Development of a TaqMan Array Card for Pneumococcal Serotyping on Isolates and Nasopharyngeal Samples

Suporn Pholwat,a Fuminori Sakai,b Paul Turner,c,d Jorge E. Vidal,b Eric R. Houpta
Division of Infectious Diseases and International Health, Department of Medicine, University of Virginia, Charlottesville, Virginia, USA; Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA; Cambodia Oxford Medical Research Unit, Angkor Hospital for Children, Siem Reap, Cambodia; Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom

Streptococcus pneumoniae is both a commensal and a major pathogen that causes invasive disease in people of all ages. The introduction of serotype-specific pneumococcal vaccines has reduced the burden of disease but has also led to replacement with new strains; thus, serotyping remains important for vaccine-related disease surveillance. Conventional serotyping methods are laborious and expensive. We developed an easy-to-perform genotypic TaqMan array card (TAC) to identify S. pneumoniae strains, including ltyA-based sequences, and 53 sequence-specific PCRs to identify 74 serotypes/serogroups covering all current vaccine types as well as prevalent nonvaccine types. The TAC method was evaluated on 146 clinical S. pneumoniae isolates and 13 nonpneumococcal species that naturally inhabit the upper respiratory tract and yielded 97% (142/146) sensitivity and 100% (13/13) specificity versus results of standard Quellung serotyping. The calculated limit of detection was 20 to 200 fg (8–8 to 84 genome equivalents) per reaction. On 23 blinded nasopharyngeal specimens that were pneumococcus culture positive, the TAC pan-pneumococcus ltyA assay was positive in 21 (91% sensitivity versus culture). On TAC ltyA-positive specimens, a serotype result was obtained on 86%, and the result was 95% accurate versus the subsequent culture’s Quellung result. TAC also detected mixed serotypes in two specimens where Quellung detected only the predominant serotype. This TAC method yields fast and comprehensive serotyping compared to the standard method and may be useful on direct specimens.

S. pneumoniae (the pneumococcus) is a leading invasive pathogen of children and older adults, principally causing pneumonia, otitis media, and meningitis. The precursor to invasive disease is upper airway colonization (1). Existing vaccines are based upon capsular polysaccharide and are highly effective only against vaccine types (2–5). Serotype replacement occurs with increased colonization and disease caused by nonvaccine strains (6, 7). With over 90 different capsular serotypes, there is a constant race to add more capsular types to further expand coverage to reduce disease burden amid a headwind of changing strain replacement.

In this context it is important to epidemiologically follow pneumococcal serotypes, both in invasive strains to detect emergence of virulent serotypes and also in the upper airway to monitor strain replacement (1, 8). However, serotyping of pneumococci with the Quellung method is technically difficult, requires expensive panels of polyclonal antisera and precise inocula (9), and may yield visually ambiguous reactions (10). Furthermore, a limited number of subcultured colonies are typed, limiting the ability to detect mixed infections, particularly from nasopharyngeal specimens (11, 12).

Molecular serotyping methods are therefore emerging. After elucidation of the capsular biosynthetic locus (13), PCR assays for the capsular polysaccharide gene cluster have been devised. Sequencing-based assays of the cps and wzi genes (14, 15) have been published, as have real-time PCR assays to detect 21 serotypes/serogroups (16, 17). Nanofluidic, microarray, and Lumex-based systems have also been developed (18–21). Recently, we optimized 53 singleplex reactions to discern most serotypes/serogroups, including all vaccine types (22). However, performing that many reactions per specimen is onerous and difficult to implement in field settings; therefore, in this work we further optimized and configured the reactions to a single TaqMan array card (TAC).

MATERIALS AND METHODS

Bacterial strains. All bacterial strains utilized in this study were cultured at Emory University on blood agar plates and incubated at 37°C with 5% CO2 overnight (~16 h) prior to DNA extraction. Strains from 70 S. pneumoniae included serotypes 1, 2, 3, 4, 5, 6A, 6B, 6C, 7A, 7B, 7F, 8, 9L, 9N, 9V, 10A, 10B, 10F, 11A, 11B, 11C, 11F, 12B, 12F, 13, 14, 15A, 15B, 16A, 16F, 17A, 17F, 18C, 19A, 19B, 19C, 19F, 19°F (atypical), 20, 21, 22A, 22F, 23A, 23B, 23F, 24A, 24B, 25A, 27, 28A, 28F, 29, 31, 33A, 33B, 33D, 33F, 34, 35A, 35B, 35F, 36, 38, 41A, 41F, 43, 45, 46, and 47A, as described previously (22). For specificity testing, we included 20 streptococci naturally found in the nasopharynx, including S. infantis, S. oralis, S. anginosus, S. intermedius, S. sobrinus, S. pseudopneumoniae, S. mitis, S. parasanguinis, S. australis, S. mutans, S. peroris, S. oligormentans, S. intestinalis, S. vestibularis, S. cristanus, S. salivarius, S. gordonii, S. sanguinis, S. sinensis, Dolosigranulum pigrum, and three other bacterial species Neisseria meningitidis, Haemophilus influenzae, and Staphylococcus aureus.

Received 22 March 2016 Returned for modification 15 April 2016 Accepted 30 April 2016 Accepted manuscript posted online 11 May 2016

Citation Pholwat S, Sakai F, Turner P, Vidal JE, Houpt ER. 2016. Development of a TaqMan array card for pneumococcal serotyping on isolates and nasopharyngeal samples. J Clin Microbiol 54:1842–1850. doi:10.1128/JCM.00613-16.

Editor: S. S. Richter, Cleveland Clinic

Address correspondence to Eric R. Houpt, erh6k@virginia.edu.

