 93 *S. pneumoniae* bacterial lysates tested in the UAD-2 assay, 19 had S/N ratios >5 (serotypes 2, 8, 9N, 10A, 11A, 11D, 11F, 12F, 15B, 15C, 17A, 17F, 20A, 20B, 22A, 22F, 33A, 33F, and 39; data not shown). Subsequently, purified PnPS of these 19 serotypes were then used to make mock samples by spiking them individually into assay buffer at 10 ng per well (approximately 40–200 times the saturating concentration), which were then tested using the UAD-2 assay. In all cases, homologous PnPS mAb-coated microspheres produced S/N ratios greater than the assay buffer (130–405-fold; Table 2); S/N ratios >5 to PnPS were also obtained between the following anti-PnPS mAb-coated microspheres: 10A with serotype 39, 11A with 11D and 11F, 15B with 15C, 17F with 17A, 20 with serotype 20A and 20B, 22F with 22A, and 33F with 33A (Table 2). Of the 413 non–*S. pneumoniae* organisms tested with the UAD-2 assay, lysates from 2 *Streptococcus mitis* isolates were serotype 10A positive. Whole-genome sequencing of the 2 *S. mitis* isolates demonstrated multiple sequence alignments to the 10A capsular polysaccharide operon (data not shown).

Assay Qualification

To classify a test sample as positive using the UAD-2 assay requires a fluorescent signal above the positivity cutoff, because the UAD-2 is a limit assay. Relevant to the validation of a limit assay are sensitivity, precision, and specificity according to published nomenclature for bioanalytical validation [20, 21]. Because the positivity cutoffs are defined by the reference standard, the accuracy of the reference standard could also be evaluated. For precision, the low and high assay arbitrarily assigned Pneumococcal polysaccharide Units/mL (PnPS U/mL) ranges were extrapolated from a 25% relative standard deviation limit and presented as an assay range based on precision (Table 3). The assay range for sample linearity was determined similarly, except that bias to an expected value was plotted, and the assay range was calculated within an 80%–125% bias interval (Table 3). The reference curves from all 89 assay plates in the precision and sample linearity analyses were used to determine accuracy by comparing each point on the reference curve's back-calculated concentration value to the expected value. Each serotype's standard curve bias range was within 80%–125% (Table 3).

Table 1. Specificity of the Urinary Antigen Detection 2 Assay With Crude Bacterial Antigens of 413 Non–*Streptococcus pneumoniae* Organisms[a]

Strain	Gram Staining	Number Analyzed
Acinetobacter species[b]	Negative	7
Aerococcus viridans	Positive	2
Alcaligenes faecalis	Negative	1
Bacillus species[b]	Positive	4
Candida albicans	Yeast	15
Candida species[b]	Yeast	2
Citrobacter koseri	Negative	1
Comamonas acidovorans	Negative	1
Corynebacterium accolens	Positive	2
Enterobacter cloacae	Negative	14
Enterobacter species[b]	Negative	3
Enterococcus species[b]	Negative	2
Escherichia coli	Negative	15
Haemophilus haemolyticus	Negative	1
Haemophilus influenzae	Negative	63
Haemophilus parahaemolyticus	Negative	3
Haemophilus parainfluenzae	Negative	39
Klebsiella oxytoca	Negative	7
Klebsiella ozaenae	Negative	1
Klebsiella pneumoniae	Negative	47
Kocuria rosea	Positive	2
Lactobacillus species	Positive	1
Leuconostoc species	Positive	1
Moraxella catarrhalis	Negative	11
Neisseria species[b]	Negative	8
Pantoea species[b]	Negative	3
Proteus species[b]	Negative	3
Pseudomonas aeruginosa	Negative	14
Ralstonia pickettii	Negative	1
Salmonella group D	Negative	1
Serratia liquefaciens	Negative	1
Serratia marcescens	Negative	5
Shewanella algae	Negative	1
Staphylococcus aureus	Positive	45
Staphylococcus epidermidis	Positive	14
Staphylococcus hominis	Positive	6
Staphylococcus species	Positive	8
Staphylococcus warneri	Positive	5
Stentrophomonas maltophilia	Negative	2
Streptococcus mitis	Positive	11
Streptococcus oralis	Positive	10
Streptococcus parasanguis	Positive	5
Streptococcus species (other)[a,b]	Positive	24
Yersinia spp.	Negative	1

[a]No cross-reaction was observed among the 413 non–*Streptococcus pneumoniae* organisms except for 2 of the 11 isolates of *S. mitis* in the urinary antigen detection 2 assay.
[b]Including ≥2 unique species. A total of 83 unique organisms were tested in the urinary antigen detection 2 assay.

