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PCR-Based Serotyping of *Streptococcus pneumoniae* from Culture-Negative Specimens: Novel Primers for Detection of Serotypes within Serogroup 18

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Six multiplex-compatible PCR primers were designed to distinguish *Streptococcus pneumoniae* serotypes within serogroup 18 from culturable/nonculturable pneumococcal specimens, with no cross-reactivity with other serotypes and respiratory organisms. These primers will aid in the generation of better data on vaccine/nonvaccine serotypes in invasive and carriage pneumococcal surveillance and contribute to future vaccine formulation and impact studies.

Streptococcus pneumoniae is a major cause of infectious disease burden worldwide, especially in children (1). It has a highly diverse polysaccharide capsule that forms the basis of more than 90 different serotypes, whose distribution varies geographically (2, 3). The available pneumococcal conjugate vaccines (PCVs) were designed to provide immunity against the most prevalent invasive serotypes worldwide (3, 4). Understanding the geographical distribution and shifts in prevalence over time of every serotype is important for optimizing vaccine design and understanding post-vaccine impact on disease burden.

The current gold standard for serotyping is the Quellung reaction (5). This method is expensive and demands expertise; most importantly, this method cannot discern serotypes in culture-negative specimens (6). This limitation has serious implications in South Asia and Africa, where more than 50% of all meningitis cases are culture negative due to the common practice of antibiotic use prior to seeking care and specimen collection (7, 8). To overcome these limitations, a PCR-based serotyping scheme was optimized to determine serotypes from culture-negative specimens (6). However, the available conventional primers cannot distinguish serotypes within some serogroups, like 18 and 6. Few quantitative PCR (qPCR) schemes are available (9, 10), but they lack completeness (can detect 18B/C only) and were not validated completely due to a lack of culture-negative serogroup-18-positive clinical specimens. This limits our knowledge about serotype distribution and vaccine coverage. This is specifically true for serogroup 18, one of the predominant invasive serogroups worldwide (7, 11–13), which has four different serotypes 18A, 18B, 18C, and 18F. Only serotype 18C has been included in all PCVs (14–16). Distribution data of these serotypes are lacking for all culture-negative cases. Moreover, PCR serotyping directly from respiratory specimens has received attention recently (17–19). Considering the nasopharynx as the key reservoir for transmission (20), the trend of pneumococcus in carriage is crucial for surveying herd immunity and replacement of vaccine serotypes after PCV introduction (21, 22). However, the current PCR serotyping algorithm remains incomplete, as it is unable to detect serotypes within serogroup 18 (23). Although previous attempts to design serotype-specific primers for serogroup 18 were unsuccessful due to the high sequence similarity (23, 24), in this study, we designed new primers to identify and distinguish the serogroup 18 serotypes.

The pneumococcal capsular genes on the capsule polysaccharide (*cps*) locus are flanked by the conserved *dexB* and *aliA* genes (25). The first four genes are highly conserved among all serotypes, but genes in the central part of the locus are more serotype specific and serve as the basis of differentiation between serotypes (23). We designed PCR primers to distinguish serogroup 18 serotypes through manual analysis of a multiple-sequence alignment of all four published *cps* locus sequences (26).

For each selected region of the locus, blastn was used to check its specificity. Once a region was validated to be specific for the serogroup 18 serotypes (i.e., no significant match was found with *cps* loci of other serotypes), primers were designed and checked for physical properties (melting temperature [T_m], G+C% content, hairpins, and dimers) and multiplex PCR compatibility using OligoAnalyzer version 3.1 (Integrated DNA Technologies, USA). Overall, six primers were designed to differentiate between the serogroup 18 serotypes 18A, 18B/C, and 18F (Fig. 1). Primers to distinguish 18B and 18C could not be designed using their CPS sequence due to high (99.99% [21,817/21,819]) sequence identity.

