

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. Houpt^a

Division of Infectious Diseases and International Health, Department of Medicine, University of Virginia, Charlottesville, Virginia, USA^a; Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA^b; Cambodia Oxford Medical Research Unit, Angkor Hospital for Children, Siem Reap, Cambodia^c; Centre for Tropical Medicine and Global Health, Nuffield Department of Medicine, University of Oxford, Oxford, United Kingdom^d

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 *lytA*-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 to 84 genome equivalents) per reaction. On 23 blinded nasopharyngeal specimens that were pneumococcus culture positive, the TAC pan-pneumococcus *lytA* assay was positive in 21 (91% sensitivity versus culture). On TAC *lytA*-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.

Streptococcus 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 synthesis gene clusters have been devised. Sequencing-based assays of the *cps* and *wzh* genes (14, 15) have been published, as have real-time PCR assays to detect 21 serotypes/serogroups (16, 17). Nanofluidic, microarray, and Luminescence-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 opti-

mized 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, 39, 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. oligofermentans*, *S. intestinalis*, *S. vestibularis*, *S. cristatus*, *S. salivarius*, *S. gordonii*, *S. sanguinis*, *S. sinensis*, *Dolosigranulum pigrum*, and three other bacterial species *Neisseria meningitidis*, *Haemophilus influenzae*, and *Staphylococcus aureus*.

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

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

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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 $1\times$ phosphate-buffered saline (PBS) and then mixed with antiserum on a glass slide and read microscopically at a magnification of $\times 100$. Pneumococcus Neufeld antiserum 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 μl of each forward and reverse primer, 0.025 μl 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 $2\times$ 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 $2\times$ 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 \pm 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 $10^{(CT - 33.701)/-3.4262}$.

RESULTS

Analytical assay performance. The 53 serotype/serogroup-specific singleplex PCR assays as well as the *S. pneumoniae lytA* assay were tested against 54 serotyped pneumococcal isolates as well as several nonpneumococcal species, and 100% specificity with no cross-reactivity was observed (see Fig. 1 in the supplemental material). The PCR performance of each primer/probe assay was determined on both 384-well plates and the TaqMan array card formats. DNA from both individual and pooled serotypes was tested in serial dilution. The overall linearity of the 53 serotype assay targets including *lytA* was 0.997 ± 0.01 and 0.986 ± 0.02 , and the PCR efficiencies were $93\% \pm 4.9\%$ and $97\% \pm 9.7\%$ using the 384-well plates and TaqMan array card formats, respectively. The limit of detection on 384-well plates was 10 to 100 fg/reaction while that of TAC was 20 to 200 fg/reaction (Table 2).

Evaluation of TaqMan array card serotyping on clinical isolates. The performance of the TaqMan array card was evaluated on 54 *S. pneumoniae* serotypes (previously serotyped by Quellung reactions) and then on 92 blinded isolates, which included serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 10F, 11A, 11B, 11C, 12F, 14, 15B, 16A, 17A, 17F, 18C, 19A, 19B, 19C, 19F, 20, 22A, 22F, 23F, 24B, 28A, 28F, 33F, and 13 non-*S. pneumoniae* strains. TAC yielded 97% (155/159) agreement compared with the Quellung serotype result (Table 3). There was 100% specificity, and all four discordant samples were indicated as serotype 22F by Quellung (performed twice) but negative with the TAC 22AF assay. This was not due to 22AF assay failure since it was positive for three serotype 22A strains.