Table 2. Specificity of *Streptococcus pneumoniae* Urinary Antigen Detection 2 Assay

Streptococcus pneumoniae Serotype[a]	Signal Produced by mAb-Coated Microspheres, by Serotype																					
	2		8		9N		10A		11A		12F		15B		17F		20		22F		33F	
	MFI	S/N	MFI	S/N	MFI	S/N	MFI	S/N	MFI	S/N	MFI	S/N	MFI	S/N	MFI	S/N	MFI	S/N	MFI	S/N	MFI	S/N
2	31 379[b]	264[b]	125	1	156	1	175	1	176	1	165	1	149	1	211	1	58	1	76	1	72	1
8	119	1	40 364[b]	215[b]	214	1	223	1	277	1	297	1	239	1	226	1	100	1	129	1	120	1
9N	121	1	193	1	46 264[b]	213[b]	215	1	274	1	293	1	220	1	233	1	99	1	126	1	116	1
10A	125	1	239	1	241	1	44 340[b]	209[b]	312	1	304	1	271	1	229	1	99	1	155	1	119	1
11A	141	1	204	1	239	1	232	1	41 537[b]	165[b]	294	1	246	1	211	1	102	1	150	1	130	1
12F	119	1	189	1	235	1	240	1	314	1	36 941[b]	130[b]	231	1	227	1	99	1	129	1	106	1
15B	102	1	161	1	205	1	219	1	226	1	242	1	40 514[b]	180[b]	212	1	78	1	105	1	93	1
17F	122	1	219	1	235	1	222	1	275	1	300	1	372	2	45 191[b]	210[b]	98	1	137	1	137	1
20[c]	133	1	212	1	268	1	218	1	249	1	280	1	219	1	207	1	36 435[b]	368[b]	131	1	113	1
22F	121	1	188	1	227	1	205	1	264	1	284	1	251	1	194	1	98	1	30 236[b]	243[b]	127	1
33F	136	1	185	1	230	1	215	1	302	1	306	1	229	1	212	1	103	1	428	3	45 742[b]	405[b]
39	102	1	141	1	162	1	39 893[b]	188[b]	206	1	170	1	161	1	151	1	66	1	103	1	87	1
11D	125	1	200	1	645	3	205	1	41 112[b]	163[b]	283	1	218	1	207	1	103	1	126	1	117	1
11F	128	1	197	1	228	1	225	1	27 157[b]	108[b]	268	1	226	1	294	1	105	1	379	3	152	1
15C	128	1	686	4	271	1	481	2	562	2	280	1	21 176[b]	94[b]	206	1	94	1	129	1	107	1
17A	139	1	214	1	274	1	235	1	278	1	322	1	246	1	2377[b]	11[b]	107	1	135	1	198	2
20A	113	1	169	1	226	1	212	1	222	1	235	1	204	1	189	1	5963[b]	60[b]	162	1	96	1
22A	131	1	201	1	221	1	222	1	258	1	303	1	231	1	236	1	105	1	27 277[b]	219[b]	120	1
33A	128	1	195	1	234	1	215	1	252	1	281	1	239	1	232	1	104	1	140	1	30 833[b]	273[b]

Abbreviations: mAb, monoclonal antibody; MFI, median fluorescence intensity; S/N, signal-to-noise ratio.

[a] *S. pneumoniae* serotypes consisted of 10 ng/well purified pneumococcal polysaccharide spiked in urine that was tested negative for any *S. pneumoniae* pneumococcal polysaccharide.

[b] Serotype reactivity with S/N ratio >5.

[c] Structural analysis indicates serotype 20B.