S. P. F., J. E., and E. R. H. contributed equally to this article.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.00613-16.

Copyright © 2016 Pholwat et al. This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International license.
Nasopharyngeal samples from children. Nasopharyngeal (NP) samples (n = 28) belonged to our laboratory collection, and pneumococcal carriage had been analyzed in our previous studies (10, 23). NP samples were stored at −80°C in skim milk-tryptone-glucose-glycerin (STGG) transport medium prior to DNA extraction.

Quellung standard serotyping. Quellung results were determined as described previously (22). Briefly, a fresh overnight bacterial culture in a blood agar plate was suspended in 1× phosphate-buffered saline (PBS) and then mixed with antisum on a glass slide and read microscopically at a magnification of ×100. Pneumococcus Neufeld antisum was obtained from the Statens Serum Institute (Copenhagen, Denmark).

DNA extraction from bacterial cultures and nasopharyngeal specimens. A bacterial colony was suspended in 200 μl of lysis buffer (Tris-EDTA [TE] buffer containing 0.04g/ml lysozyme and 75 U/ml mutanolysin), or 200 μl of nasopharyngeal specimens (in STGG medium) was mixed with 100 μl of lysis buffer. Samples were incubated for 1 h at 37°C. DNA was then purified using a QIAamp DNA minikit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions and eluted in 100 μl. The quality and quantification of DNA preps obtained from bacterial cultures were further evaluated using a NanoDrop system (NanoDrop Technologies, Wilmington, DE).

Assay development on 384-well plates. We adopted 53 serotype/serogroup-specific primers and probes from published sources (16, 22, 24–26) (Table 1) and, if needed, made modifications to accommodate the common cycling condition of the TaqMan array card (TAC) using Primer Express, version 3 (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA). We also included one pan-pneumococcus assay (lytA) (26) and an assay for an internal control (27). Optimization of conditions and probe specificity testing were performed using the 384-well format of the ViiA7 platform (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA). Each primer/probe set (0.09 μM of each forward and reverse primer, 0.025 μM of probe of a 50 μM stock) was amplified in singleplex in a total of 5 μl of PCR mixture containing 2.5 μl of 2× TaqMan universal master mix II with uracil-N glycosylase (UNG) (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA), 1.295 μl of nuclease-free water, and 100 pg of genomic DNA. Cycling conditions included UNG activation at 50°C for 2 min and initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 1 min. We included 54 previously characterized serotypes in each run for specificity testing, and nuclease-free water was used for a nontemplate control.

Evaluation of the TaqMan array card. Primer and probe oligonucleotides were synthesized and spotted onto the TaqMan array card by Applied Biosystems (Life Technologies Corp., Carlsbad, CA, USA) as laid out in Fig. 1. Twenty microliters of input DNA (1 ng/μl for isolates) was mixed with 50 μl of 2× TaqMan universal master mix II with UNG (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA) and 30 μl of nuclease-free water to a 100-μl final volume. This was loaded into each port of the card, whereby each card included seven clinical samples and one synthetic positive-control plasmid (Genewiz, Inc., South Plainfield, NJ, USA) that we designed to contain the primer and probe region of all 55 assays (53 serotype-specific assays plus lytA and phocine herpesvirus [PhHV]). The loaded card was centrifuged twice at 1,200 rpm for 1 min and then sealed; the loading ports were excised, and the card was inserted into a ViiA7 instrument (Life Technologies Corp., Carlsbad, CA, USA) and run under the same cycling conditions as described above for 40 cycles.

Statistical analysis. Means or medians were compared using Student’s t test or a Mann-Whitney test. Data are shown as means ± standard deviations unless otherwise stated. A standard curve of lytA was generated with known DNA concentrations and plotted against the threshold cycle (C_T) to yield the copy number, calculated as