TaqMan array card serotyping on direct specimens. Twenty-eight nasopharyngeal specimens underwent DNA extraction and TAC testing, and the results were compared with the culture and Quellung results (22). In 23 of these nasopharyngeal specimens an *S. pneumoniae* strain had been isolated whereas five were culture negative. The TAC yielded a *lytA*-positive result in 21/23 culture-positive specimens and was positive in 1/5 culture-negative specimens (C_T of 34), corresponding to a TAC *lytA* sensitivity of 91% and specificity of 80% versus culture (Table 4). The serotype assays were exclusively positive in *lytA*-positive specimens (TAC serotype assays, 100% specificity versus *lytA*). In the 22 *lytA*-positive specimens, a serotype result was obtained for 19 specimens (TAC serotype assays, 86% sensitivity versus *lytA*). The TAC serotype result matched the Quellung result in 18 specimens (95% accuracy), the exception being one pneumococcus specimen non-typeable by Quellung that was serotype 4 by TAC (with a somewhat late C_T of 30 compared with the other serotype 4 strains). There were two culture-positive specimens undetectable by *lytA* on the TAC, one of which was *lytA*-positive when larger volumes of DNA were used (1 μl ; C_T of 37) in a plate format and one of which remained negative. Likewise, we retested the three *lytA*-positive/serotype-negative samples with the serotype assays with larger volumes of DNA, and one became positive (1 μl ; C_T of 36). We used the TAC *lytA* C_T to extrapolate the pneumococcal concentration in the nasopharyngeal specimens, and the two discrepant results (e.g., *lytA* positive/serotype negative) were both at the lowest concentration of 3×10^3 to 4×10^3 CFU/ml.

DISCUSSION

In this work we describe the development of a TaqMan array card that compartmentalizes 53 reactions to detect 74 pneumococcal serotypes and that can be used on isolates or nasopharyngeal spec-

TABLE 1 Primer and probe sequences of the 53 PCRs corresponding to 74 serotypes

Serotype or sample type	Target	Sequence (5'–3') ^a	Reference(s) or source
1	<i>wchD</i>	F-CGTGCGGTAATTGAAGCTATGA R-TGTGGCCCCAGCAACTCT P-TGCTTGCCCTTGTATAGGGT	24
2	<i>wzy</i>	F-TTATGGACTGGCTGATGGTTCTC R-AAATCCTGACCAATAATAGCCTTT P-AGGTCAACGTATTGGAACCTTAGAAATTGGGAAA	25
3	<i>tnp</i>	F-GGTCAGCAGAAAGTATGCATTGG R-TCGTTTATCCAGGGTCTGATGA P-TATTGGATGTGGTTTATCGTGAAGA	22, 24
4	<i>wzy</i>	F-GCATCAGCGACGGTTGTTAT R-CACCACCATAGTAACCAAGTTCC P-TTACCTGTAGGCTCTTCTTTTG	This study 16 16 (modified)
5	<i>wzy</i>	F-CATGATTATGCCCTCTTGCAA R-GACAGTATAAGAAAAGCAAGGGCTAA P-CTTCTTCTCATCGTTTCCGCAT	16 (modified)
6ABCD	<i>wciP</i>	F-AAGTTTGCRCTAGAGTATGGGAAGGT R-ACATTATGTCCATGTCTTCGATACAAG P-TGTTCTGCCCTGAGCAACTGG	22, 24 (modified)
6CD	<i>wciN_{beta}</i>	F-CAATCAGGCAGTTCTTTCTCG R-ACCTGACTCACCATCAATAACC P-AAATGGGAGGGCTTTGGATTGGC	22
7AF	<i>wcwH</i>	F-ATGAAGGCTTTGGTTTGACAGG R-ATTCTCGCCATCAATTGCATATTC P-TGAGACTAACGCACAGCCA	16 (modified)
7BC-40	<i>wcxU</i>	F-TCCAGATATAGTCATTCCCAATCAG R-AAAGAAGGTAAATCCCATGATGAATT P-TCCCTCATTATCGATTACTGACCCACCA	22 (modified)
8	<i>wzx</i>	F-CCACTCATCAGTTTCCCATATGTTT R-TCAATAATTGAAGAAGCGAACGTT P-TGATGGCAGATGGGTTGGGACGAG	22, 24
9AV	<i>wzx</i>	F-AGGTATCCTATATACTGCTTAGG R-CGAATCTGCCAATATCTGAAAG P-ACACATTGACAACCGCTACA	16 (modified)
9LN	<i>wzx</i>	F-CGTGGAATTTTCTATACTGCAATAGG R-CTACTGCTACGATACCATAITCTACAG P-CAATTCTTAGCCGGATTCTCTC	22 (modified)
10A	<i>wcrD</i>	F-AGAGGCCCTAAGAAAAGATTCTG R-CCCAGTCATCCCCATCAATAAC P-AGGTCATGGCTCAACAATT	22 (modified)
10B	<i>wcrD</i>	F-AAATATGAGATTGGTAAGGAATTTCTGG R-GTCTTTTCACTTAAACGAATTCCATT P-AACGGATTCCAATGCACTCGGTAAC	22
11AD	<i>wchK</i>	F-CGGCCCAGCTACATTTATGG R-TGATCATTCACATGCTCACCAA P-AAATACCAATAGTTGTTCCGAGATTAAAGAAGT	This study
11F*	<i>wchK</i>	F-TGGTCCAGCTACTTTTATGGC R-TGATCATTCACATGCTCCCC P-ACTCCAATAGTTGTTCCGAGGCAAAAAGA	22
12ABF-44-46	<i>mnaB</i>	F-GCACCACGGGTAAATATTCTAC R-CAACTAAGAACCAAGGATCCACAG P-ATACAATGCCACCAACACC	16 (modified)
12B	<i>wzx</i>	F-GGTTGCTGATCAAAAGGTCTATG R-AGGTTCAAAGTAAGATTTTAGCAA P-AGATAAAAATCTTTCCAAATCATCAAAGTGA	This study This study 22 (modified)
13	<i>wzy</i>	F-AGACTACCATTTTTTGTATCAGTTAGATT R-CAGAAAACATATTTTGTTCATAAATCCATC P-AAGCAGCACTTCCAAGTCGTAATCTACC	22
14	<i>wchL</i>	F-CGACTGAAATGTCACTAGGAGAAGAT R-AATACAGTCCATCAATTACTGCAATACTC P-TCATTGCTTTGCCAATACITGATGGTCTC	22, 24 (modified)

