

# Validation of an Immunodiagnostic Assay for Detection of 13 *Streptococcus pneumoniae* Serotype-Specific Polysaccharides in Human Urine

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**To improve the clinical diagnosis of pneumococcal infection in bacteremic and nonbacteremic community-acquired pneumonia (CAP), a Luminex technology-based multiplex urinary antigen detection (UAD) diagnostic assay was developed and validated. The UAD assay can simultaneously detect 13 different serotypes of *Streptococcus pneumoniae* by capturing serotype-specific *S. pneumoniae* polysaccharides (PnPSs) secreted in human urine. Assay specificity is achieved by capturing the polysaccharides with serotype-specific monoclonal antibodies (MAbs) on spectrally unique microspheres. Positivity for each serotype was based on positivity cutoff values calculated from a standard curve run on each assay plate together with positive- and negative-control urine samples. The assay is highly specific, since significant signals are detected only when each PnPS was paired with its homologous MAb-coated microspheres. Validation experiments demonstrated excellent accuracy and precision. The UAD assay and corresponding positivity cutoff values were clinically validated by assessing 776 urine specimens obtained from patients with X-ray-confirmed CAP. The UAD assay demonstrated 97% sensitivity and 100% specificity using samples obtained from patients with bacteremic, blood culture-positive CAP. Importantly, the UAD assay identified *Streptococcus pneumoniae* (13 serotypes) in a proportion of individuals with nonbacteremic CAP, a patient population for which the pneumococcal etiology of CAP was previously difficult to assess. Therefore, the UAD assay provides a specific, noninvasive, sensitive, and reproducible tool to support vaccine efficacy as well as epidemiological evaluation of pneumococcal disease, including CAP, in adults.**

Community-acquired pneumonia (CAP) is associated with substantial morbidity and mortality in children and older adults (18), and *Streptococcus pneumoniae* is the most common cause of CAP in older adults. In the adult population, the increase in CAP morbidity and mortality is significant after age 50 years due to the number of people with one or more risk factors and/or chronic conditions and is consistent with the increased incidence and severity of invasive pneumococcal disease (IPD) seen in this age group (37). In the United States, it is estimated that 24 to 32% of the population 50 to 64 years of age has one or more of these risk factors and/or chronic conditions, such as diabetes, chronic obstructive lung disease, cardiovascular disease, cancer, or immunosuppression (46). In the United States, CAP is the leading cause of death from infectious diseases and overall the sixth most common cause of death (1).

Medically, CAP is defined as an infection of the lungs that develops outside the hospital setting. CAP can be caused by a variety of pathogens, including both bacteria and viruses (28; Harrison's Practice Answers on Demand). As an etiologic agent can rarely be identified in more than 50% of patients with CAP, further development of sensitive non-culture-based identification methods has been encouraged. The contribution of *S. pneumoniae* in the etiology of CAP varies according to the published literature and may reflect the inherent variability in study design and laboratory isolation of *S. pneumoniae* and the difficulty with detection of *S. pneumoniae* in nonbacteremic CAP. To gain an understanding of the disease burden and the serotype distribution of pneu-

mococcal pneumonia in adults is very important, especially in the light of assessment of the efficacy of pneumococcal conjugate vaccines under development for adult populations.

Over the years, numerous methods for detecting and typing pneumococci have been developed. The "gold standard" and clinically approved method for confirming and serotyping *S. pneumoniae* IPD cases has been the capsular swelling/Quellung reaction (4, 35). This technique has relatively low sensitivity, as it requires viable bacteria in a blood sample, and it is time-consuming (3, 34). More recently, a rapid non-culture-based screening method for evaluating *S. pneumoniae* infection was introduced in 1999 and is marketed as the BinaxNOW *S. pneumoniae* test (Inverness Medical, Scarborough, ME; now marketed by Alere North America, Orlando, FL). This assay tests for the presence of pneumococcal C polysaccharide (C-PS) antigen in the urine of patients with pneumonia and in the cerebrospinal fluid of patients with

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meningitis using an immunochromatographic membrane (33, 43, 45). While this assay is rapid (15-min performance time), the BinaxNOW *S. pneumoniae* urine antigen test measures the presence only of the C-PS antigen, which is present on all *S. pneumoniae* strains, and does not distinguish between the different serotypes of this organism. In addition, the sensitivity of this method has also been called into question by users in the field (27, 43).

Other, non-culture-based methods have also been developed to detect and serotype pneumococcal antigens in biological fluids, such as urine, serum, and sputum (reviewed in references 15 and 30), including latex agglutination (39, 40), radioimmunoassay (29), and countercurrent immunoelectrophoresis (14, 48). Overall, these techniques are relatively low in sensitivity, require large sample volumes, and are slow and tedious to perform. Other methods, such as molecular typing methods (e.g., PCR), to detect *S. pneumoniae* in blood have shown limited value due to their low sensitivity related to sampling issues (31, 42, 44). A traditional sandwich enzyme-linked immunosorbent assay (ELISA) for detecting and serotyping pneumococcal polysaccharide antigens in urine has also been described (27). However, this assay can detect only one serotype per test well, requires a large sample volume, and is labor-intensive when examining multiple serotypes.

Multiplex immunoassays, based on the Luminex xMAP bead technology, can potentially overcome some of the assay limitations previously described. The advantage of the Luminex bead-based assay lies in its ability to combine multiple spectrally distinct microspheres, each conjugated to a different serotype-specific monoclonal antibody (MAb), in a single well to allow the detection of all antigens simultaneously using only a small volume of sample. This assay would also reduce the amount of sample required as well as the time, labor, and costs associated with testing for each antigen separately. Findlow et al. developed a microsphere-based competitive inhibition assay; however, this assay has several limitations, as discussed by the authors (16). We have developed a Luminex platform-based multiplex urinary antigen detection (UAD) assay for identification of 13 serotype-specific polysaccharides of *S. pneumoniae* in urine samples from patients with pneumococcal disease, including X-ray-confirmed CAP, in support of epidemiological investigation of pneumococcal infection and efficacy studies of Pfizer's 13-valent pneumococcal conjugate (13vPnC) vaccine in adults. The 13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F) covered in the UAD assay correspond to the 13 polysaccharide antigens used in Pfizer's 13vPnC vaccine. This paper describes the development and validation of this assay. Even though the assay is used as a limit assay, performance parameters examined in this study, in addition to specificity and positivity cutoff limits, included accuracy, precision, and sample linearity, as the positivity cutoff limit is determined by a reference standard run in each test.

## MATERIALS AND METHODS

**Monoclonal and polyclonal antibodies.** All purified serotype-specific capture MAbs were generated in-house (Pfizer Inc.) by immunizing mice (Swiss Webster, BALB/c, or SJL) with each of the 13 pneumococcal polysaccharides conjugated to the CRM<sub>197</sub> carrier protein (except for serotype 19A, which was primed with a tetanus toxoid conjugate and boosted with the CRM<sub>197</sub> conjugate). Splenocytes were fused with the nonsecreting myeloma cell line X63Ag8.653 to generate hybridomas that were subsequently screened for the appropriate serotype specificity. All MAbs were of the IgG1 subclass (clones PN 26-2, serotype 1; PN 459-1, serotype 3; PN

31-1, serotype 4; PN 55-1, serotype 5; PN 10-2.1, serotype 6A/C; PN 36-1, serotype 6B; PN 503-1, serotype 7F/A; PN 771-20, serotype 9V/A; PN 730-1, serotype 14; PN 56-1, serotype 18; PN 177-7, serotype 19A; PN 571-1, serotype 19F; PN 53-2, serotype 23F). Ascitic fluid, generated in ICR-SCID mice, was purified by 50% ammonium sulfate precipitation, followed by passage over protein G, protein A, or DEAE ion-exchange columns (Pharmacia). Serogroup-specific *S. pneumoniae* polysaccharide (PnPS) polyclonal antibodies, used as secondary detection antibodies, were generated by immunizing rabbits with the polysaccharide conjugates using tetanus toxoid as a carrier for all serotypes except serotype 5, which was obtained from the Statens Serum Institute (SSI, Denmark). All polyclonal antisera were purified using protein A columns (Satorius Biotech GmbH, Germany).