\[10^{(\frac{1}{\text{C}_T} - 33.7013)} - 3.4262\]
| Serotype or sample type | Target | Sequence (5′–3′)* | Reference(s) or source |
|------------------------|--------|------------------|------------------------|
| 1                      | wchD   | F-CGTTCGGTAAATTTGAAGCTATGAGR-TGTGGGGCCGCAATACCT| 24                     |
|                        |        | P-TGCTTCGCTTGTTCAATGATGAT |                     |
| 2                      | wzy    | F-CTTATGAGCTTGCTTAGTTGTCCTC | 25                     |
|                        |        | R-AAATCTGTCGAAAAATATGATGATGAT |                     |
|                        |        | P-AAGGTCGAATTTCTGAAATATTCTGAA |                     |
| 3                      | tsp    | F-GGTCAGCAAGAATATGACTTGG | 22, 24                 |
|                        |        | R-TGGTTTATTCCAGGGGCTTAGTA |                     |
|                        |        | P-TATTGGATGTTGTTTACGTTGAAGA |                     |
| 4                      | wzy    | F-GCCATCAGCGGAGGTGTTTCTAG | This study             |
|                        |        | R-CACCACATAGTCAAGAGCTTCC | 16                     |
|                        |        | P-CTTCTGCTCAGTCGTTTCCGAAT | 16 (modified)          |
| 5                      | wzy    | F-CATGATTTAATGCGCCCTCTTGGAAG | 16 (modified)          |
|                        |        | R-GACAGTATAAGGAAAGAAGGCTTACG |                     |
|                        |        | P-CTTCTCTATCGTTTCCGGAAT |                     |
| 6ABCD                  | wciP   | F-AAAGTTCTTCTGCTAGATGATGGAAGG | 22, 24 (modified)     |
|                        |        | R-ACATTATGTCATTTCCGATATAGCAG |                     |
|                        |        | P-TGGTTTATGACGGCGACAACTGGAAT |                     |
| 6CD                    | wciN("ota") | F-CAATCGAGAGCAGTTTCTTCTCG | 22                     |
|                        |        | R-ACCTGACTACATCAGTGAATGATGGAATG |                     |
|                        |        | P-AATGGGAGGCTGCTATGGAATGATGGAATG |                     |
| 7AF                    | wcwH   | F-ATGGACGCTTTGTTGGACAGG | 16 (modified)          |
|                        |        | R-ATTGCGCGATCTAATGCTAATATCC |                     |
|                        |        | P-TGAACTCAAGAGCAGCACTTCA |                     |
| 7BC-40                 | wcxU   | F-TCCAGATATAGCTATTTCCAAATCAG | 22 (modified)         |
|                        |        | R-AAAGAAAGGTTAATTTCCGATGTAATG |                     |
|                        |        | P-TCCCTATTATATGATATGACCCACA |                     |
| 8                      | wx     | F-CCACCTCATATTTCGTTTCTTCTC | 22                     |
|                        |        | R-TCAATTATGGAAGAAGGCAAGCTT |                     |
|                        |        | P-TGATGGCAGATGGGTTCCGCGAG |                     |
| 9AV                    | wx     | F-AGGATCTTCCTTTATATCTGCTTTTACG | 16 (modified)         |
|                        |        | R-CAATATCGCCTAATATGCTAAAG |                     |
|                        |        | P-AACAGATTGCAAAAGGCTTCA |                     |
| 9LN                    | wx     | F-CGTTGAAATTTTCTTATATCTGCAATAGG | 22                     |
|                        |        | R-CTACTGCTACGATACAGATTATCTCATACG |                     |
|                        |        | P-CAATTTGCTGGCGATATGCTTCTC |                     |
| 10A                    | wcrD   | F-AGAAGGCCCTAAGAAGAGATCGG | 22                     |
|                        |        | R-CCAGCTATTTCCATGTAAATACG |                     |
|                        |        | P-AGGTCATGCTGCTCAACATT |                     |
| 10B                    | wcrD   | F-AAATATGAGATGGTTAGGAATATATGCTT | 22                     |
|                        |        | R-GTCCTTTCTACATTGGAAGATATTCTC |                     |
|                        |        | P-AACGGATTCTGAATGCTGGTAACT |                     |
| 11AD                   | wchK   | F-CGGCCCGACGCTACATTATTG | 22                     |
|                        |        | R-TGATGACTTACATGCTCACCACAA |                     |
|                        |        | P-AAATACCAATATGTTGTTCCGAGATTAAAGAATG |                     |
| 11F*                   | wchK   | F-TGGTTCAGCTACTTTTATGCG | This study             |
|                        |        | R-TGATGACTTACATGCTCACCACAA |                     |
|                        |        | P-AACGCATTGCTTTCCGAGAAAGGAAGAAGA |                     |
| 12ABF-44-46            | mnaB   | F-GCCACCCACGCTTAAATATTCTAC | 16 (modified)         |
|                        |        | R-CAACTAAGAAGCAAGGATCCACAG |                     |
|                        |        | P-ATACAAATGGCCACCAACACC |                     |
| 12B                    | wx     | F-CTTTGCTGGTCAACAGAAGGCTTATG | This study             |
|                        |        | R-AAGGTTCAAAAGGTAATTGTTTATTAGGAA |                     |
|                        |        | P-AGATGAAATATCTTCCAAATCATCAAGGTAATG |                     |
| 13                     | wzy    | F-AGAATGACATTGTTTGGATGACTT | 22                     |
|                        |        | R-CAGAAAAAATATTTTGTATTGATAAAATCCATC |                     |
|                        |        | P-AAGAAGCTCCGCTACGATGTTAACTTACCC |                     |
| 14                     | wchL   | F-CTGACTAAATGTTGCTCAATGGGAGG | 22, 24 (modified)     |
|                        |        | R-ATAACAGCTGATATTACATGCTGAAATCTC |                     |
|                        |        | P-TGATGGCTTTGCCAATATGATGTTCTC |                     |
TABLE 1 (Continued)