(Continued on following page)

TABLE 1 (Continued)

Serotype or sample type	Target	Sequence (5'–3') ^a	Reference(s) or source
15	<i>wzx</i>	F-TTGAATCAGGTAGATTGATTTCTGCTA R-CTCTAGGAATCAAATACTGAGTCCTAATGA P-CTCCGGCTTTTGTCTTCTCTGT	22, 24
16F	<i>wzy</i>	F-TAATGTTATGACCTTGGAATCTTCCC R-TCCCAAAGGATAATCAATAACTTTTAGAAG P-TCTTCCAAATGCTTAACCGC	16 (modified)
17F	<i>abp2</i>	F-GGAACTGTTGATCATCTTAGCGTA R-TTTTGATCCCGTACTCGGAAG P-TCTTCGTATGCTAGTTCTAAGAGAGCTACTGA	This study This study 25 (modified)
18ABCF	<i>wzy</i>	F-TCGATGGCTAGAACAGATTTATGG R-CCATTGTCCCTGTAAGACCATTG P-TTGAATCAACCTATAATTTGCCCC	16 (modified)
19A	<i>wzy</i>	F-GCTCATTGATATCCAATTCGGAA R-CATGGCTAAGTGCAAGATTATGAATC P-AGCTCTTACTATTATAGTTGACCTCATTATTCT	This study
19F	<i>wzy</i>	F-CGGGGTCAAATATTCAGTGG R-CACGAATGAGAAGTCGAATAAAAG P-TTCGCACTGTCAATTCACCT	This study 16 16 (modified)
19 ^{“F”b}	<i>wzy</i>	F-GTCCTTAGTTCTGGTTATTCGGG R-GGATGAGGAACCGAATCGAAG P-CCAGTTATGAAGGTGAGCTAACAGTGCG	22
20	<i>wciL</i>	F-AAAGATACTGGCTGAGGAGCTATCTATT R-AGTCAAAAGTACTCAACCAATTCGATATATTC P-AGGATAAGGTCTACTTTGTGGGAGTTC	22, 24
21*	<i>wzy</i>	F-GGTTTAAATATCGTCCGGGTAT R-CAAAAAAAGGGCTTGTAGACGAA P-TGTGAATTGGACACGTTATGGAGC	25
22AF	<i>wcwA</i>	F-TCTCTGAAATGGTTGTTGAAGGAA R-TCGCATCCGATAGTTCTTGTGA P-TGGCAATCCCAGGACAA	22, 24 (modified)
23A	<i>wzy</i>	F-CTCCCCTCCATTACCCATTGG R-TGAAGAAAGTGCTGTTTGTGAACC P-TCCCACACTCCCTACTCCCA	16 (modified)
23B	<i>wzx</i>	F-TTGAAGAAATTGCTCCAGAAACAT R-CCAAAAGACTAGCCTCAACCACTAA P-TAGAGCTATTTATCTTTCTGTTGTTTT	22, 25
23F	<i>wzy</i>	F-AAGTGATAGTGAAGTTGGGATTGTCT R-GATTCTATTTGCAAACACGTTGAGA P-TGTTAAAAATACACACAACATCAACA	This study
24A	<i>wzx</i>	F-CTTGGAGTTGCTAATTATGGGAAG R-ATCTCTTACACGTGCACATC P-CACAGCATATCGTAAAAATACCCGCA	22 (modified)
25AF*	<i>wcyE</i>	F-ATACCAACTAGAATCAGCAGGAC R-AAATGGAATATCTTTGATAATTTACTCGC P-CCGCTGGACTTACTGCAATA	22 (modified)
27	<i>whaK</i>	F-AGCGATTAGCGACTGATATCC R-TCTCAAAATCGATCTCGCGTG P-TGTGGAAGGCGTTTGAAGGTGACT	22
29*	<i>wcrJ</i>	F-TTCGAGTTGTGCCGTTTTTACA R-GGCGTACCCACCTCTAAAATTTT P-TGAATCCTAGTCTTTTCTCTGCG	25 (modified)
31	<i>wzy</i>	F-GCAGAAGTTTAAAGTCACGGAC R-AGCATTACAGATGTCACTAAGGG P-CCCCACGTAAAACCGCAAGG	22
33AF-37	<i>wzy</i>	F-GGAACTGGTTCAGCAACTATACG R-GGTTCTAAGACCGTCTGAAATACC P-TAGGACTTTTCTGCCATGCC	16 (modified)
33B	<i>wciN</i>	F-CCTGTTAGTGCACCTGTATTTAAC R-GCATTCAAAACTCCTTCATCTCC P-TTCGTTGTTACAGCCATTTA	22 (modified)