These new primers were evaluated for cross-reactivity with pneumococcal isolates of other serotypes confirmed through Quellung reactions. A library of 167 DNA samples from pneumococcal isolates (pneumococcal DNA library), obtained from invasive and carriage sources, was used to validate these primers. This

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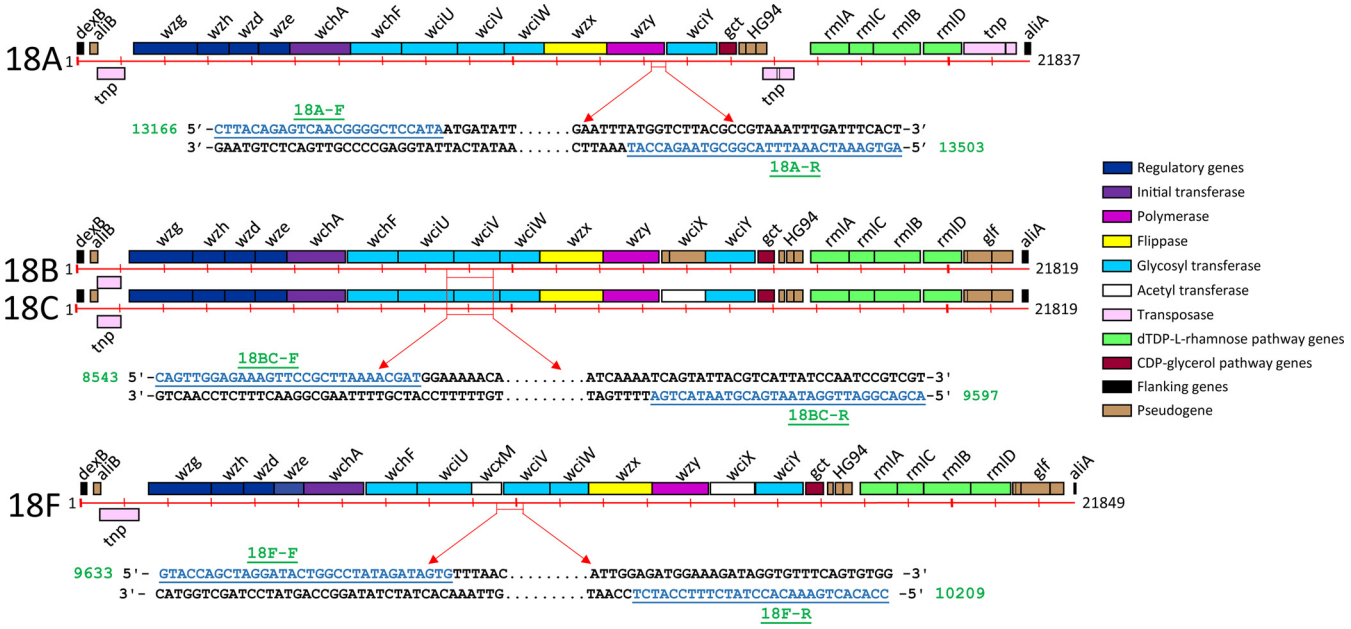

FIG 1 New primers to detect serogroup 18 serotypes (18A, 18B/C, and 18F) and their positions in the *cps* locus. The schematic of the serogroup 18 serotype *cps* locus and organization of the genes with color keys has been adapted from the study by Bentley et al. (26). Genes are represented by boxes and colored according to the gene key, with gene designations indicated above each box. The *cps* loci of 18B and 18C are shown together, as their primers (18BC-F and 18BC-R) were common due to very high sequence identity. The primers used in this study are shown along with their position in the locus (with arrows). Primer sequences are colored and underlined with their designation shown above/below; the genomic position in the *cps* locus is presented beside the primer.

library contained 67 different serotypes, and 42 isolates belonged to serogroup 18 (11 from 18A, 22 from 18C, and nine from 18F). Further, cross-reaction with other microbial species found in the same niche as *S. pneumoniae* was appraised using 79 DNA specimens isolated from nasopharyngeal (NP) swabs containing multiple species of bacteria (NP-DNA library). Fifty-four of 79 specimens contained *S. pneumoniae* strains of 25 different serotypes (one 18A, four 18C, and one 18F), determined by Quellung reactions, along with other bacterial species. Finally, to verify the compatibility with DNA from culture-negative clinical specimens, all new primers were used with 10 culture-negative but serogroup-18-positive clinical specimens (cerebrospinal fluid, $n = 9$; ascitic fluid, $n = 1$); those were confirmed using published sequential multiplex PCR (culture-negative library) (6). Additional information on all DNA samples used here is found in File S1 in the supplemental material.