| Serotype or sample type | Target | Sequence (5′–3′)* | Reference(s) or source |
|-------------------------|--------|------------------|------------------------|
| 15                      | wzx    | F-TTGAATCAGGTAGATTGATTTCTGCTA R-CTCTAGGAACTTAACTGAGTCTGTTAAG P-TGCCGCTTTGTGCTCTCTGTT | 22, 24 |
| 16F                    | wzy    | F-TAATTTATGAGCCTTGGTAACTCTTCCC R-TCCCAAAGGATAAATCAATACATTTTAGGA P-TCTTTCAATGGTTAAACCCG | 16 (modified) |
| 17F                    | alp2   | F-GGAACGTGTAGCATTCTTTAGGTA R-TTTTTGTCGGTACCTCGGAAAG P-TCTTTGATGCTATGCTAGAAGCTCAGTGA | This study |
| 18ABCDF                | wzy    | F-TCGATGGGCATAAGACAGATTATGGA R-CCATTGTCCTTGTAAGACCACTTGG P-TGAAATCAACCTATATTAAGTTGACCATCTATTCT | 16 (modified) |
| 19A                    | wzy    | F-GCTCAGTATATCCAAATTTCTGGA R-CATGGAATAGGAAAGATTGATGATATCACT | This study |
| 19F                    | wzy    | F-CGGGTGCAATATATTGCACTGG R-CAGGAATGAGAATGTCAAGAATGAAGG P-TCTTTCCTACTATTAT | 16 |
| 19°F<sup>b</sup>       | wzy    | F-GTCTTATGTCGGTATGTTTTGCGP R-GGATGAGGAACGGAATCGAAGAGG P-CCAGTTATGAGGTTGAGCTAAGTGG | This study |
| 20                      | wciL   | F-AAAGATACGTGGTCAGGACTATCTATT R-AGTCAGAAATGCTAATCCACCTATTCTATTC | 22, 22 |
| 21*                    | wzy    | F-GGTATTTAATATGCTGCACGGGTTAT R-CAAAAAGAGGCTGTGAGAGAAGG | 25 |
| 22AF                   | wcwA   | F-TCTCAGTCAAATTTCTGTGGA R-TCGGATCCGATGTTAGGACGAGA P-TGGAATCAGGAGGACA | 22, 24 (modified) |
| 23A                    | wzy    | F-CTCCGGCCATTTACCTCATTTTGGA R-TGAAGAAGATGCTGTTGTTGAGAAC P-TCCCAACCTCCATTTCCCA | 16 (modified) |
| 23B                    | wzx    | F-CTGAAAGAATAGGAAAGATATTGGAAGG P-TCCCAACCTCCATTTCCCA | 22, 25 |
| 23F                    | wzy    | F-AAAGTGTGCTGACTTGGATGATGTC R-GATTAAATTATGCTGAGAGAAGG P-TGTTGAATCAGGAGGACGAGA | This study |
| 24A                    | wzx    | F-CTGAGCTGGTGAATATTAGGGAAG P-TCCCAACCTCCATTTCCCA | 22, 24 (modified) |
| 25AF*                  | wcyE   | F-ATACCAACTAGATCCAGGAGGAC P-RAAATGGGAATATCTTTTTTATCAGTACCGC P-CCCGTGCACTTTACCAATA | 22 |
| 27                      | whaK   | F-AGCGATTTAGAGCAGTATGAC R-TCTCAAAATGCTGACGGTTG P-TGTTGAAGGACGAGGACTGTTG | 22 |
| 29*                    | wcrJ   | F-TFCCGATTTGCGGCTTTTACA R-GGGTACCCACCTCAAAATTATTTT P-TGAATTCCTGTTTCTTCTTGG | 25 (modified) |
| 31                      | wzy    | F-GCAGAAGTTTATAGGCACGACG P-AGATACGATGACGATGACGAGG P-CGCCACGATTTAACAGGCGCAGG | 22 |
| 33AF-37                | wzy    | F-GGAACGTGGTGGAGGACTATACG R-GGTTCAACAGGCGTGAATAACCG P-TAGAATCTGTTGCGGACTG | 16 (modified) |
| 33B                    | wciN   | F-CCTGTTATGGCAGCTGATTACCTTAC P-RGACATTTCAACCTCCTCTACTC | This study |
|                         |        |                  |                        |

(Continued on following page)
imens. Once developed, the TAC assay is as simple to perform as a single PCR. The TAC assays exhibited excellent linearity and limits of detection, albeit they were slightly less sensitive than the assays in a plate format, where more DNA template can be added. This slight sensitivity loss may not be clinically deleterious, and certainly the procedural advantage of the TAC versus setting up 54 singleplex PCRs is enormous.

For isolates where abundant DNA is available, performance remained excellent, with 97% accuracy versus the Quellung result. Indeed, the card had 100% accuracy on blinded isolates from a wide variety of 24 serotypes, including all of the PCV13 strains.

For isolates where abundant DNA is available, performance remained excellent, with 97% accuracy versus the Quellung result. Indeed, the card had 100% accuracy on blinded isolates from a wide variety of 24 serotypes, including all of the PCV13 strains.