(Continued on following page)

TABLE 1 (Continued)

Serotype or sample type	Target	Sequence (5'–3') ^a	Reference(s) or source
33D*	<i>wciN</i>	F-CGTATAGTCTTGCGACATTTCA R-TTCCACATGCGTTACCTCAC P-CACAACTAGTTTTTTATCAAAAAGACCTTGGC	22 (modified)
34	<i>wzy</i>	F-CGGTGGAGTAGGTCAAGATG R-GTCTGTTCTCCCCAATACTGAG P-ACGGAGCGCCAATGTACTTGAATAGTT	22
35AC-42	<i>wcrK</i>	F-TGTTTCAAGCTTCCCTTTAGA R-AAATGAAATCAAAGTATCACGTATCG P-TTCAAAATACCCAGGACACCCGTTCA	This study 22 (modified) 22
35B	<i>wcrJ</i>	F-GCATGGAGGTGGAGCATACA R-TGTAAAGACTGCACAACCTCGATATAAAA P-AACAATATTAGTAAAGCGCAGGTC	22, 24 (modified)
35F-47F	<i>wzy</i>	F-GTGGTCTATATACTTGATGAATAAATCG R-ACATACAAATTATCAACATACAGATAGGTC P-TTCAACTGGTCGTCGGAATA	22 (modified)
36*	<i>wzy</i>	F-CTTGCTATTTCAGCCCTTCTGG R-CGCGATTATATTGTAAATTGGGAACT P-AGAATGCCCGCTACAATGAG	22 (modified)
38-25AF	<i>wciI</i>	F-GTCTTACGTAGAACCTCTCTGGATGA R-TGGTCTCTACAAGCGACATGTG P-TTGCCACAGATTTGGAATATTTTGGTCGG	22, 24
39*	<i>wcrG</i>	F-CAAAAAAATGAACCTAACTCAAATAGTAACG R-ATACTGTAATTTTCTTGTTTATTTGCGG P-AAGTCAGGCGTATTCTTCACAAGGGAAA	22
41A	<i>wciB</i>	F-GCAAATAGATGTATCCCAGTTAACAC R-GGTAGCTCTTTTGGTTAATGTCC P-CGACCGAATAGTCTAGCTTCAAAGG	22 (modified)
41F	<i>wzx</i>	F-TTTTTGGGAGGAAGTGCTTTT R-AACCGCTTTCTCATGATTCATAACT P-CTTCTGTGCTAACAGTGGAGAT	22 This study 22 (modified)
43	<i>wzx</i>	F-AGAGGCTACATCAAATAGTTGGC R-GAATCACACCGTAACCTCCAAAG P-TCCAATAGTACTCACCCCTACCGAGC	22
45*	<i>wzy</i>	F-TCTAGCTACTTGACTAAAAATTGAACTG R-GACGAGTCGATTTGCTGTAT P-CTTTTAGTGACCTCGCTCCC	22 (modified)
47AF*	<i>whaI</i>	F-AGGAATTGGTAGAGAGTTGTGG R-GAAAGTTGAACCATCATCCGTC P-CACTTGATGGAATGCCTGCTGCC	22
<i>lytA</i>	<i>lytA</i>	F-TCGTGCGTTTTAATTCCAGCT R-ACGCAATCTAGCAGATGAAGCA P-CTCCCTGTATCAAGCGTTTTTCGGCA	26 (modified)
PhHV	<i>gB</i>	F-GGGCGAATCACAGATTGAATC R-GCGGTTCCAAACGTACCAA P-TATGTGTCCGCCACCATCT	27