Table 3. *Streptococcus pneumoniae* Urinary Antigen Detection 2 Assay Serotype Ranges Based on Precision, Sample Linearity, and Accuracy

Serotype	Positivity Cutoff Value, PnPS U/mL	Assay Range, PnPS U/mL		
		Precision[a]	Sample Linearity[b]	Accuracy[c]
2	60.6	0.7–3189.0	0.8–2140.0	6.3–1541.7
8	38.3	0.2–393.8	0.3–310.0	1.3–108.3
9N	49.4	0.4–442.2	0.5–280.0	0.7–159.7
10A	48.1	0.2–58.0	0.6–717.3	2.2–181.3
11A	48.0	0.3–336.8	0.6–340.0	1.3–325.0
12F	61.4	0.7–1139.5	0.8–709.1	2.6–631.9
15B	79.1	4.5–587.3	8.1–167.9	1.3–322.9
17F	103.2	0.5–314.5	0.5–680.0	1.3–322.9
20	13.9	0.8–1345.2	3.3–823.9	3.6–32.2
22F	29.0	0.7–369.6	0.3–600.0	1.9–473.1
33F	27.5	0.5–1260.5	0.3–810.0	2.6–625.0

Abbreviation: PnPS U/mL, pneumococcal polysaccharide units/mL.

[a]Lower and upper limits represent well concentrations with RSD ≤25%.

[b]Lower and upper limits represent expected well concentration values for which the mean sample linearity was within the acceptance limits of 80%–125%

[c]Lower and upper limits represent expected concentrations for which ≥80% of the reference standard curve bias values fall within the acceptance limits of 80%–125%

The final assay range of the UAD-2 assay for each serotype was based on the most conservative values obtained for accuracy, precision, and sample linearity. The serotype-specific final lower and upper limits ranged from 0.7 to 8.1 and from 32.2 to 1541.7 PnPS U/mL, respectively. For all serotypes, the positivity cutoffs fell within the linear range of the UAD-2 assay (Table 4).

Clinical Validation of the UAD-2 Assay

To determine the diagnostic accuracy of the UAD-2 assay, we analyzed 11 087 urine specimens obtained from patients with radiographically confirmed CAP. The cause of CAP was determined for 867 bacteremic patients with a corresponding BC-positive result. Of 867 patients with CAP, 144 had BC-confirmed *S. pneumoniae*. Of 144 BCs, 64 were positive for UAD-2 *S. pneumoniae* serotypes and used to determine sensitivity. Of the 64 corresponding urine samples, 59 were positive with the UAD-2 assay for the exact *S. pneumoniae* serotype shown by BC (Table 5), and 5 were negative with the UAD-2 assay (92.2% sensitivity). To determine specificity, all BC-positive sample results negative for a UAD-2 serotype were compared with UAD-2 test results. Of the 867 BC samples from patients with CAP, 803 had results that were either nonserotyped (n = 17), non–UAD-2 *S. pneumoniae* serotypes (n = 63), or non–*S. pneumoniae* organisms (n = 723). The nonserotyped results were removed from specificity calculations because discrimination between UAD-2 and non–UAD-2 serotypes could not be determined; therefore, specificity was based on the remaining 786 samples.

Overall, the UAD-2 assay identified 32 positive samples from this population (n = 786), which resulted in a specificity of 95.9% against a reference standard of bacteremic pneumonia (Table 5). Within the 32 UAD-2–positive results, 5 urine samples from the 63 patients with a corresponding BC positive for an *S. pneumoniae* serotype not covered by the UAD-2 assay were also positive with the UAD-2 assay (Table 5). In addition, 27 of the 723 samples from patients with bacteremic CAP with BCs positive for etiological agents other than *S. pneumoniae* were positive with the UAD-2 assay (Table 5). Of 723 non–*S. pneumoniae* organisms, 260 were potential contaminates. Removal of these samples from the analysis did not appreciably affect specificity (96.6%). By comparison, the BinaxNOW test was positive for 32 of 64 samples that were BC positive for serotypes covered with the UAD-2 assay and was positive for 33 of 63 samples that had corresponding BC-positive results for *S. pneumoniae* serotypes not covered by the UAD-2 assay, resulting in sensitivities of 50% and 52.4%, respectively (Table 5). Of 867 CAP cases with bacteremic pneumonia, 11.5% were UAD-2 serotypes (22F [3.58%], 20 (1.27%], 33F [1.38%], 11A [1.38%], 9N [1.04%], 12F [0.92%], 8 [0.81%], 17F [0.58%], 15B [0.46%], and 10A [0.35%]) (Table 6). There were no BC-positive results for serotype 2.