**Specificity of capture monoclonal antibodies.** The specificity of the 13 capture MAbs was analyzed via flow cytometry using bacterial cells corresponding to 91 *S. pneumoniae* organisms and 407 non-*S. pneumoniae* organisms. All *S. pneumoniae* bacterial strains were obtained from MiraVista/SSI, except for serotype 6C, which was obtained from Moon Nahm (University of Alabama). The non-*S. pneumoniae* organisms were cultured from patients enrolled in phase 3 clinical trials of tigecycline for the following indications: complicated intra-abdominal infections, CAP, and hospital-acquired pneumonia (5, 36). Bacterial strains were grown in their appropriate media and subsequently fixed in 1% (vol/vol) paraformaldehyde in 1× phosphate-buffered saline (PBS). The anti-PnPS capture MAbs were added to the bacterial pellets. Bound capture MAbs were detected by biotinylated goat anti-mouse IgG (subclasses 1, 2a, 2b, and 3; Jackson ImmunoResearch Labs, Inc.), followed by detection of the complex by streptavidin-phycoerythrin (PE; BD Biosciences). Mouse IgG instead of the capture MAb was included as a negative control. Samples were analyzed using a BD LSR II flow cytometer and FlowJo (version 7) software (Treestar, Ashland, OR). The mean fluorescence intensity (MFI) of the PE channel was determined for each sample after gating on bacterial/microorganism cells in the logarithmic forward scatter-versus-side scatter dot plot. For all MAbs, the concentrations had previously been optimized with their homologous strains to determine the optimal concentration. An MFI was considered positive if the MFI was greater than 4 times that of the control mouse IgG MFI.

**Coupling of monoclonal antibodies to Luminex xMAP microspheres.** All 13 serotype-specific MAbs were passively coated individually onto spectrally unique Luminex xMAP carboxylated microspheres. During optimization of the assay, certain serotypes displayed improved sensitivity when LumAvidin microspheres were used instead of the carboxylated microspheres. Correspondingly, for 6 of the 13 serotype-specific MAbs, assay components utilized the biotinylated MAb-LumAvidin microsphere combination. All coated microspheres were stored in bead storage buffer (Dulbecco's-PBS containing 1% [wt/vol] bovine serum albumin [BSA] plus 0.05% [vol/vol] NaN<sub>3</sub>, pH 7.4) and refrigerated in amber vials protected from light.

**Positivity cutoff limits.** The UAD assay is considered a limit assay. To determine the positivity cutoff limits, 48 reference standard curves were first generated for each serotype to ensure the suitability of the reference standard. For the UAD assay, positivity cutoff limits, based on antigen concentrations read off 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 pneumococcal disease. Nonparametric tolerance intervals were computed from these concentrations, giving a range predicted to contain 98% of negative urine samples with 99% confidence, thus achieving at least 97% assay specificity for each serotype (19).

**UAD reference standard and positive controls.** The UAD assay reference standard is comprised of 13 purified PnPSs spiked into a negative urine pool at a starting concentration of 2,060 pg/ml for serotypes 1, 3, 4, 5, 6A, 6, 7F, 18C, 19A, 19F, and 23F, one of 6,175 pg/ml for serotype 9V, and one of 685 pg/ml for serotype 14. Positive controls are mixtures of the

13 PnPSs spiked into a negative urine pool at low and high concentrations relative to the linear range of each serotype. The negative urine control consists of a pooled urine sample (treated with 0.5 M PIPES to a final concentration of 25 mM) obtained from healthy adult donors (ages 50 to 67 years) with no evidence of pneumococcal disease. Stocks of the reference standard mixture, positive controls, and negative urine pool were stored at  $-70^{\circ}\text{C}$  until use.

It is known that the PnPSs excreted in the urine of patients with pneumococcal disease differ in their physical properties from the PnPSs of vaccine-grade purified PnPSs (7). Specifically, the molecular masses of vaccine-grade purified PnPSs range from approximately 350 to 820 kDa, while polysaccharides in the urine of infected individuals are lower-molecular-mass fragments of approximately 70,000 Da or less (7). Therefore, to reflect this difference, arbitrary unitages of PnPSs (U/ml) instead of absolute PnPS amounts were assigned to the reference standard and controls.

**Multiplex serotype-specific UAD assay.** Opaque filter plates (Multi-screen<sub>HTS</sub> filter plates; catalog no. MSBVN1B50; Millipore, Watford, United Kingdom) were pretreated with blocking buffer (D-PBS containing 1% [wt/vol] BSA, pH 7.4) and aspirated by vacuum on a vacuum manifold. One hundred microliters of the coated microsphere mixture (pooled mixture of  $\sim 2,500$  coated beads per serotype) was then added into each well of the filter plates and aspirated by vacuum. One hundred microliters of the positive controls was added to the plate, as was 100  $\mu\text{l}$  of a reference standard that was serially diluted 3-fold to create a 10-point standard curve. Negative (negative urine) and buffer controls were also added to the assay plate. Finally, urine samples of unknown PnPS status were added to the plates in triplicate for testing. The assay plates were covered with adhesive foil to protect the microspheres from photobleaching and placed overnight ( $20 \pm 4$  h) at  $4^{\circ}\text{C}$  on a shaker. Following the overnight incubation, the plates were washed three times with wash buffer (D-PBS containing 0.05% [vol/vol] Tween 20, pH 7.4) and aspirated by vacuum between each wash. One hundred microliters of the detection antibody mixture was then added to each well, and the plate was covered with an adhesive foil and left shaking at room temperature for 1 h. The plates were then washed three times and aspirated by vacuum between each wash. After washing, 100  $\mu\text{l}$  of goat anti-rabbit IgG R-phycoerythrin conjugate (catalog no. 111-116-144; Jackson ImmunoResearch Labs, Inc.) was added to each well of the plates. The plates were covered with adhesive foil and placed on a shaker at room temperature for 1 h. The plates were washed three times and aspirated by vacuum between each wash, followed by two washes with D-PBS, with aspiration by vacuum between each wash. After the final wash and vacuum, 100  $\mu\text{l}$  of D-PBS was added to each well and the plates were read using a qualified Bio-Plex reader (Bio-Plex 200 systems; Bio-Rad Laboratories, Hercules, CA). For each well, a minimum of 50 microspheres per serotype were counted. Signals were expressed as MFIs and read against the reference standard. Clinical samples were tested in triplicate and scored as positive for a given serotype if the values for at least 2 out of the 3 triplicates were greater than the serotype-specific positivity cutoff limit. All statistical analyses and data processing were carried out utilizing a validated SAS program.

**Validation of *S. pneumoniae* UAD assay.** Validation of the UAD assay consisted of a series of assay runs to address accuracy, precision, and sample linearity. Accuracy was formally addressed through a series of 2-fold dilutions, made independently from the reference standard, in negative urine to create a set of mock samples. The reference standard was serially diluted 2-fold into pooled negative urine for a series of 12 dilutions. Mock samples were prepared so that at a minimum the values for two mock samples fell above and below the designated serotype-specific positivity cutoff limit. Accuracy measurements were performed eight times (eight separate plates) on 1 day by one operator. The resulting observed concentrations were compared to the expected concentrations (in PnPS U/ml) to determine the accuracy of each mock sample dilution and the percent bias, which was equal to  $100 \cdot$

(observed concentration/expected concentration). Accuracy was considered acceptable if the bias was between  $\geq 70\%$  and  $\leq 143\%$ . Additionally, accuracy was considered acceptable if the percent bias for any one dilution was outside the acceptance criterion but the percent bias for the dilutions immediately above and below this dilution was within the acceptance criterion. The ranges of the assay by accuracy were determined from the geometric mean concentration (GMC) and percent bias of the dilutions. The upper limit was the GMC of the lowest dilution (highest concentration) with an acceptable percent bias, and the lower limit was the GMC of the highest dilution (lowest concentration) with an acceptable percent bias.