**TABLE 1 (Continued)**

| Serotype or sample type | Target | Sequence (5’–3’)a | Reference(s) or source |
|------------------------|--------|-------------------|------------------------|
| 33D*                   | wciN   | F-CGTATAGTCTTCGGCAATTC 22 (modified) |                        |
|                        |        | R-TCACGTTCGATCATCACAG |                        |
|                        |        | P-GACACACCATATTTGAAATGG |                        |
| 34                     | wzy    | F-CGGTGGATCGACAATATGG | 22                     |
|                        |        | R-GTCTGGTCTTCCCAATATACCTGAG |                        |
|                        |        | P-ACGGAGCCCAATGTCATTGAATGTTT |                        |
| 35AC-42                | wc1K   | F-TGTTTCCAGCTTCCTCTTGA |                        |
|                        |        | R-AAATGAAATCAAAGTATACGTAG | 22 (modified) |
|                        |        | P-TTCAAAATACCGGACACCTGCTGTC |                        |
| 35B                    | wc1J   | F-GCATTGAGGTGAGCACATA | 22, 24 (modified) |
|                        |        | R-TGTAAGAAGCTGAAACCTGATATAAA |                        |
|                        |        | P-AACCAATATGGAAAGGCAAGGTC |                        |
| 35F-47F                | wzy    | F-GTGGTGTATATACCTGATGAAATAGGG | 22 (modified) |
|                        |        | R-ACTCTAATAATTCATATCTGAGA |                        |
|                        |        | P-AGAAATGGGCTCTACATATA |                        |
| 36*                    | wzy    | F-CITGCTATTTCAGGCCCTTTCTGG | 22 (modified) |
|                        |        | R-CGGGATTATATTTGAAATGGGAACCT |                        |
|                        |        | P-AGAATGGCAGCTCTACATATA |                        |
| 38-25AF                | wc1L   | F-GTCATTGTCGAGAACTTCTGGAATG | 22, 24 |
|                        |        | R-TGGTCTTACAGAAGGACATGG |                        |
|                        |        | P-TTGGCCAGATGTGGAATATTTGTCGG |                        |
| 39*                    | wc1G   | F-CAAAAATAATCAACTCATAATTAGGACCT | 22 |
|                        |        | R-ATACGGATATTATTTCTTGGGG |                        |
|                        |        | P-AGCTCAGGGCTTTTCTTTATGACGGA |                        |
| 41A                    | wc1B   | F-GCAATAATAGTATCCCGAGTTAACAC | 22 (modified) |
|                        |        | R-GTTAGCTCTTTTGGTTTAATGTC |                        |
|                        |        | P-GAGGCAATAGTCTAGCTCAGGAA |                        |
| 41F                    | wzx    | F-TTTTTGGGAGGAGTGCTTTT | 22                     |
|                        |        | R-AAACGGTCTTCTATGATTCTCATACT | This study |
|                        |        | P-CTTCTGTGTCAACCAGTGGAGAT | (modified) |
| 43                     | wzx    | F-AGAGGGCATCATAAAATAGTGGGC | 22                     |
|                        |        | R-GAATCAACAGTGATTCTCMAAG |                        |
|                        |        | P-TCCATAATATCGTGAAAGCTGGG |                        |
| 45*                    | wzy    | F-TCTAGATCCTTCTGAAATAATATTGGAACG | 22 (modified) |
|                        |        | R-GACGAGGTGAATTCTGGTATGAT |                        |
|                        |        | P-CITTTTAGTGCCTGGTCC |                        |
| 47AF*                  | whaL   | F-AGGATATTGGGAGAATTTG | 22                     |
|                        |        | R-GAACCTTACCACATCTCCGGTC |                        |
|                        |        | P-CTCACATAGGAGTGCTGCTG |                        |
| lytA                   | lytA   | F-TGGTGGTATTTTGCATTGACC | 26 (modified) |
|                        |        | R-ACGGCAATGCTCAGGATGAAAGCA |                        |
|                        |        | P-CTCAGGTATCAAGCGTTTTTGGGCA |                        |
| PhHV                   | gB     | F-GGCGGAATCAGAATGATGATG | 27                     |
|                        |        | R-GCCGGTCTAAAAGTACCAAA |                        |
|                        |        | P-TATGGTCTCGCCACCATCT |                        |

a F, forward primer; R, reverse primer; P, probe labeled with FAM (6-carboxyfluorescein) except for the probes for the serotypes marked with asterisks, which are labeled with VIC at the 5’ end. All are 3’ minor groove binder probes.
b Atypical 19F.
The assay also worked well on direct nasopharyngeal specimens, with a 91% sensitivity versus culture and an 86% accuracy of the serotype result versus the Quellung reaction on \textit{lytA}-positive specimens. A few samples had low levels of DNA at the \textit{lytA} or serotype level that could be rescued with larger amounts of DNA. Thus, the serotype result was 100% accurate for any \textit{lytA} result of a CT of 34 or below (corresponding to a nasopharyngeal density of \(8 \times 10^3\) CFU/ml), which is how we would propose using the assay. This assay is suitable for monitoring pneumococcus density and mixed infections in nasopharyngeal specimens, which is of great interest in the effort to better document the phenomenon of serotype replacement in the nasopharynx after vaccination (6). Regarding mixed infections, there was one discrepant nasopharyngeal specimen which was nontypeable by Quellung but serotype 4 by TAC, which we hypothesize was mixed. It is also plausible that this

![FIG 1 Streptococcus pneumoniae serotyping TaqMan array card layout. The TaqMan array card includes eight sample ports, whereby each sample is aliquoted into 48 PCRs. Serotypes in the form AB or A-B indicate a common assay that detects multiple serotypes/serogroups. Serotypes in the form A/B* indicate a duplex assay.](image)

| Serotype or sample | 384-well plate TaqMan array card | TaqMan array card |
|---|---|---|
| Linearity \(R^2\) | LOD (fg) | Linearity \(R^2\) | LOD (fg) |
| 1 | 0.953 (87.2) | 10 (4.2) | 0.994 (85.7) | 20 (8.4) |
| 2 | 0.998 (85.9) | 10 (4.2) | 0.940 (87.3) | 20 (8.4) |
| 3 | 0.999 (97.7) | 10 (4.2) | 0.998 (98.8) | 20 (8.4) |
| 4 | 0.998 (90.0) | 100 (42) | 0.995 (95.6) | 200 (84) |
| 5 | 0.997 (89.9) | 10 (4.2) | 1.000 (92.8) | 20 (8.4) |
| 6ABCD | 0.999 (92.4) | 10 (4.2) | 0.995 (98.9) | 20 (8.4) |
| 6CD | 0.999 (91) | 10 (4.2) | 0.998 (98.6) | 20 (8.4) |
| 7AF | 1.000 (92.9) | 10 (4.2) | 0.998 (89.5) | 20 (8.4) |
| 7BC-40 | 0.999 (91.1) | 10 (4.2) | 0.968 (80.4) | 20 (8.4) |
| 8 | 0.999 (100) | 10 (4.2) | 0.996 (99.0) | 20 (8.4) |
| 9AV | 0.992 (95.7) | 10 (4.2) | 0.993 (109.1) | 20 (8.4) |
| 9LN | 0.993 (90.6) | 10 (4.2) | 0.957 (87.4) | 20 (8.4) |
| 10A | 1.000 (97.0) | 10 (4.2) | 0.996 (108.1) | 20 (8.4) |
| 10B | 0.999 (100) | 100 (42) | 0.979 (84) | 200 (84) |

\(a\) Values in parentheses represent PCR efficiency (%).