Table 4. Assay Ranges for Final *Streptococcus pneumoniae* Urinary Antigen Detection 2 Assay

Serotype	Positivity Cutoff Value, PnPS U/mL	Final Assay Ranges, PnPS U/mL
2	60.6	6.3–1541.7
8	38.3	1.3–108.3
9N	49.4	0.7–159.7
10A	48.1	2.2–58.0
11A	48.0	1.3–325.0
12F	61.4	2.6–631.9
15B	79.1	8.1–167.9
17F	103.2	1.3–314.5
20	13.9	3.6–32.2
22F	29.0	1.9–369.6
33F	27.5	2.6–625.0

Abbreviation: PnPS, pneumococcal polysaccharide.

UAD-2 Serotype Distribution in Nonbacteremic CAP

After determining sensitivity and specificity in bacteremic CAP, the UAD-2 assay was used to assess serotype distribution in nonbacteremic CAP. The assay identified all 11 serotypes in an additional 3.72% of urine samples obtained from patients with nonbacteremic CAP (380 of 10 220; Table 6). The BinaxNOW test demonstrated *S. pneumoniae* in 547 of 10 220 samples (5.4%) from patients with nonbacteremic CAP. Of these 547 samples, 100 were also positive with the UAD-2 assay. Within the nonbacteremic CAP population, the percentages of individual UAD-2 serotypes positive with the UAD-2 assay were as follows: 22F, 0.89%; 11A, 0.74%; 9N, 0.45%; 10A, 0.43%; 33F, 0.25%; 8, 0.25%; 17F, 0.23%; 12F,

Table 5. Clinical Validation of the Urinary Antigen Detection 2 Assay

BC Results	Cases, Number	UAD-2 Assay				BinaxNOW Assay[a]	
		Negative, Number	Positive, Number	Sensitivity vs BC, %	Specificity vs BC, %	Positive, No.	Sensitivity vs BC, %
BC positive[b] (bacteremic CAP)							
Total	867	772	95	…	…	106	…
UAD-2 serotypes	64	5	59	92.2	NA	32	50.0
Non–UAD-2 serotypes	63	58	5	NA	92.1	33	52.4
Non–*Streptococcus pneumoniae*	723	696	27	NA	96.3	35	NA
Total non–UAD-2 *S. pneumoniae* and non–*S. pneumoniae*[c]	786	754	32	NA	95.9	68	NA
BC negative (nonbacteremic CAP)	10 220	9840	380[d]	NA	NA	547	NA
Total CAP (bacteremic and nonbacteremic)	11 087	10 612	475	NA	NA	653	NA

Abbreviations BC, blood culture; CAP, community-acquired pneumonia; NA, not applicable; UAD-2, urinary antigen detection 2.

[a]The BinaxNOW assay does not discriminate between UAD-2 and non–UAD-2 serotypes.

[b]BC positive, including *S. pneumoniae* results not serotyped.

[c]These totals do not include *S. pneumoniae* results not serotyped.

[d]This total includes double serotype positives in the UAD-2 assay.

0.22%; 20, 0.18%; 15B, 0.16%; and 2, 0.03% (Table 6). Overall, 4.33% (480 of 11 087; Table 6) of all patients with chest radiographically confirmed bacteremic and nonbacteremic CAP tested positive for the 11 serotypes.

DISCUSSION

The causes of IPD, including CAP, vary according to the published literature and reflect study design variability and the sensitivity and specificity of laboratory methods to detect and type pneumococci. In addition, the introduction of PCVs into routine immunization programs has substantially altered pneumococcal epidemiology, including serotype distribution, over time. Lack of a reference standard test to determine the pneumococcal etiological agent makes performance assessments of any diagnostic test challenging [22]. For example, lung puncture has been used but requires an accessible consolidation and is an invasive procedure. Bronchoalveolar lavage has been used but is limited to patients ill enough to require intubation and may not access bacteria in the lung periphery.

The standard method for confirming and serotyping IPD cases is the capsular swelling/Quellung reaction [10, 23]; however, this technique requires isolation of viable bacteria from blood, has low sensitivity, does not identify nonbacteremic CAP [24], and relies on the subjective interpretation of assay results [10]. The BinaxNOW test, reported as 86% sensitive for IPD, detects pneumococcal C-polysaccharide in urine by means of an immunochromatographic membrane assay; however, lower sensitivities (52%–82%) have been reported in studies involving adults with CAP [14, 25–27]. More importantly, the BinaxNOW is not a serotype-specific test. Therefore, UAD assays that identify pneumococcal serotypes in urine samples from patients with CAP have clear advantages [14, 15].