Precision was established over a range of PnPS concentrations in a factorial design experiment run over multiple days and with multiple operators. Repeatability and intermediate precision, as well as the sample linearity of the assay, were determined using mock samples prepared from urine samples obtained from subjects with X-ray-confirmed CAP. All precision samples were diluted 2-fold into pooled negative urine for a total of 6 dilutions. Samples were prediluted so that the last dilution was below the positivity cutoff limit for any given serotype. In order to have sufficient data at each concentration, PnPS measurements of mock urine samples were combined into bins (i.e., groups) according to their GMCs. The highest concentration was assigned to bin 1, and subsequent concentrations were assigned to bins in order. Observations from two different dilutions of the same urine sample were not allowed into the same bin. Thus, each bin contained independent observations within a relatively narrow concentration range. Each bin was characterized by the GMC of all samples in the bin. Relative bias and precision analyses were performed within each bin. Precision was acceptable if the relative standard deviation (RSD) was less than or equal to 30%. Sample linearity was considered acceptable if the relative bias was between  $\geq 70\%$  and  $\leq 143\%$ . Additionally, precision and sample linearity were considered acceptable if the percent RSD or percent relative bias for any one dilution was outside the acceptance criterion but the percent RSD or percent relative bias for the dilutions immediately above and below this dilution was within the acceptance criterion.

**Clinical validation of UAD assay.** To clinically validate the diagnostic potential of the UAD assay, urine specimens from patients with X-ray-confirmed CAP from a pilot clinical study conducted in The Netherlands were analyzed. This study was set up to evaluate diagnostic procedures for patients with suspected CAP and was approved by all local research ethics committees. Written informed consent was obtained from all enrolled subjects. In total, 1,114 urine samples were analyzed in the UAD assay. Of these 1,114 samples, 338 were excluded in the final analysis for various reasons (1 duplicate sample, 8 subjects did not have CAP, informed consent could not be verified for 10 subjects, 319 were ineligible due to various study exclusion criteria). In total, samples from 776 subjects with X-ray-confirmed CAP were used for the study analysis. The etiology of a CAP case was supported by positive cultures of blood from bacteremic CAP patients. Of the 776 CAP patients, 54 had blood culture-confirmed *S. pneumoniae* infection and 15 had blood cultures positive for organisms that were identified to be non-*S. pneumoniae* strains. Urine samples (treated with 0.5 M PIPES to a final concentration of 25 mM) were collected between January 2008 and April 2009 and stored at  $-70^{\circ}\text{C}$  prior to testing. Additional information on the study population and corresponding samples is described by Huijts et al. (S. M. Huijts, M. W. Pride, K. U. Jansen, W. G. Boersma, D. Saijders, J. A. J. W. Kluytmans, M. F. Peeters, I. van der Lee, B. A. F. Kuipers, A. van der Ende, and M. J. M. Bonten, submitted for publication).

**BinaxNOW *S. pneumoniae* urine antigen test.** Urine samples were analyzed in the BinaxNOW *S. pneumoniae* urine antigen test (Inverness Medical, Scarborough, ME) according to the manufacturer's instructions. The assay results were interpreted by two analysts. A third analyst interpreted the results only when the results of the first two analysts did not agree.



**TABLE 1** Specificity of UAD assay's 13 serotype-specific monoclonal antibodies to 91 strains of *S. pneumoniae* bacteria

| <i>S. pneumoniae</i> serotype | MFI for MAb specific for the following <i>S. pneumoniae</i> serotype: |                           |               |               |              |               |               |               |               |              |               |               |               |               |
|-------------------------------|---|---------------------------|---------------|---------------|--------------|---------------|---------------|---------------|---------------|--------------|---------------|---------------|---------------|---------------|
|                               | Control mouse   |                           |               |               |              |               |               |               |               |              |               |               |               |               |
|                               | IgG   | 1                         | 3             | 4             | 5            | 6A            | 6B            | 7F            | 9V            | 14           | 18C           | 19A           | 19F           | 23F           |
| 1                             | 123   | <b>16,569<sup>a</sup></b> | 334           | 339           | 315          | 335           | 315           | 264           | 343           | 328          | 350           | 380           | 426           | 366           |
| 3                             | 55  | 46                        | <b>17,647</b> | 58            | 40           | 48            | 56            | 65            | 47            | 54           | 52            | 52            | 51            | 46            |
| 4                             | 344   | 260                       | 269           | <b>52,681</b> | 255          | 269           | 265           | 285           | 305           | 297          | 281           | 265           | 467           | 263           |
| 5                             | 122   | 78                        | 68            | 69            | <b>8,186</b> | 77            | 64            | 68            | 64            | 66           | 70            | 65            | 129           | 70            |
| 6A                            | 184   | 53                        | 64            | 61            | 73           | <b>10,868</b> | 62            | 49            | 70            | 69           | 59            | 57            | 72            | 55            |
| 6B                            | 127   | 92                        | 79            | 113           | 75           | -37           | <b>20,255</b> | 90            | 67            | 94           | 94            | 61            | 172           | 100           |
| 7F                            | 150   | 91                        | 113           | 154           | 144          | 104           | 61            | <b>11,986</b> | 95            | 57           | 130           | 75            | 139           | 82            |
| 9V                            | 157   | 122                       | 80            | 106           | 14           | 103           | 115           | 114           | <b>16,617</b> | 164          | 76            | 88            | 107           | 67            |
| 14                            | 202   | 159                       | 156           | 186           | 168          | 201           | 191           | 187           | 136           | <b>6,308</b> | 205           | 161           | 213           | 123           |
| 18C                           | 184   | 133                       | 173           | 177           | 181          | 144           | 145           | 170           | 190           | 173          | <b>7,387</b>  | 121           | 213           | 129           |
| 19A                           | 198   | 107                       | 66            | 66            | 315          | 81            | 56            | 49            | 59            | 61           | 134           | <b>18,778</b> | 60            | 58            |
| 19F                           | 125   | 57                        | 46            | 39            | 42           | 46            | 45            | 52            | 56            | 70           | 44            | 48            | <b>16,545</b> | 45            |
| 23F                           | 182   | 218                       | 196           | 225           | 228          | 185           | 158           | 199           | 175           | 114          | 195           | 205           | 204           | <b>12,842</b> |
| 6C                            | 73  | 40                        | 31            | 25            | 43           | <b>4,770</b>  | 38            | 31            | 32            | 35           | 50            | 3             | 47            | 31            |
| 7A                            | 139   | 57                        | 32            | 47            | 40           | 47            | 48            | <b>45,187</b> | 55            | 46           | 47            | 47            | 109           | 40            |
| 9A                            | 68  | 50                        | 49            | 51            | 46           | 46            | 53            | 55            | <b>973</b>    | 57           | 51            | 49            | 75            | 46            |
| 18A                           | 321   | 100                       | 90            | 89            | 128          | 98            | 106           | 149           | 122           | 118          | <b>16,031</b> | 85            | 112           | 115           |
| 18B                           | 37  | 77                        | 32            | 46            | 47           | 52            | 42            | 53            | 46            | 58           | <b>16,930</b> | 51            | 71            | 33            |
| 18F <sup>b</sup>              | 69  | 48                        | 263           | 268           | 47           | 36            | 38            | 40            | 38            | 43           | <b>6,344</b>  | 40            | 67            | 41            |

<sup>a</sup> Values in bold indicate positive MFI reactivity (>4 times control mouse IgG MFI).

<sup>b</sup> For all other *S. pneumoniae* strains ( $n = 72$ ), values were  $\leq 4$  times the MFI of the control mouse IgG MFI.