\(b\) LOD, limit of detection. Values in parentheses are the numbers of copies per reaction.

The genome size of \textit{S. pneumoniae} serotype 4 TIGR4 (2,160,842 bp) was used for calculations.
specimen represents a weakly expressing strain. We think that applying TAC to nasopharyngeal colonization will be particularly useful to monitor vaccine effectiveness in communities over time, ensuring that vaccine types are being eliminated as expected. Nasopharyngeal specimens in children with pneumonia could be used as a surrogate for vaccine effectiveness (11).

Limitations of our study are that the number of direct specimens with culture- and serotype-confirmed results was small; thus, the sensitivity and specificity estimates of the TAC assay are approximate, and additional evaluation will be beneficial. Other investigators have found that nonpneumococcal streptococcal species can interfere with serotyping assays (29), so more direct investigation of these problems is needed. Although the limit of detection by TAC was within the range of other reported assays (26), it was 2-fold higher than that of the regular real-time PCR format (22).

The Quellung reaction was performed with pure culture colonies from the same isolate condition and Quellung serotype or sample type. For example, to infer serotype 6AB we must detect serotypes 6AB and 6BC in the absence of serotypes 6CD (Fig. 1). We certainly suspect that the serotype reactions may need to be modified over time to include alternate types.

We embarked on this project because we have demonstrated excellent performance and reproducibility of the TAC platform in multisite field studies in Africa and Asia (30), areas of high pneumococcal carriage, coinfection, and variable serotype distributions. While the real-time PCR instrument is costly (~$75,000), it also performs routine real-time PCR. To our knowledge, the TAC platform exists in at least 13 countries across sub-Saharan Africa and South Asia. The TAC cards are stable at 4°C for at least 2 years and cost about $50 per specimen, or approximately $1 per reaction, which compares favorably with conventional Quellung testing, which can costs up to $100 per colony (12).

In conclusion, the

#### TABLE 3 Performance of TaqMan array card serotyping on isolates versus the Quellung standard

| Isolate condition and Quellung serotype or sample type | No. of isolates tested | No. of concordant results | No. of discordant results | % Accuracy |
|--------------------------------------------------------|------------------------|---------------------------|---------------------------|------------|
| Unblinded                                              | 54                     | 54                        | 0                         | 100        |
| All serotypes from Table 2                            |                        |                           |                           |            |
| Blinded<sup>a</sup>                                    |                        |                           |                           |            |
| 1                                                      | 4                      | 4                         | 0                         | 100        |
| 2                                                      | 2                      | 2                         | 0                         | 100        |
| 3                                                      | 3                      | 3                         | 0                         | 100        |
| 4                                                      | 4                      | 4                         | 0                         | 100        |
| 5                                                      | 2                      | 2                         | 0                         | 100        |
| 6A                                                     | 3                      | 3                         | 0                         | 100        |
| 6B                                                     | 5                      | 5                         | 0                         | 100        |
| 7F                                                     | 4                      | 4                         | 0                         | 100        |
| 8                                                      | 3                      | 3                         | 0                         | 100        |
| 9N                                                     | 4                      | 4                         | 0                         | 100        |
| 9V                                                     | 4                      | 4                         | 0                         | 100        |
| 10A                                                    | 3                      | 3                         | 0                         | 100        |
| 11A                                                    | 3                      | 3                         | 0                         | 100        |
| 12F                                                    | 1                      | 1                         | 0                         | 100        |
| 14                                                     | 4                      | 4                         | 0                         | 100        |
| 15B                                                    | 2                      | 2                         | 0                         | 100        |
| 17F                                                    | 3                      | 3                         | 0                         | 100        |
| 18C                                                    | 4                      | 4                         | 0                         | 100        |
| 19A                                                    | 3                      | 3                         | 0                         | 100        |
| 19F                                                    | 5                      | 5                         | 0                         | 100        |
| 20                                                     | 3                      | 3                         | 0                         | 100        |
| 22A                                                    | 2                      | 2                         | 0                         | 100        |
| 22F                                                    | 4                      | 0                         | 4                         | 0          |
| 23F                                                    | 4                      | 4                         | 0                         | 100        |
| 33F                                                    | 3                      | 3                         | 0                         | 100        |
| Serotypes not included in Table 2                     | 10                     | 10                        | 0                         | 100        |
| Nonpneumococcal bacteria                               | 13                     | 13                        | 0                         | 100        |
| Total                                                  | 159                    | 155                       | 4                         | 97         |

<sup>a</sup> Serotypes in bold are those included in PCV13.

<sup>b</sup> Positive with lytA but negative with any serotype-specific probe on the TAC.

<sup>c</sup> Negative with any probe on the TAC, including lytA.