Although it is desirable to have high assay sensitivity to accurately assess serotype-specific disease burden, the focus of the assay was to achieve high specificity to ensure that the regulatory end point of vaccine efficacy (and potential effectiveness) could be obtained with high validity. Therefore, positivity cutoffs achieving ≥97% assay specificity for each serotype were established from donors without evidence of PD. In particular, samples from patients with COPD were included in this assessment, because these individuals demonstrate higher carriage rates and approximately 35%–47% of patients with CAP are assumed to

Table 6. Distribution of Urinary Antigen Detection 2 Serotypes (Bacteremic and Nonbacteremic Community-acquired Pneumonia)

	Nonbacteremic CAP (n = 10 220)[a]	Bacteremic CAP (n = 867)[b,c]	Bacteremic and Nonbacteremic CAP (N = 11 087)
2	3 (0.03)	0 (0.00)	3 (0.03)
8	26 (0.25)	7 (0.81)	33 (0.30)
9N	46 (0.45)	9 (1.04)	55 (0.50)
10A	44 (0.43)	3 (0.35)	47 (0.42)
11A	76 (0.74)	12 (1.38)	88 (0.79)
12F	22 (0.22)	8 (0.92)	30 (0.27)
15B	16 (0.16)	4 (0.46)	20 (0.18)
17F	24 (0.23)	5 (0.58)	29 (0.26)
20	18 (0.18)	11 (1.27)	29 (0.26)
22F	91 (0.89)	31 (3.58)	122 (1.10)
33F	26 (0.25)	12 (1.38)	38 (0.34)
Total	380[d] (3.72)	100[e] (11.53)	480 (4.33)

The "No. (%)" in Table 6 refers to Patients No. (%).

Abbreviations: CAP, community-acquired pneumonia; UAD-2, urinary antigen detection 2.

[a]The UAD-2 assay was the only diagnostic test used in this population.

[b]Includes non-serotyped *Streptoccus pneumoniae* blood culture with a UAD-2 result.

[c]Includes results from both UAD-2 test and blood culture.

[d]A total of 12 patients had 2 UAD-2 serotypes: 10A/11A (n = 2), 8/11A (n = 1), 10A/17F (n = 7), 2/20 (n = 1), 10A/12F (n = 1).

[e]A total of 2 patients were positive for 2 different serotypes by either blood culture or UAD-2 assay: 8 (blood culture)/22F (UAD-2) and 22F (blood culture)/11A(UAD-2).

have COPD [28]. Further studies are needed to evaluate the sensitivity of the UAD-2 assay when used in other populations (ie, patients from other geographic locations, patients in different age groups [eg, infants and toddlers], those with immunodeficiencies, and those living in high-transmission settings, such as crowded households), especially in regions where carriage rates are higher than in the United States.

Like the UAD-1 assay, the UAD-2 assay demonstrated excellent robustness, analytical specificity, accuracy, precision, and sample linearity around the positivity cutoffs (Tables 2–4 and Supplementary Tables 1 and 2), and its sensitivity was better than that of other urine based *S. pneumoniae* tests [25–27, 29–31]. The high specificity and sensitivity of the UAD-2 assay are primarily due to the use of serotype-specific mAbs. Cross-reactivity between some serotypes was observed, particularly for closely related serotypes; in particular, mAb cross-reactivity between serotypes 10A and 39 is based on structural similarities and was observed elsewhere [31]. Despite these cross-reactivities, the UAD-2 assay's usefulness is not diminished substantially, because the proportion of CAP cases due to these cross-reacting serotypes is low in IPD, except for serogroup 15, where both serotypes 15B and 15C are identified.

Clinical validation is often assessed by comparison to a predicate method or reference standard. For the UAD-2 assay, a measured result was compared with what was considered a "true result" from BC/typed samples. During clinical validation, the UAD-2 assay was 92.2% sensitive and 95.9% specific to BC results (Table 5). However, the sensitivity for nonbacteremic pneumonia is unknown because of the lack of a reference standard. Our clinical validation study suggests that there is a large burden of pneumonia due to the 11 serotypes in the UAD-2 assay that cannot be detected by BC. This is likely to be an underestimate, because the cutoffs were assigned to drive specificity, which may result in some nonbacteremic pneumonia cases, for UAD-2 serotypes being classified as negative.