## RESULTS

**Specificity of monoclonal antibodies.** The specificity of the monoclonal antibodies was analyzed by flow cytometry. The 13 MAbs used as capture antibodies in the UAD assay exhibited a high degree of specificity to their corresponding homologous serotype among the 91 *S. pneumoniae* strains (Table 1) and 407 non-*S. pneumoniae* organisms (see Table S1 in the supplemental material) tested. Additional reactivity above background was observed only between the following: (i) anti-PnPS 6A MAb and serotype 6C, (ii) anti-PnPS 7F MAb and serotype 7A, (iii) anti-PnPS 9V MAb and serotype 9A, and (iv) anti-PnPS 18C MAb and serotypes 18A, 18B, and 18F (Table 1). Heterologous MAb reactivity was confirmed by inhibition experiments using the heterologous PnPS (data not shown).

**Robustness of UAD assay.** Robustness is the ability of an assay to remain unaffected by small but deliberate variations in the test method. To this end, a 16-run fractional factorial design of experiment (DOE) was established to study eight assay robustness factors. The eight robustness factors were operator; day; primary, secondary, and reporter incubation times; temperature; and shaker speed. The reference standard and 13 positive clinical urine samples, 1 for each serotype, were analyzed in the DOE. For each serotype, a variance component analysis was carried out to estimate the variability of each factor and selected interactions. Results were used to estimate the RSD for each factor and the total RSD over all factors. Overall, results of the DOE demonstrated that no significant differences were observed when the various robustness factors studied were assessed (data not shown).

Another potential source of assay variability is the use of different MAb-coated bead lots. Comparisons of two different MAb-coated microsphere lots were performed on 2 days, by two analysts, using a panel of 60 urine samples that contained PnPS concentrations spanning above and below the 13 serotype-specific

positivity cutoff values. No meaningful differences between the MAb-coated microsphere lots were observed, in that the geometric mean ratios of sample concentrations measured using the two lots were within acceptable comparability limits (80% to 125%; data not shown).

**Specificity of UAD assay.** The specificity of the UAD assay was assessed by two different methods. In the first method, 24 polysaccharide types were spiked individually into negative urine at 100 ng/ml (approximately 50 times the saturating concentration) and tested in the UAD assay. The resulting signal (MFI) produced by a homologous polysaccharide-MAb pair was compared to the signal (signal-to-noise ratio [S/N]) produced from the negative urine control samples (Table 2). Additionally, the signal produced by a homologous polysaccharide-MAb pair was compared to the signals from the other 12 microspheres (nonhomologous MAb-coated microspheres) (Table 2). For example, spiking PnPS 1 into a negative urine sample at 100 ng/ml elicited high MFI (21,067) and S/N (744) values in the PnPS 1 channel (microspheres coated with anti-PnPS 1). Values in the remaining 12 channels were equivalent to those for the negative urine control samples (S/N = 1). In all cases, an S/N substantially greater than that for the pooled negative urine sample ( $\approx 300$ - to  $>1,000$ -fold) was achieved for the homologous PnPS-MAb-coated microsphere pair compared to the other 23 heterologous PnPSs (Table 2).

In the second specificity method, a mixture of the 13 different MAb-coated microspheres was tested against 13 different PnPS pools comprised of only 12 PnPSs (each pool was missing a different PnPS). In all cases, the MFI signal and S/N produced from MAb-coated microspheres were similar to those for the negative urine sample when paired with samples that lacked the homologous polysaccharide (data not shown). Overall, these results demonstrate the high specificity of the *S. pneumoniae* UAD assay.

**Assay positivity cutoff limits.** To determine the positivity cut-

TABLE 2 Specificity of *S. pneumoniae* UAD assay

| <i>S. pneumoniae</i> serotype | Signal produced by Mab-coated microspheres |            |               |            |               |              |               |            |               |              |               |            |               |            |               |            |               |            |               |            |               |            |               |            |               |            |   |
|-------------------------------|--|------------|---------------|------------|---------------|--------------|---------------|------------|---------------|--------------|---------------|------------|---------------|------------|---------------|------------|---------------|------------|---------------|------------|---------------|------------|---------------|------------|---------------|------------|---|
|                               | 1  |            | 3             |            | 4             |              | 5             |            | 6A            |              | 6B            |            | 7F            |            | 9V            |            | 14            |            | 18C           |            | 19A           |            | 19F           |            | 23F           |            |   |
|                               | 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        | MFI           | S/N        | MFI           | S/N        |   |
| 1                             | <b>21,067<sup>a</sup></b>                  | <b>744</b> | 24            | 1          | 9             | 1            | 1             | 49         | 1             | 22           | 1             | 39         | 1             | 29         | 1             | 56         | 1             | 29         | 1             | 18         | 1             | 30         | 1             | 40         | 1             | 59         | 1 |
| 3                             | 32   | 1          | <b>22,335</b> | <b>967</b> | 10            | 1            | 46            | 1          | 24            | 1            | 37            | 1          | 29            | 1          | 47            | 1          | 33            | 1          | 19            | 1          | 30            | 1          | 42            | 1          | 76            | 1          |   |
| 4                             | 30   | 1          | 25            | 1          | <b>11,629</b> | <b>1,264</b> | 46            | 1          | 25            | 1            | 48            | 1          | 27            | 1          | 49            | 1          | 33            | 1          | 18            | 1          | 32            | 1          | 44            | 1          | 79            | 1          |   |
| 5                             | 41   | 1          | 26            | 1          | 11            | 1            | <b>15,841</b> | <b>362</b> | 35            | 2            | 38            | 1          | 31            | 1          | 45            | 1          | 30            | 1          | 19            | 1          | 30            | 1          | 44            | 1          | 79            | 1          |   |
| 6A                            | 31   | 1          | 27            | 1          | 11            | 1            | 52            | 1          | <b>22,817</b> | <b>1,061</b> | 39            | 1          | 30            | 1          | 49            | 1          | 33            | 1          | 19            | 1          | 33            | 1          | 42            | 1          | 82            | 1          |   |
| 6B                            | 32   | 1          | 28            | 1          | 11            | 1            | 47            | 1          | 39            | 2            | <b>23,040</b> | <b>705</b> | 29            | 1          | 56            | 1          | 33            | 1          | 21            | 1          | 30            | 1          | 42            | 1          | 105           | 1          |   |
| 7F                            | 32   | 1          | 26            | 1          | 11            | 1            | 46            | 1          | 25            | 1            | 38            | 1          | <b>21,107</b> | <b>800</b> | 47            | 1          | 31            | 1          | 19            | 1          | 29            | 1          | 41            | 1          | 112           | 1          |   |
| 9V                            | 27   | 1          | 25            | 1          | 11            | 1            | 44            | 1          | 22            | 1            | 35            | 1          | 25            | 1          | <b>19,962</b> | <b>476</b> | 30            | 1          | 21            | 1          | 30            | 1          | 37            | 1          | 91            | 1          |   |
| 14                            | 29   | 1          | 25            | 1          | 10            | 1            | 46            | 1          | 22            | 1            | 35            | 1          | 27            | 1          | 42            | 1          | <b>23,065</b> | <b>759</b> | 18            | 1          | 28            | 1          | 51            | 1          | 95            | 1          |   |
| 18C                           | 30   | 1          | 26            | 1          | 10            | 1            | 46            | 1          | 22            | 1            | 33            | 1          | 28            | 1          | 44            | 1          | 29            | 1          | <b>16,354</b> | <b>962</b> | 28            | 1          | 39            | 1          | 99            | 1          |   |
| 19A                           | 28   | 1          | 26            | 1          | 10            | 1            | 48            | 1          | 24            | 1            | 34            | 1          | 26            | 1          | 40            | 1          | 29            | 1          | 19            | 1          | <b>23,555</b> | <b>850</b> | 43            | 1          | 83            | 1          |   |
| 19F                           | 26   | 1          | 25            | 1          | 9             | 1            | 43            | 1          | 23            | 1            | 32            | 1          | 26            | 1          | 44            | 1          | 30            | 1          | 18            | 1          | 30            | 1          | <b>22,579</b> | <b>591</b> | 96            | 1          |   |
| 23F                           | 32   | 1          | 22            | 1          | 10            | 1            | 43            | 1          | 24            | 1            | 33            | 1          | 28            | 1          | 42            | 1          | 29            | 1          | 17            | 1          | 29            | 1          | 38            | 1          | <b>24,240</b> | <b>316</b> |   |
| <sup>2b</sup>                 | 36   | 1          | 9             | 1          | 6             | 1            | 42            | 1          | 40            | 1            | 72            | 1          | 23            | 1          | 46            | 1          | 30            | 1          | 8             | 1          | 30            | 1          | 87            | 1          | 11            | 1          |   |
| <sup>8b</sup>                 | 36   | 1          | 15            | 1          | 6             | 1            | 42            | 1          | 37            | 1            | 80            | 1          | 27            | 1          | 47            | 1          | 31            | 1          | 6             | 1          | 34            | 1          | 86            | 1          | 12            | 1          |   |
| 9N <sup>b</sup>               | 37   | 1          | 12            | 1          | 5             | 1            | 42            | 1          | 41            | 1            | 76            | 1          | 29            | 1          | 47            | 1          | 33            | 1          | 7             | 1          | 33            | 1          | 89            | 1          | 10            | 1          |   |
| 10A <sup>b</sup>              | 36   | 1          | 11            | 1          | 6             | 1            | 40            | 1          | 39            | 1            | 72            | 1          | 27            | 1          | 44            | 1          | 30            | 1          | 7             | 1          | 31            | 1          | 82            | 1          | 10            | 1          |   |
| 11A <sup>b</sup>              | 36   | 1          | 11            | 1          | 6             | 1            | 43            | 1          | 39            | 1            | 79            | 1          | 27            | 1          | 50            | 1          | 33            | 1          | 8             | 1          | 38            | 1          | 93            | 1          | 11            | 1          |   |
| 12F <sup>b</sup>              | 39   | 1          | 12            | 1          | 6             | 1            | 55            | 1          | 39            | 1            | 78            | 1          | 27            | 1          | 47            | 1          | 32            | 1          | 8             | 1          | 33            | 1          | 90            | 1          | 13            | 1          |   |
| 15B <sup>b</sup>              | 45   | 1          | 13            | 1          | 7             | 1            | 41            | 1          | 45            | 1            | 66            | 1          | 27            | 1          | 63            | 1          | 35            | 1          | 12            | 1          | 32            | 1          | 204           | 3          | 16            | 1          |   |
| 17F <sup>b</sup>              | 41   | 1          | 12            | 1          | 7             | 1            | 37            | 1          | 43            | 1            | 59            | 1          | 21            | 1          | 61            | 1          | 27            | 1          | 10            | 1          | 24            | 1          | 71            | 1          | 17            | 1          |   |
| 20 <sup>b</sup>               | 36   | 1          | 11            | 1          | 6             | 1            | 41            | 1          | 37            | 1            | 70            | 1          | 24            | 1          | 220           | 5          | 98            | 3          | 8             | 1          | 31            | 1          | 87            | 1          | 16            | 1          |   |
| 22F <sup>b</sup>              | 33   | 1          | 10            | 1          | 5             | 1            | 47            | 1          | 57            | 2            | 71            | 1          | 22            | 1          | 44            | 1          | 28            | 1          | 8             | 1          | 30            | 1          | 79            | 1          | 12            | 1          |   |
| 33F <sup>b</sup>              | 37   | 1          | 11            | 1          | 7             | 1            | 42            | 1          | 39            | 1            | 75            | 1          | 25            | 1          | 47            | 1          | 31            | 1          | 7             | 1          | 33            | 1          | 80            | 1          | 12            | 1          |   |