^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.

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 simplex 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.

Discrepancies were observed only with 22F strains. Curiously, *wcwA* sequences, according to GenBank accession numbers [CR931681.1](#) and [CR931682.1](#), are identical between 22A and 22F in the primer region, yet the 22F strains did not amplify, while 22A did, suggesting that there is a disconnect between the available 22F GenBank sequence and these four strains (see Fig. 2 in the supplemental material). Future iterations of the assays can attempt to understand and improve this. This caveat aside, the assay is clearly robust for use on cultured isolates from invasive sites and should provide an important tool to document whether serotype replacement is occurring in invasive strains (28).

		Port	
24. Serotype 18ABCF	48. <i>phHV</i>	24	48
23. Serotype 17F	47. <i>phHV</i>	23	47
22. Serotype 16F	46. Serotype 43/45*	22	46
21. Serotype 15	45. Serotype 41F	21	45
20. Serotype 14	44. Serotype 41A/47AF*	20	44
19. Serotype 13/21*	43. Serotype 38-25AF/39*	19	43
18. Serotype 12B	42. Serotype 35F-47F	18	42
17. Serotype 12ABF-44-46	41. Serotype 35B	17	41
16. Serotype 11AD	40. Serotype 35AC-42	16	40
15. Serotype 10B/11F*	39. Serotype 34/36*	15	39
14. Serotype 10A	38. Serotype 33B	14	38
13. Serotype 9LN	37. Serotype 33AF-37	13	37
12. Serotype 9AV	36. Serotype 31/33D*	12	36
11. Manufacturing control	35. <i>lytA</i>	11	35
10. Serotype 8	34. Serotype 27/29*	10	34
9. Serotype 7BC-40	33. Serotype 24A/25AF*	9	33
8. Serotype 7AF	32. Serotype 23F	8	32
7. Serotype 6CD	31. Serotype 23B	7	31
6. Serotype 6ABCD	30. Serotype 23A	6	30
5. Serotype 5	29. Serotype 22AF	5	29
4. Serotype 4	28. Serotype 20	4	28
3. Serotype 3	27. Serotype 19"F"	3	27
2. Serotype 2	26. Serotype 19F	2	26
1. Serotype 1	25. Serotype 19A	1	25

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.

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 *lytA*-positive specimens. A few samples had low levels of DNA at the *lytA* or serotype level that could be rescued with larger amounts of DNA. Thus, the serotype result was 100% accurate for any *lytA* result of a C_T of 34 or below (corresponding to a nasopharyngeal density of 8×10^3 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