Evidence for this possibility comes from a public health analysis of a large randomized, controlled trial of PCV13 among Dutch adults aged ≥65 years [32, 33]. The study's prespecified primary outcome was vaccine type, radiographically confirmed CAP, with vaccine type determined primarily based on UAD-1 assay positivity. For the primary outcome, the rate reduction for vaccine-preventable disease was 25.1 cases per 100 000 person-years of observation. By comparison, for clinical CAP, based only on criteria with no requirement for etiological or radiographic confirmation, the rate reduction was 72.2 per 100 000 person-years (2.8-fold greater).

This increase in rate reduction likely resulted largely from a lack of chest radiographic sensitivity; however, lower UAD assay sensitivity (to achieve high specificity) could also be a contributing factor. Consequently, radiographically confirmed CAP with UAD-1 and/or UAD-2 assay results should be considered minimum estimates of the contribution of these serotypes to pneumonia. In addition, sensitivity and specificity may be underestimated because the UAD-2 assay can type >1 *S. pneumoniae* serotype in a sample, whereas serotyping using the Quellung reaction relies on sampling morphologically distinct *S. pneumoniae* colonies on a BC plate and is prone to analyst bias. Likewise, accurate typing methods, like UAD assays, are needed because more than one serotype can cause IPD [34–36]. Overall, clinical validation results demonstrated high sensitivity and specificity and indicate that the UAD-2 serotype-specific positivity cutoffs are appropriate. By comparison, the BinaxNOW test was less sensitive (50.0%; Table 5) than the UAD-2 assay in identifying *S. pneumoniae* in individuals with an *S. pneumoniae* BC result.

Other urine-based PnPS serotype-specific multiplex immunoassays have been described [31, 37]. The method described by Eletu et al [31] is significantly different from the UAD-2 assay. First, positivity is based on a test-to-negative control value being ≥2. In contrast, the positivity cutoffs of the UAD-2 assay are diagnostic cutoffs calibrated to a serotype-specific reference standard. Second, the analytical specificity for each serotype was broad and could limit the accuracy of serotype-specific epidemiological assessments of PD burden or vaccine effectiveness. Third, the assay described is not designed to adjust the positivity cutoffs to account for potential differences in colonization state or urine compositions in geographically diverse non-CAP populations that may require such adjustments. For example, nonpneumococcal organisms that express *S. pneumoniae* capsule have been isolated from individuals without CAP [38, 39]. Carriage of such nonpneumococcal organisms in healthy individuals may elevate urine polysaccharide levels. Therefore, for this reason and others previously discussed, geographically relevant "healthy control" urine samples are needed for each study to confirm that the serotype positivity cutoffs accurately separate carriage from disease.

In addition, several studies have demonstrated that *S. pneumoniae* antigens can be detected in the urine up to 3 months after infection [29, 40] and 7 days after vaccination with PnPS vaccine [41]. Thus, UAD assay results should take into account clinical and chest radiographic findings at the time urine specimens are obtained, and samples for UAD assays should not be collected shortly after *S. pneumoniae* vaccination. In addition, PnPS has been reported in the urine of healthy infants (2–60 months) with upper respiratory tract colonization [29]. Urinary PnPS detection in young children may not discriminate between carriage and disease when adult cutoffs are used. Therefore, these tests are not recommended for use in pediatric populations unless cutoffs are adjusted to allow differentiation between carriage and disease in these pediatric populations.

In conclusion, this article describes the development/qualification and clinical validation of a highly sensitive and specific UAD-2 assay for diagnosing 11 clinically relevant *S. pneumoniae* serotypes. Because BC is likely to remain the reference standard for diagnosing IPD, the main value of the UAD-2 assay will be for assessing serotype-specific nonbacteremic CAP, which accounts for the majority of PD cases [9]. In addition, Luminex technology allows flexibility to add additional serotypes to a UAD assay, which may be important to assess future serotype-specific PD epidemiology not covered by UAD-1 and UAD-2 assays. In combination with the UAD-1 assay, the UAD-2 assay may be useful in monitoring PD epidemiology after the introduction of future pneumococcal vaccines, determining the efficacy and effectiveness of future vaccines, and providing the data needed for decision makers to arrive at evidence-based decisions regarding pneumococcal vaccine choice and target populations.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. All authors contributed to research and development of the urinary antigen detection 2 assay, associated studies, and drafting of the manuscript, and all authors have read and approved the final manuscript.

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