<sup>a</sup> Values in bold indicate serotype-specific reactivity.  
<sup>b</sup> Additional 23v serotypes.

TABLE 3 Accuracy of UAD assay

| Serotype | Positivity cutoff value (PnPS U/ml) | Assay range based on accuracy |         |             |         |
|----------|-------------------------------------|-------------------------------|---------|-------------|---------|
|          |                                     | Lower limit                   |         | Upper limit |         |
|          |                                     | GMC                           | RSD (%) | GMC         | RSD (%) |
| 1        | 17.8                                | 1.1                           | 8.8     | 1,034.1     | 2.3     |
| 3        | 330.5                               | 1.0                           | 8.8     | 997.2       | 2.8     |
| 4        | 27.3                                | 0.7                           | 7.1     | 917.0       | 11.8    |
| 5        | 2.5                                 | 1.2                           | 10.3    | 746.8       | 5.5     |
| 6A       | 106.7                               | 1.1                           | 10.0    | 1,033.2     | 3.8     |
| 6B       | 79.7                                | 1.3                           | 11.1    | 796.6       | 5.5     |
| 7F       | 6.6                                 | 1.2                           | 4.5     | 502.0       | 7.3     |
| 9V       | 15.6                                | 8.4                           | 8.8     | 2,984.8     | 9.1     |
| 14       | 1.7                                 | 0.5                           | 11.6    | 344.9       | 4.8     |
| 18C      | 4.3                                 | 0.6                           | 9.5     | 724.0       | 5.5     |
| 19A      | 9.8                                 | 2.3                           | 21.2    | 760.1       | 6.0     |
| 19F      | 150.0                               | 1.2                           | 7.5     | 504.8       | 1.6     |
| 23F      | 8.0                                 | 1.4                           | 6.6     | 906.3       | 3.5     |

off limits, 48 reference standard curves were first generated for each serotype to ensure the suitability of the reference standard. Clinical positivity diagnostic cutoff limits were then calibrated to the reference standard curve for each of the 13 *S. pneumoniae* serotypes using 400 control urine specimens obtained from the following subjects: 107 samples from patients undergoing elective surgery (ages  $\geq 65$  years with no underlying respiratory disease, no suspicion of pneumococcal disease or CAP at the time of surgery, and no documented clinical suspicion of CAP in the 2 months before surgery), 184 samples from otherwise healthy patients with stable COPD who exhibited no symptoms of pneumococcal disease (ages  $\geq 50$  years with no COPD exacerbation at the time of sampling, no suspicion of CAP at the time of study inclusion, and no documented clinical suspicion of CAP or exacerbation of COPD in the 2 previous months), and 109 samples from healthy adult donors (ages 50 to 67 years). Control urine samples associated with the 10 largest MFI values per serotype from the original set of 400 MFI measurements were retested for the purpose of

establishing the positivity cutoff limits. All samples were retested for a total of 8 independent determinations (2 analysts over 4 days). Nonparametric statistics were applied to determine the positivity cutoff limits achieving at least 97% assay specificity for each serotype. The resulting positivity cutoff limits for each serotype are listed in Table 3.

**Validation of UAD assay.** To classify a test sample as positive in the UAD assay requires a fluorescent signal that is above a predetermined level, i.e., the positivity cutoff limit. As such, the UAD assay is categorized as a limit assay, according to published nomenclature for bioanalytical validation (21, 47). Relevant to this type of assay format is assay sensitivity, precision, and specificity. As a reference standard is used to define the positivity cutoff limits, the accuracy of the reference standard was also evaluated.

For determination of accuracy, a series of mock samples was made from the reference standard that was serially 2-fold diluted into pooled negative urine for a total of 12 dilutions. The observed concentrations were compared to the expected concentrations in PnPS U/ml to determine the accuracy of each mock sample dilution. Overall, percent bias was acceptable (between  $\geq 70\%$  and  $\leq 143\%$ ) around the positivity cutoff limits of the UAD assay for all serotypes (minimum of 2 dilutions above and below the positivity cutoff limits). Depending on the serotypes, the lower- and upper-limit assay ranges based on accuracy were from 0.5 to 8.4 PnPS U/ml and 344.9 to 2,984.8 PnPS U/ml, respectively (Table 3).