TABLE 2 PCR performance of each serotype/serogroup-specific assay

Serotype or sample	384-well plate		TaqMan array card	
	Linearity (R^2) ^a	LOD (fg) ^b	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)	0.998 (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)	0.983 (102.6)	20 (8.4)
10B	1.000 (94.4)	10 (4.2)	0.996 (108.1)	20 (8.4)
11AD	1.000 (92.4)	10 (4.2)	0.965 (86.5)	20 (8.4)
11F	0.994 (84.8)	10 (4.2)	0.943 (120.2)	20 (8.4)
12ABF-44-46	0.999 (99.2)	10 (4.2)	0.997 (102.3)	20 (8.4)
12B	1.000 (91.5)	100 (42)	0.961 (76.1)	200 (84)
13	0.999 (90.9)	100 (42)	0.861 (94.8)	200 (84)
14	0.999 (95.7)	10 (4.2)	0.994 (95.7)	20 (8.4)
15	1.000 (93.7)	10 (4.2)	0.994 (102.6)	20 (8.4)
16F	0.998 (86.9)	100 (42)	0.979 (92.5)	200 (84)
17F	0.998 (88.5)	10 (4.2)	0.996 (103.3)	20 (8.4)
18ABCF	0.999 (91.0)	10 (4.2)	0.998 (78.4)	20 (8.4)
19A	1.000 (92.8)	10 (4.2)	0.994 (99.5)	20 (8.4)
19F	0.999 (99.3)	10 (4.2)	0.998 (95.0)	20 (8.4)
19"F"	1.000 (94.4)	10 (4.2)	0.993 (94.4)	20 (8.4)
20	0.998 (95.3)	10 (4.2)	0.996 (90.6)	20 (8.4)
21	0.999 (93.4)	10 (4.2)	0.993 (98.2)	20 (8.4)
22AF	0.998 (100)	10 (4.2)	0.999 (103.5)	20 (8.4)
23A	0.999 (89.3)	10 (4.2)	0.995 (92.8)	20 (8.4)
23B	0.998 (103)	10 (4.2)	0.992 (103.7)	20 (8.4)
23F	0.996 (88.5)	10 (4.2)	0.998 (93.6)	20 (8.4)
24A	0.998 (100)	10 (4.2)	0.973 (82.8)	20 (8.4)
25AF	0.997 (88.5)	10 (4.2)	0.997 (103.6)	20 (8.4)
27	0.998 (89.9)	10 (4.2)	0.996 (106.7)	20 (8.4)
29	0.992 (85.5)	10 (4.2)	0.997 (70.0)	20 (8.4)
31	0.999 (93.1)	10 (4.2)	0.996 (89.7)	20 (8.4)
33AF-37	1.000 (90.7)	10 (4.2)	0.998 (101.3)	20 (8.4)
33B	0.993 (97.8)	10 (4.2)	0.996 (98.9)	20 (8.4)
33D	1.000 (96.5)	10 (4.2)	0.985 (95.1)	20 (8.4)
34	0.996 (92.6)	100 (42)	1.000 (103.4)	200 (84)
35AC-42	0.997 (105)	10 (4.2)	0.996 (103.8)	20 (8.4)
35B	0.998 (94.0)	10 (4.2)	0.979 (100.2)	20 (8.4)
35F-47F	0.997 (90.1)	10 (4.2)	0.996 (94.3)	20 (8.4)
36	0.999 (88.7)	10 (4.2)	0.997 (103.1)	20 (8.4)
38-25AF	0.999 (96.8)	10 (4.2)	1.000 (94.3)	20 (8.4)
39	0.999 (96.4)	10 (4.2)	0.986 (108.8)	20 (8.4)
41A	1.000 (91.7)	10 (4.2)	0.994 (97.9)	20 (8.4)
41F	1.000 (79.0)	100 (42)	0.997 (105.7)	200 (84)
43	0.998 (95.4)	10 (4.2)	0.929 (96.8)	20 (8.4)
45	1.000 (94.7)	10 (4.2)	0.998 (95.0)	20 (8.4)
47AF	1.000 (93.4)	100 (42)	0.995 (104.3)	200 (84)
<i>lytA</i>	0.998 (94.8)	10 (4.2)	0.999 (97.9)	20 (8.4)
<i>PhHV</i>	0.994 (94.7)		0.986 (114.9)	

^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 *S. pneumoniae* serotype 4 TIGR4 (2,160,842 bp) was used for calculations.

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				
All serotypes from Table 2	54	54	0	100
Blinded ^a				
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 ^b	0
23F	4	4	0	100
33F	3	3	0	100
Serotypes not included in Table 2	10	10 ^b	0	100
Nonpneumococcal bacteria	13	13 ^c	0	100
Total	159	155	4	97

^a Serotypes in bold are those included in PCV13.
^b Positive with *lytA* but negative with any serotype-specific probe on the TAC.
^c Negative with any probe on the TAC, including *lytA*.