#### TABLE 4 Performance of TaqMan array card serotyping method on nasopharyngeal specimens

| Quellung serotype or culture result<sup>a</sup> | lytA Ct<sub>T</sub> | Serotype(s) Ct<sub>Y</sub> | DNA (copies/reaction) | Predicted bacterial load from lytA ct<sub>T</sub> (CFU/ml) |
|-----------------------------------------------|--------------------|---------------------------|-----------------------|----------------------------------------------------------|
| 4                                            | 27                 | 4 (29), 23B (30)          | 9.3E3                 | 9.3E3                                                    |
| 4                                            | 28                 | 4 (28)                    | 4.7E0                 | 4.7E0                                                    |
| 6A                                           | 27                 | 6ABCD (29)                | 6.7E7                 | 6.7E7                                                    |
| 10A                                          | Negative           | Negative                  | NA<sup>b</sup>        | NA                                                       |
| 10A                                          | 31                 | 10A (33)                  | 6.4E9                 | 6.4E9                                                    |
| 11A                                          | 23                 | 11AD (23)                 | 4.9E6                 | 4.9E6                                                    |
| 11A                                          | 28                 | 11AD (28)                 | 5.9E1                 | 5.9E1                                                    |
| 11A                                          | 32                 | 11AD (35)                 | 4.1E0                 | 4.1E0                                                    |
| 11A                                          | 34                 | 11AD (34)                 | 8.5E9                 | 8.5E9                                                    |
| 17F                                          | 30                 | 17F (30)                  | 1.3E2                 | 1.3E2                                                    |
| 17F                                          | 33                 | 17F (36)                  | 1.6E0                 | 1.6E0                                                    |
| 19A                                          | 29                 | 19A (31), 6CD (33)        | 1.7E9                 | 1.7E9                                                    |
| 19F                                          | 28                 | 19A (30)                  | 3.7E1                 | 3.7E1                                                    |
| 19F                                          | 29                 | 19F (29)                  | 2.8E5                 | 2.8E5                                                    |
| 19F                                          | 28                 | 19°F (30)                 | 4.2E0                 | 4.2E0                                                    |
| 19F                                          | 30                 | 19F (30)                  | 1.4E1                 | 1.4E1                                                    |
| 19F                                          | 28                 | 19F (28)                  | 5.0E4                 | 5.0E4                                                    |
| 19F                                          | 35                 | Negative                  | 3.86E                   | 3.86E                                                    |
| 23F                                          | 28                 | 23F (31)                  | 3.6E3                 | 3.6E3                                                    |
| 23F                                          | 27                 | 23F (28)                  | 7.4E5                 | 7.4E5                                                    |
| 23F                                          | Negative           | Negative                  | NA                    | NA                                                       |
| 35F                                          | Negative           | NA                        | NA                    | NA                                                       |
| NT                                           | 27                 | 4 (30)                    | 3.0E8                 | 3.0E8                                                    |

<sup>a</sup> The Quellung reaction was performed with pure culture colonies from the same nasopharyngeal samples. NT, not typeable.

<sup>b</sup> NA, not applicable.
TaqMan array card is a fast, high-throughput, serotyping method for pneumococcal that is suitable to field studies.

ACKNOWLEDGMENTS

This work was supported by NIH grant K24 AI102972 (to E.R.H.) and by the Murdoch Children's Research Institute (to J.V.) which received funds from the Bill and Melinda Gates Foundation (grant 52099). P.T. is funded by the Wellcome Trust as part of the Wellcome Trust-Mahidol University-Oxford Tropical Medicine Research Programme.

We thank Catherine Satzke for helpful discussions and Yiming Lin from Emory University for his assistance in some laboratory procedures.

We also thank Lesley McGee and Bernard Beall from the CDC for providing most nonpneumococcal streptococci utilized in this study.

FUNDING INFORMATION

This work, including the efforts of Eric Houpt, was funded by HHS | National Institutes of Health (NIH) (K24 AI102972). This work, including the efforts of Paul Turner, was funded by Wellcome Trust. This work, including the efforts of Jorge Eugenio Vidal, was funded by Bill and Melinda Gates Foundation (52099).

REFERENCES

1. Bogaert D, De Groot B, Hermans PW. 2004. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis 4:144–154. http://dx.doi.org/10.1016/S1473-3099(04)00938-7.

2. Klugman KP, Madhi SA, Huebner RE, Kohberger R, Mbelle N, Prince N, Vaccine Trials Group. 2003. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. N Engl J Med 349:1341–1348. http://dx.doi.org/10.1056/NEJMoa035060.

3. Black SB, Shinefield HR, Ling S, Hansen J, Fireman B, Spring D, Noyes J, Lewis E, Ray P, Lee J, Hackell J. 2002. Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than five years of age for prevention of pneumonia. Pediatr Infect Dis J 21:810–815. http://dx.doi.org/10.1097/01.inf.000004654-20020900.00005.

4. Cutts FT, Zaman SM, Enwere G, Jaffar S, Levine OS, Okoko JB, Oluwalana C, Vaughan A, Obaro SK, Leach A, McAdam KP, Biney E, Thomas KE, van de Pol I, Witteveen S, van der Heide HG, Schot CS, van Dijk A, van der Ende A, Schous L. 2011. Population structure of invasive Streptococcus pneumoniae in The Netherlands in the pre-vaccination era assessed by MLVA and capsular sequence typing. PLoS One 6:e202390. http://dx.doi.org/10.1371/journal.pone.0020239.

5. Leung MH, Bryson K, Freystatter K, Pichon B, Edwards G, Charalam- bous BM, Gillespie SH. 2012. Sequotyping: serotyping Streptococcus pneumoniae by a single PCR sequencing strategy. J Clin Microbiol 50:2419–2427. http://dx.doi.org/10.1128/JCM.06384-11.

6. Pimenta FC, Roundtree A, Soysal A, Bakir M, du Plessis M, Wolter N, von Gottberg A, McGee L, Carvalho MDG, Beall B. 2013. Sequential triplex real-time PCR assay for detecting 21 pneumococcal capsular serotypes that account for a high global disease burden. J Clin Microbiol 51:647–652. http://dx.doi.org/10.1128/JCM.02927-12.