For precision/sample linearity studies, urine specimens (a minimum of three positive samples for each serotype) were selected to create the precision sample panel. All samples were diluted 2-fold into negative urine for a total of 6 dilutions so that at a minimum the last dilution was below the positivity cutoff limits for any given serotype. Acceptable precision (RSD, 2.9% to 27.8%) and sample linearity bias (between  $\geq 70\%$  and  $\leq 143\%$ ) were obtained for all serotypes in the UAD assay. Depending on the serotypes, the lower- and upper-limit assay ranges based on precision were from 0.5 to 9.8 PnPS U/ml and 15.2 to 1,480.5 PnPS U/ml, respectively (Table 4). Sample linearity was demon-

TABLE 4 Precision and sample linearity of UAD assay

| Serotype | Positivity cutoff value (PnPS U/ml) | Assay range based on: |             |             |                  |             |                           |
|----------|-------------------------------------|-----------------------|-------------|-------------|------------------|-------------|---------------------------|
|          |                                     | Precision             |             |             | Sample linearity |             |                           |
|          |                                     | GMC (PnPS U/ml)       |             | % RSD range | GMC (PnPS U/ml)  |             | % bias <sup>a</sup> range |
|          |                                     | Lower limit           | Upper limit |             | Lower limit      | Upper limit |                           |
| 1        | 17.8                                | 9.1                   | 477.2       | 5.5–25.5    | 9.1              | 477.2       | 82.6–117.5                |
| 3        | 330.5                               | 8.6                   | 1,153.3     | 5.7–24.1    | 8.6              | 1,153.3     | 71.4–114.0                |
| 4        | 27.3                                | 1.9                   | 192.5       | 6.5–13.2    | 1.9              | 192.5       | 94.6–110.6                |
| 5        | 2.5                                 | 0.8                   | 32.9        | 8.5–15.2    | 1.1              | 32.9        | 82.2–119.8                |
| 6A       | 106.7                               | 4.8                   | 644.7       | 2.9–12.3    | 4.8              | 472.1       | 77.1–123.8                |
| 6B       | 79.7                                | 4.3                   | 504.2       | 5.7–21.3    | 4.3              | 504.2       | 89.6–114.8                |
| 7F       | 6.6                                 | 0.5                   | 211.3       | 5.1–14.5    | 0.6              | 165.4       | 80.4–121.1                |
| 9V       | 15.6                                | 4.3                   | 1,480.5     | 10.3–27.8   | 5.9              | 1,480.5     | 72.5–122.5                |
| 14       | 1.7                                 | 0.6                   | 16.4        | 5.7–15.1    | 0.6              | 16.4        | 90.2–110.6                |
| 18C      | 4.3                                 | 0.6                   | 15.2        | 11.0–15.1   | 0.6              | 15.2        | 88.8–116.2                |
| 19A      | 9.8                                 | 9.8                   | 174.3       | 10.6–26.7   | 2.0              | 174.3       | 68.1*–115.3               |
| 19F      | 150.0                               | 5.3                   | 428.6       | 5.4–6.4     | 5.3              | 428.6       | 85.3–111.5                |
| 23F      | 8.0                                 | 2.9                   | 122.4       | 8.1–14.5    | 2.9              | 85.6        | 72.9–119.0                |

<sup>a</sup> Sample linearity is considered acceptable if the percent relative bias for any one dilution is outside of the acceptance criterion but the percent relative bias for the dilutions immediately above and below this dilution is within the acceptance criterion.

TABLE 5 Final UAD assay range

| Serotype | Positivity cutoff value (PnPS U/ml) | Final assay GMC range (PnPS U/ml) |             |
|----------|-------------------------------------|-----------------------------------|-------------|
|          |                                     | Lower limit                       | Upper limit |
| 1        | 17.8                                | 9.1                               | 477.2       |
| 3        | 330.5                               | 8.6                               | 997.2       |
| 4        | 27.3                                | 1.9                               | 192.5       |
| 5        | 2.5                                 | 1.2                               | 32.9        |
| 6A       | 106.7                               | 4.8                               | 472.1       |
| 6B       | 79.7                                | 4.3                               | 504.2       |
| 7F       | 6.6                                 | 1.2                               | 165.4       |
| 9V       | 15.6                                | 8.4                               | 1,480.5     |
| 14       | 1.7                                 | 0.6                               | 16.4        |
| 18C      | 4.3                                 | 0.6                               | 15.2        |
| 19A      | 9.8                                 | 9.8                               | 174.3       |
| 19F      | 150.0                               | 5.3                               | 428.6       |
| 23F      | 8.0                                 | 2.9                               | 85.6        |

strated for all serotypes and spans the cutoff limits for all serotypes (Table 4).

The final assay range of the UAD assay for each serotype was based on acceptable accuracy (bias ratio for geometric mean,  $\geq 70\%$  and  $\leq 143\%$ ), precision (RSD,  $\leq 30\%$ ), and sample linearity (relative bias,  $\geq 70\%$  and  $\leq 143\%$ ). Depending on the serotype, the final lower- and upper-limit assay ranges were from 0.6 to 9.8

PnPS U/ml and 15.2 to 1,480.5 PnPS U/ml, respectively. For all serotypes, the positivity cutoff limits fell within the range of the UAD assay, showing acceptable accuracy, precision, and sample linearity (Table 5).

**Clinical validation of UAD assay.** To clinically validate the positivity cutoff limits and diagnostic potential of the UAD assay, 776 urine specimens obtained from patients with X-ray-confirmed CAP were analyzed. The etiology of CAP was determined for 69 bacteremic patients with corresponding positive blood cultures. Of these 69 CAP patients, 54 had blood culture-confirmed *S. pneumoniae* infection. As shown in Table 6, 34 positive blood cultures results were positive for *S. pneumoniae* serotypes covered by the UAD assay. The urine samples of 33 out of 34 of these patients were positive for the exact *S. pneumoniae* serotype also obtained by blood culture. One of the 34 samples was negative in the UAD assay, resulting in an overall clinical specificity and sensitivity for bacteremic CAP of 100% and 97.1%, respectively. Assay specificity was, furthermore, confirmed, as the UAD assay was negative for any urine sample from 17 patients with a corresponding blood culture positive for non-13-valent (13v) *S. pneumoniae* serotypes (0/17 urine samples; Table 6). In addition, 15 samples from bacteremic CAP patients with blood cultures positive for etiological agents other than *S. pneumoniae* were also negative in the UAD assay (see Table S2 in the supplemental material). In contrast, the BinaxNOW *S. pneumoniae* urine antigen test was positive for only 23/34 samples that had corresponding blood