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 materials are much easier to obtain than invasive isolates, particularly in children and in resource-limited settings where the burden is highest. It has been proposed that the absence of a vaccine type in 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 specimen testing 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). Some serotypes could not be identified individually by single assay

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

TaqMan array card				
Quellung serotype or culture result ^a	<i>lytA</i> C _T	Serotype result(s) (C _T)	DNA (copies/reaction)	Predicted bacterial load from <i>lytA</i> C _T (CFU/ml)
4	27	4 (29), 23B (30)	9.33E1	9.33E5
4	28	4 (28)	4.70E1	4.70E5
6A	27	6ABCD (29)	6.77E1	6.77E5
6A	Negative	Negative	NA ^b	NA
10A	31	10A (33)	6.49E0	6.49E4
11A	23	11AD (23)	4.96E2	4.96E6
11A	28	11AD (28)	5.94E1	5.94E5
11A	32	11AD (35)	4.16E0	4.16E4
11A	34	11AD (34)	8.59E−1	8.59E3
17F	30	17F (30)	1.32E1	1.32E5
17F	33	17F (36)	1.66E0	1.66E4
19A	29	19A (31), 6CD (33)	1.79E1	1.79E5
19A	28	19A (30)	3.71E1	3.71E5
19F	29	19F (29)	2.85E1	2.85E5
19F	28	19“F” (30)	4.20E1	4.20E5
19F	30	19F (30)	1.41E1	1.41E5
19F	28	19F (28)	5.04E1	5.04E5
19F	35	Negative	3.86E−1	3.86E3
23F	28	23F (31)	3.63E1	3.63E5
23F	27	23F (28)	7.45E1	7.45E5
23F	Negative	Negative	NA	NA
35F	35	Negative	3.16E−1	3.16E3
NT	27	4 (30)	3.08E1	3.08E5
Pneumococcal culture negative				
With positive TAC result (n = 1)	34	Negative	9.62E−1	9.62E3
With negative TAC result (n = 4)	Negative	Negative	NA	NA

^a The Quellung reaction was performed with pure culture colonies from the same nasopharyngeal samples. NT, not typeable.
^b NA, not applicable.

sets. For example, to infer serotype 6AB we must detect serotypes 6ABCD 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 cost up to \$100 per colony (12). In conclusion, the

TaqMan array card is a fast, high-throughput, serotyping method for pneumococci that is suitable to field studies.