7. Duve FS, van Mens SP, Robberts L, Wolter N, Nicol P, Mafofo J, Africa S, Zar HJ, Nicol MP. 2015. Comparison of a real-time multiplex PCR assay and pneumococcal serotyping assay for pneumococcal serotyping. PLoS One 10:e0157349. http://dx.doi.org/10.1371/journal.pone.0157349.

8. Tomita, Y., Okamoto, A., Yamada, K., Yagi, T., Hasegawa, Y., Ohta, M. 2011. A new microarray system to detect Streptococcus pneumoniae serotypes. J Biomed Biotechnol 2011:352736. http://dx.doi.org/10.1155/2011/352736.

9. Satzke C, Dunne EM, Porter BD, Klugman KP, Mulholland EK, Pneumococcal Project Group. 2015. The PneuCarriage Project: a multi-centre comparative study to identify the best serotyping methods for examining pneumococcal carriage in vaccine evaluation studies. PLoS Med 12:e1001903. http://dx.doi.org/10.1371/journal.pmed.1001903.

10. Dhoubhadel BG, Yasunami M, Yoshida LM, Tha HA, Thi TH, Thi TA, Watanabe K, Suzuki M, Morimoto K, Dang DA, Ariyoshi K. 2014. A novel high-throughput method for molecular serotyping and serotype-specific quantification of Streptococcus pneumoniae using a nanoplastic real-time PCR system. J Med Microbiol 63:528–539. http://dx.doi.org/10.1099/jmm.0.061944-0.

11. Yu J, Lin J, Kim KH, Benjamin WH, Jr, Nahm MH. 2011. Development of an automated and multiplexed serotyping assay for Streptococcus pneumoniae. Clin Vaccine Immunol 18:1900–1907. http://dx.doi.org/10.1128/CVI.05312-11.

12. Sakai F, Chouchua S, Satzke C, Dunne EM, Mulholland K, Klugman KP, Vidal JE. 2015. Single-plex quantitative assays for the detection and quantification of most pneumococcal serotypes. PLoS One 10:e0121064. http://dx.doi.org/10.1371/journal.pone.0121064.

13. Sakai F, Talekar SJ, Klugman KP, Vidal JE, for the Investigators G. 2013. Expression of virulence-related genes in the nasopharynx of healthy Children. PLoS One 8:e76147. http://dx.doi.org/10.1371/journal.pone.0076147.

14. Azzari C, Moriondo M, Indolfi G, Cortimiglia M, Canessa C, Becciolini L, Lippi F, de Martino M, Resti M. 2010. Realtime PCR is more sensitive than multiple PCR for diagnosis and serotyping in children with culture negative pneumococcal invasive disease. PLoS One 5:e9282. http://dx.doi.org/10.1371/journal.pone.0009282.

15. Azzari C, Moriondo M, Cortimiglia M, Valleri M, Canessa C, Indolfi G, Ricci S, Nieddu F, de Martino M, Resti M. 2012. Potential serotype carriage of three pneumococcal conjugate vaccines against invasive pneumococcal infection in Italian children. Vaccine 30:2701–2705. http://dx.doi.org/10.1016/j.vaccine.2011.12.008.

16. Carvalho MdA G, Tondella ML, McAusland K, Weidlich L, McGee L, Mayer LW, Steigerwalt A, Whaley M, Facklam RR, Fields B, Carlone G, Ades EW, Dagan R, Sampson JS. 2007. Evaluation and improvement of real-time PCR assays targeting lytA, pspA, and pspC genes for detection of pneumo-
mococcal DNA. J Clin Microbiol 45:2460–2466. http://dx.doi.org/10.1128/JCM.02498-06.

27. Liu J, Gratz J, Amour C, Kibiki G, Becker S, Janaki L, Verweij JJ, Taniuchi M, Sobuz SU, Haque R, Haverstick DM, Houpt ER. 2013. A laboratory-developed TaqMan Array Card for simultaneous detection of 19 enteropathogens. J Clin Microbiol 51:472–480. http://dx.doi.org/10.1128/JCM.02658-12.

28. Feikin DR, Kagucia EW, Loo JD, Link-Gelles R, Puhan MA, Cherian T, Levine OS, Whitney CG, O’Brien KL, Moore MR. 2013. Serotype-specific changes in invasive pneumococcal disease after pneumococcal conjugate vaccine introduction: a pooled analysis of multiple surveillance sites. PLoS Med 10:e1001517. http://dx.doi.org/10.1371/journal.pmed.1001517.

29. Carvalho Mda G, Pimenta FC, Moura I, Roundtree A, Gertz RE, Jr, Li Z, Jagero G, Bigogo G, Junghae M, Conklin I, Feikin DR, Breiman RF, Whitney CG, Beall BW. 2013. Non-pneumococcal mitis-group streptococci confound detection of pneumococcal capsular serotype-specific loci in upper respiratory tract. PeerJ 1:e97. http://dx.doi.org/10.7717/peerj.97.

30. Liu J, Kabir F, Manneh J, Lertsethtakarn P, Begum S, Gratz J, Becker SM, Operario DI, Taniuchi M, Janaki L, Platts-Mills TA, Haverstick DM, Kabir M, Sobuz SU, Nakjarung K, Sakpaisal P, Silapong S, Boddhidatta I, Qureshi S, Kalam A, Saidi Q, Swai N, Mujaga B, Maro A, Kwambana B, Dione M, Antonio M, Kibiki G, Mason CJ, Haque R, Iqbal N, Zaidi AK, Houpt ER. 2014. Development and assessment of molecular diagnostic tests for 15 enteropathogens causing childhood diarrhoea: a multicentre study. Lancet Infect Dis 14:716–724. http://dx.doi.org/10.1016/S1473-3099(14)70808-4.