TABLE 6 Clinical validation of UAD assay

| Blood culture-confirmed serotype | No. of urine samples | UAD assay               |                         |                          | UAD clinical sensitivity compared to blood culture (%) | No. of samples positive by BinaxNOW <i>S. pneumoniae</i> urine antigen test |
|----------------------------------|----------------------|-------------------------|-------------------------|--------------------------|--|---|
|                                  |                      | No. of positive samples | No. of negative samples | Clinical specificity (%) |  |   |
| Pneumoniae serotypes             |                      |                         |                         |                          |  |   |
| 1                                | 7                    | 7                       | 0                       | 100                      | 100  | 3   |
| 3                                | 1                    | 0                       | 1                       | 100                      | 0  | 0   |
| 4                                | 3                    | 3                       | 0                       | 100                      | 100  | 2   |
| 5                                | 1                    | 1                       | 0                       | 100                      | 100  | 1   |
| 6A/C                             | 1                    | 1                       | 0                       | 100                      | 100  | 0   |
| 6B                               | 0                    | 0                       | 0                       | NA <sup>a</sup>          | NA   | 0   |
| 7F/A                             | 4                    | 4                       | 0                       | 100                      | 100  | 3   |
| 9V/A                             | 2                    | 2                       | 0                       | 100                      | 100  | 2   |
| 14                               | 7                    | 7                       | 0                       | 100                      | 100  | 5   |
| 18                               | 0                    | 0                       | 0                       | NA                       | NA   | 0   |
| 19A                              | 6                    | 6                       | 0                       | 100                      | 100  | 5   |
| 19F                              | 0                    | 0                       | 0                       | NA                       | NA   | 0   |
| 23F                              | 2                    | 2                       | 0                       | 100                      | 100  | 2   |
| Total                            | 34                   | 33                      | 1                       | 100                      | 97.1   | 23  |
| Non-Pneumoniae serotypes         |                      |                         |                         |                          |  |   |
| 8                                | 3                    | 0                       | 3                       | 100                      |  | 3   |
| 8A                               | 1                    | 0                       | 1                       | 100                      |  | 0   |
| 9N                               | 2                    | 0                       | 2                       | 100                      |  | 1   |
| 10A                              | 1                    | 0                       | 1                       | 100                      |  | 1   |
| 11A                              | 1                    | 0                       | 1                       | 100                      |  | 0   |
| 16F                              | 3                    | 0                       | 3                       | 100                      |  | 2   |
| 20                               | 1                    | 0                       | 1                       | 100                      |  | 0   |
| 22F                              | 5                    | 0                       | 5                       | 100                      |  | 1   |
| Total                            | 17                   | 0                       | 17                      | 100                      |  | 8   |

<sup>a</sup> NA, not applicable.

TABLE 7 Comparison of results from UAD assay and BinaxNOW assay in bacteremic and nonbacteremic CAP

| CAP cases  | No. of cases | UAD assay               |                         |   | BinaxNOW <i>S. pneumoniae</i> urine antigen test |   |
|--|--------------|-------------------------|-------------------------|---|--|---|
|  |              | No. of positive samples | No. of negative samples | Sensitivity compared to blood culture (%) | No. of positive samples <sup>d</sup>             | Sensitivity compared to blood culture (%) |
| Total blood culture positive (bacteremic CAP)    | 69           | 34                      | 35                      |   | 34   |   |
| <i>S. pneumoniae</i> positive                    |              |                         |                         |   |  |   |
| Pneumovax 13 serotypes                           | 34           | 33                      | 1                       | 97  | 23   | 67.6                                      |
| Non-Pneumovax 13 serotypes                       | 17           | 0                       | 17                      | NA <sup>e</sup>                           | 8  | 47.1                                      |
| Nonsensitizable                                  | 3            | 1                       | 2                       | NA  | 2  | NA  |
| Non- <i>S. pneumoniae</i>                        | 15           | 0                       | 15                      | NA  | 1  | NA  |
| Total blood culture negative (nonbacteremic CAP) | 707          | 141                     | 566                     | NA  | 126 <sup>c</sup>                                 | NA  |
| Indeterminate                                    | 1/707        | NA                      | NA                      | NA  | 1/707  | NA  |
| Total CAP (bacteremic and nonbacteremic)         | 776          | 175 <sup>a</sup>        | 601                     | NA  | 160 <sup>b</sup>                                 | NA  |

<sup>a</sup> 22.6% (175/776) of total CAP samples were UAD assay positive.

<sup>b</sup> 20.6% (160/776) of total CAP samples were BinaxNOW assay positive.

<sup>c</sup> Seventy-one of the 126 BinaxNOW-positive samples (nonbacteremic CAP) were also UAD assay positive.

<sup>d</sup> 12.1% (23 + 71/776) of all BinaxNOW-positive samples were also UAD positive (Pneumovax 13 serotypes).

<sup>e</sup> NA, not applicable.

samples that were culture positive for serotypes covered in the UAD assay and positive for 8/17 samples that had corresponding blood samples that were culture positive for *S. pneumoniae* serotypes not covered in the UAD assay, resulting in clinical sensitivities of 67.6% and 47.1%, respectively (Table 7).

In addition to accurately detecting all 13v serotypes detected by blood culture, the UAD assay also identified 13v PnPS in an additional 19.9% of urine samples obtained from patients with nonbacteremic CAP (141/707; Table 7). The BinaxNOW *S. pneumoniae* urine antigen test detected 126 out of the 707 (17.8%) samples from patients with nonbacteremic CAP. Of these 126 samples, 71 were also positive in the UAD assay. In total, 22.6% (175/776) of all patients with X-ray-confirmed bacteremic and nonbacteremic CAP tested positive for the 13 serotypes covered in the UAD assay. Additional information on the study population and corresponding samples is described by Huijts et al. (submitted).

## DISCUSSION

Pneumococcal pneumonia and other diseases caused by *S. pneumoniae* cause substantial morbidity and mortality throughout the world (32). From a global perspective, acute lower respiratory tract infections are responsible for an estimated 3.7 million deaths annually as well as 40 million hospitalizations for pneumonia each year in children and adults. Among adults, pneumococcal pneumonia is the most common clinical presentation of pneumococcal disease. Prior to the introduction of pneumococcal conjugate vaccine and its broad use in a pediatric national immunization program, the incidence of IPD in adults older than age 65 years was second only to that in children younger than 2 years of age (26). Since the introduction of the pneumococcal conjugate vaccine in children, the incidence of IPD in adults over 50 years of age is now at least 9-fold greater than that in children younger than 2 years of

age (6, 37). However, data for community-acquired pneumococcal pneumonia, especially nonbacteremic pneumonia, in adults are limited due to the lack of a sensitive assay. To this end, we developed a sensitive multiplex assay, based on the Luminex xMAP bead technology, which can efficiently detect serotype-specific capsular polysaccharides, corresponding to the 13 polysaccharide antigens used in Pfizer's 13-valent pneumococcal conjugate (13vPnC) vaccine, in the urine of subjects with either bacteremic or nonbacteremic CAP.

Detection of pneumococcal antigen in urine was first described in the early 20th century (9) and has become more applied with the commercial availability of the BinaxNOW *S. pneumoniae* urine antigen test. The sensitivity and specificity of this assay have previously been reported to be between 52% to 82% and 83% to 97%, respectively, in studies involving adult populations with CAP (22, 33, 45). A major disadvantage of the BinaxNOW *S. pneumoniae* urine antigen test, unlike the UAD assay, is that it cannot give information on the pneumococcal capsular serotype. Such serotype-specific data are extremely important for epidemiological purposes and for assessing the extent of postvaccination CAP and potential pneumococcal serotype replacement in patients with CAP.

The UAD method was designed to increase diagnostic sensitivity and specificity. For instance, assay sensitivity could be increased by changing the source of the capture MAbs and type of microsphere used to capture the serotype-specific MAbs. To ensure the consistency of the UAD assay, a reference standard curve was developed and included in each assay plate. In addition, a quality control panel consisting of buffer blank, a negative urine control, and low- and high-dose positive controls was also added on each assay plate to monitor the consistency of the assay over time. Although the UAD assay is considered a limit assay, the UAD assay was validated to establish precision and sample linearity



across the positivity cutoff limit since the clinical trial samples were scored as positive or negative in relation to the positivity cutoff limits. As a reference standard is used to define the positivity cutoff limits, the accuracy of the reference standard was also evaluated. Although it is desirable to have high assay sensitivity, the assay was designed to achieve sensitivity without losing assay specificity. Therefore, the positivity cutoff limits achieving at least 97% assay specificity for each serotype were established using 400 control urine specimens from various donors who did not exhibit signs of pneumococcal disease. In particular, COPD patient samples were included in the panel, as it is known that these individuals demonstrate high carriage rates and approximately 35% to 47% of CAP patients are assumed to have COPD (17). Further studies will be needed to evaluate the sensitivity of the UAD assay when used in other populations (i.e., patients from other geographic regions or different age groups, such as infants and toddlers).