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REFERENCES

- Bogaert D, De Groot R, 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](http://dx.doi.org/10.1016/S1473-3099(04)00938-7).
- Klugman KP, Madhi SA, Huebner RE, Kohberger R, Mbelle N, Pierce N, Vaccine Trialists 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>.
- 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/10.1097/00006454-200209000-00005>.
- Cutts FT, Zaman SM, Enwere G, Jaffar S, Levine OS, Okoko JB, Oluwalana C, Vaughan A, Obaro SK, Leach A, McAdam KP, Biney E, Saaka M, Onwuchekwa U, Yallop F, Pierce NF, Greenwood BM, Adegbola RA, Gambian Pneumococcal Vaccine Trial Group. 2005. Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. *Lancet* 365:1139–1146. [http://dx.doi.org/10.1016/S0140-6736\(05\)71876-6](http://dx.doi.org/10.1016/S0140-6736(05)71876-6).
- Olarte L, Barson WJ, Barson RM, Lin PL, Romero JR, Tan TL, Givner LB, Bradley JS, Hoffman JA, Hulten KG, Mason EO, Kaplan SL. 2015. Impact of the 13-valent pneumococcal conjugate vaccine on pneumococcal meningitis in US children. *Clin Infect Dis* 61:767–775. <http://dx.doi.org/10.1093/cid/civ368>.
- Weinberger DM, Malley R, Lipsitch M. 2011. Serotype replacement in disease after pneumococcal vaccination. *Lancet* 378:1962–1973. [http://dx.doi.org/10.1016/S0140-6736\(10\)62225-8](http://dx.doi.org/10.1016/S0140-6736(10)62225-8).
- Gaviria-Agudelo CL, Jordan-Villegas A, Garcia C, McCracken GH, Jr. 22 February 2016. The effect of 13-valent pneumococcal conjugate vaccine on the serotype distribution and antibiotic resistance profiles in children with invasive pneumococcal disease. *J Pediatr Infect Dis Soc* <http://dx.doi.org/10.1093/jpids/piw005>.
- Weinberger DM, Bruden DT, Grant LR, Lipsitch M, O'Brien KL, Pelton SI, Sanders EA, Feikin DR. 2013. Using pneumococcal carriage data to monitor postvaccination changes in invasive disease. *Am J Epidemiol* 178:1488–1495. <http://dx.doi.org/10.1093/aje/kwt156>.
- Habib M, Porter BD, Satzke C. 2014. Capsular serotyping of *Streptococcus pneumoniae* using the Quellung reaction. *J Vis Exp* 84:e51208. <http://dx.doi.org/10.3791/51208>.
- Hanke CR, Grijalva CG, Chochua S, Pletz MW, Hornberg C, Edwards KM, Griffin MR, Verastegui H, Gil AI, Lanata CF, Klugman KP, Vidal JE. 2016. Bacterial density, serotype distribution and antibiotic resistance of pneumococcal strains from the nasopharynx of Peruvian children before and after pneumococcal conjugate vaccine 7. *Pediatr Infect Dis J* 35:432–439. <http://dx.doi.org/10.1097/INF.0000000000001030>.
- Turner P, Hinds J, Turner C, Jankhot A, Gould K, Bentley SD, Nosten F, Goldblatt D. 2011. Improved detection of nasopharyngeal colonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. *J Clin Microbiol* 49:1784–1789. <http://dx.doi.org/10.1128/JCM.00157-11>.
- Huebner RE, Dagan R, Porath N, Wasas AD, Klugman KP. 2000. Lack of utility of serotyping multiple colonies for detection of simultaneous nasopharyngeal carriage of different pneumococcal serotypes. *Pediatr Infect Dis J* 19:1017–1020. <http://dx.doi.org/10.1097/00006454-200010000-00019>.
- Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabinowitsch E, Collins M, Donohoe K, Harris D, Murphy L, Quail MA, Samuel G, Skovsted IC, Kalltoft MS, Barrell B, Reeves PR, Parkhill J, Spratt BG. 2006. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS Genet* 2:e31. <http://dx.doi.org/10.1371/journal.pgen.0020031>.
- Elberse KE, van de Pol I, Witteveen S, van der Heide HG, Schot CS, van Dijk A, van der Ende A, Schouls LM. 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:e20390. <http://dx.doi.org/10.1371/journal.pone.0020390>.
- Leung MH, Bryson K, Freystatter K, Pichon B, Edwards G, Charalambous 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>.
- 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>.
- Dube FS, van Mens SP, Robberts L, Wolter N, Nicol P, Mafoko J, Africa S, Zar HJ, Nicol MP. 2015. Comparison of a real-time multiplex PCR and sequotyping assay for pneumococcal serotyping. *PLoS One* 10:e0137349. <http://dx.doi.org/10.1371/journal.pone.0137349>.
- 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>.
- Satzke C, Dunne EM, Porter BD, Klugman KP, Mulholland EK, PneumCarriage 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>.
- Dhoubhadel BG, Yasunami M, Yoshida LM, Thi HA, Thi TH, 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 nanofluidic real-time PCR system. *J Med Microbiol* 63:528–539. <http://dx.doi.org/10.1099/jmm.0.071464-0>.
- 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>.
- Sakai F, Chochua 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>.
- 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:e67147. <http://dx.doi.org/10.1371/journal.pone.0067147>.
- 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 multiplex 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>.
- Azzari C, Moriondo M, Cortimiglia M, Valleriani C, Canessa C, Indolfi G, Ricci S, Nieddu F, de Martino M, Resti M. 2012. Potential serotype coverage 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>.
- Carvalho Mda G, Tondella ML, McCaustland 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*, *ply*, and *psaA* genes for detection of pneu-

- 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, Puhon 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 L, 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 DJ, Taniuchi M, Janaki L, Platts-Mills JA, Haverstick DM, Kabir M, Sobuz SU, Nakjarung K, Sakpaisal P, Silapong S, Bodhidatta L, 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](http://dx.doi.org/10.1016/S1473-3099(14)70808-4).