Overall, the UAD assay demonstrated excellent specificity (Table 2) as well as acceptable accuracy (bias ratio,  $\geq 70\%$  to  $\leq 143\%$ ), precision (RSD, 2.9% to 27.8%), and sample linearity (relative bias,  $\geq 70\%$  to  $\leq 143\%$ ) around the positivity cutoff limits (Tables 3 and 4). Depending on the serotype, the UAD assay can detect as little as 0.6 to 9.8 pg/ml of purified polysaccharide, which makes the UAD assay more sensitive than other assays detecting *S. pneumoniae* polysaccharides in urine samples (22, 27, 33, 39, 40, 45). The exquisite specificity and sensitivity of the UAD assay are primarily due to the serotype-specific MAbs. During development of the UAD assay, 13 capture MAbs, 1 to each of the 13 PnPS types found in the Pnevna 13 vaccine, were selected and demonstrated excellent specificity when 91 *S. pneumoniae* strains (Table 1) and 407 non-*S. pneumoniae* organisms (see Table S1 in the supplemental material) were tested. Reactivity above background was observed only between the anti-PnPS 6A MAb and serotype 6C, the anti-PnPS 7F MAb and serotype 7A, the anti-PnPS 9V MAb and serotype 9A, and the anti-PnPS 18C MAb and serotypes 18A, 18B, and 18F. Heterologous MAb reactivity was confirmed by inhibition experiments using the heterologous PnPSs (data not shown). These data were not surprising, as cross-reactive and cross-functional antibodies have been described for some of these serotypes (8). The utility of the UAD test is not diminished by these findings, as the prevalence of serotypes to which we have seen cross-reactivity is low (23, 24). However, to reflect these findings, the specificity of the UAD assay in terms of the serotypes discussed above will be presented as serotypes 6A/C, 7F/A, 9V/A, and 18, respectively.

Clinical sensitivity and specificity were determined by testing clinical specimens obtained from subjects with X-ray-confirmed CAP and comparing the results to those of a gold standard test (blood culture). The UAD assay had an overall clinical sensitivity and specificity of 97.1% and 100%, respectively, in specimens obtained from patients with bacteremic CAP who had a corresponding blood culture positive for an *S. pneumoniae* serotype covered by our assay (Table 6). One patient sample positive for serotype 3 by blood culture was not detected by the UAD, resulting in a clinical sensitivity of 97.1%. More importantly, none of these 34 samples were misclassified. Therefore, the clinical specificity of the bacteremic CAP cases positive for the 13v serotypes covered by the UAD assay was 100%. UAD assay specificity was, furthermore, confirmed, as bacteremic CAP subjects with (i) a corresponding blood culture that was positive for serotypes not covered by the

UAD assay (Table 6) and (ii) blood cultures positive for etiological agents other than *S. pneumoniae* (see Table S2 in the supplemental material) were not detected in this assay. Overall, the analysis of urine samples obtained from CAP cases with corresponding blood culture information indicated that the serotype-specific positivity cutoff limits incorporated in the UAD assay were appropriate, as excellent sensitivity and specificity were demonstrated. In contrast, the BinaxNOW *S. pneumoniae* urine antigen test identified only 23/34 samples with corresponding blood cultures positive for serotypes covered in the UAD assay and 8/17 samples with corresponding blood cultures positive for *S. pneumoniae* serotypes not covered by the UAD assay, resulting in clinical sensitivities of only 67.6% and 47.1%, respectively (Table 7). A similar multiplex immunoassay that is able to detect serotype-specific *S. pneumoniae* polysaccharide in urine samples has recently been described (41). Although our study and the study by Sheppard et al. (41) both describe the development of a multiplex immunoassay for detection of serotype-specific *S. pneumoniae* antigens in urine, there are significant differences between these two publications. (i) The positivity described in the multiplex immunoassay published by Sheppard et al. (41) is based on a test-to-negative (T/N) control value being greater than or equal to 2. In contrast, the positivity cutoff limit detailed in the UAD assay is based on diagnostic cutoff limits calibrated to a serotype-specific reference standard. (ii) The overall sensitivity of the multiplex assay described by Sheppard et al. (41) was reported to be 79.3%, with sensitivities for individual serotypes ranging from 40 to 100%. The only exception was for serotype 6B, which was demonstrated to have a relatively low sensitivity level of 20%. In comparison, the UAD assay detailed in this study was demonstrated to have a good overall sensitivity of 97.1%. (iii) The detection level of serotype-specific *S. pneumoniae* polysaccharide in the immunoassay developed by Sheppard et al. (41) was demonstrated to be in the range of 0.1 to  $< 0.01$  ng/ml, which is similar to the reported detection level of the commercially available BinaxNOW *S. pneumoniae* urine antigen test. The UAD assay, however, was able to accurately detect *S. pneumoniae* polysaccharide over the range of from 0.6 to 9.8 pg/ml. (iv) Finally, unlike the multiplex assay described by Sheppard et al. (41), the UAD assay has the potential to discriminate between disease and colonization by modification of the positivity cutoff values. Studies are under way to evaluate the feasibility of this novel approach.

BinaxNOW *S. pneumoniae* urine antigen test results from samples from nonbacteremic CAP cases, which account for approximately 80% of all cases of CAP (11, 12, 25), showed that this assay detected a total of 126/707 (17.8%) samples, whereas the UAD assay identified 141 positive samples out of 707 (19.9%). It is worth noting that of these 141 samples identified in UAD assay, only 71 of these samples were positive in the BinaxNOW *S. pneumoniae* urine antigen test (Table 7). Since the UAD assay was developed to detect only 13 serotypes, it can be concluded that the sensitivity of the UAD assay is higher than that of the BinaxNOW *S. pneumoniae* urine antigen test. Overall, the sensitivity of the UAD assay, coupled with its excellent specificity, makes the UAD assay a tool that may be used to monitor the etiology of CAP for the 13 most common *S. pneumoniae* serotypes.

It should be noted that in a small number of samples ( $n = 12$ ; 1.5% of total CAP samples), multiple 13v serotypes were detected using the UAD assay. The positivity of the samples was confirmed by retesting. In addition, inhibition experiments were conducted

by pretreatment of these samples with the specific capture MAb and the results were compared to those obtained by treatment of samples with a non-serotype-specific mouse IgG. Ten of 12 samples showed that the multiple positive results could be confirmed by inhibition experiments (data not shown). One sample was confirmed via an inhibition experiment to be positive for only one of the multiple serotypes. Upon repeat testing, the remaining sample was negative and was therefore classified indeterminate (Table 7).

Another advantage of the Luminex technology is that it provides a sensitive and precise method to simultaneously detect *S. pneumoniae* type-specific polysaccharides. Because multiple tests can be run on a single sample, the UAD assay has the advantage of being ideal for use in large-scale clinical studies. Additionally, the Luminex technology provides a platform for the incorporation of additional serotypes, which would be important in epidemiology and efficacy studies that incorporate vaccines with additional serotype coverage. There are, however, some potential circumstances that need additional careful evaluation. For example, several studies have demonstrated that *S. pneumoniae* antigens can be detected in the urine up to 3 months postinfection (2, 27, 43) and up to 7 days postvaccination with *S. pneumoniae* polysaccharide vaccine (38). Thus, assay results should take into account clinical and chest X-ray findings at the time that the urine specimen is obtained. In addition, *S. pneumoniae* polysaccharide has also been reported to be present in the urine of healthy infants (ages 2 to 60 months) with documented upper respiratory tract colonization (10, 13, 20). The BinaxNOW *S. pneumoniae* urine antigen test is not recommended for the detection of pneumococcal pneumonia or bacteremia in this pediatric age group because it does not discriminate between disease and colonization.

In conclusion, we have developed and validated a highly sensitive multiplex UAD assay that is a very useful tool for identifying the serotypes of *S. pneumoniae* present in pneumococcal infections, particularly when a blood culture isolate cannot be grown. By combining the UAD assay with the BinaxNOW *S. pneumoniae* urine antigen test, the UAD assay would provide valuable serotype information in support of epidemiological investigation of pneumococcal infection in adults and surveillance of pneumococcal CAP following introduction of the pneumococcal conjugate vaccine in adults.

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