For 167 pneumococcal isolates, DNA extraction was performed by the boiling method (6); for culture-negative and NP swab specimens, the QIAamp DNA minikit (Qiagen, Germany) was used. For multiplex PCR, two 25-μl reaction mixtures were made, (i) one for the pneumococcal DNA library using 5 μl of FIREPol mastermix (Solis BioDyne, Estonia) and 1 μl of boiled DNA lysate and (ii) one for NP swabs and culture-negative library DNA using 12.5 μl of Qiagen multiplex PCR mastermix (Qiagen, Germany) and 8 μl of DNA. Both reaction mixtures contained 0.4 μM each primer (Eurofins Genomics, USA). Primers (0.4 μM) targeting the *cpsA* locus (23) were added to the mixtures as a positive control. A water-only control was always included. The thermal cycle was 95°C for 15 min, followed by 35 cycles (37 in the case of the culture-negative library to address the challenge of low DNA concentration) of 94°C for 40 s, 61°C for 50 s, and 72°C for 60 s, and then at 72°C for 10 min and held at 4°C. The PCR products were run on a 2% agarose gel.

The multiplex PCR on the pneumococcal isolate library with 167 specimens detected all designated isolates of the serogroup 18 serotypes (see Fig. S2 and File S1 in the supplemental material), indicating 100% concordance with the Quellung results. No cross-reaction within and beyond serogroup 18 was observed. All isolates showed positive results for *cpsA* except serogroups 25 and 38, which has been described before (27, 28). For the NP swab library, our multiplex PCR assay was positive only for the pneumococcus-positive NP specimens with specific serotypes 18A/C/F, with no cross-reaction with nonpneumococcal bacterial growth (see Fig. S3 and File S1 in the supplemental material). Most excitingly, our primers amplified DNA in all 10 samples of the culture-negative library, and they revealed 18C in all nine cerebrospinal fluid (CSF) specimens and 18A in the ascitic fluid specimen. Figure 2 shows the PCR products for four samples (additional data in File S1). All reactions showed amplification for *cpsA*, and no dimers with human DNA were seen.

Overall, our results indicate that serotypes within serogroup 18 can be discerned by using the primers described herein, from both culture-positive and culture-negative, invasive and carriage specimens, without any cross-reactivity to non-serogroup-18 serotypes, nonpneumococcal respiratory bacteria, or human DNA. However, they could not be validated with serotype 18B, as none of our DNA libraries from isolates and carriage specimens contained this serotype, implying that our surveillance of multiple modalities did not detect any 18B isolates in the last 2 decades (7, 13, 29, 30). Interestingly, serotype 18B has also not been reported from other countries in this region (31–34). Therefore, we can presume that all 18B/C-positive cases (by our primers) are 18C

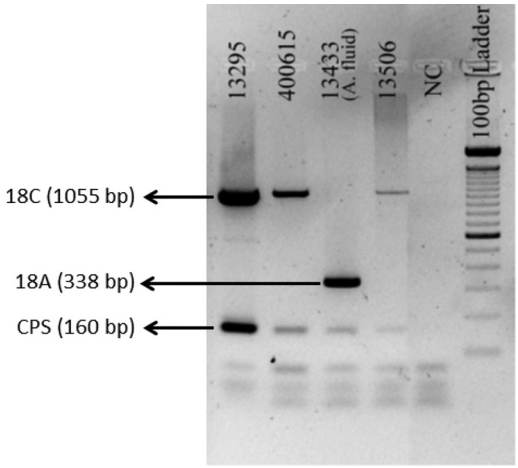

FIG 2 Primer validation with four culture-negative but serogroup-18-positive clinical specimen DNA. Amplified products of multiplex PCR with 18A, 18B/C, and 18F primers, run on 2% agarose gel (at 100 V for 50 min) showed the desired band for 18C (1,055 bp) on all three CSF samples and 18A (338 bp) on the ascitic fluid (A. fluid) specimen. A 100-bp ladder was included in the gel to determine the PCR band size. The gel was stained with SYBR Safe (Invitrogen, USA) and visualized using Gel Doc UV transilluminator (Bio-Rad, USA).

but simultaneously remain vigilant about the isolation of 18B in this region in the postvaccine era. Recently, an Indian industry, with support from the Bill & Melinda Gates Foundation (BMGF), formulated a PCV without 18C (Keith Klugman, BMGF, personal communication). This is possibly due to the limitation in detecting 18C from culture-negative cases. Therefore, it will be also important to monitor the trend of 18C, using this primer set, in India once the new vaccine is introduced.

In Bangladesh, between 2007 and 2014, serogroup 18 ranked 6th (28/442) among all invasive isolates (7). Quellung-based classification of isolates revealed that 46% of all invasive serogroup 18 strains (13/28) are nonvaccine types 18A and 18F, suggesting the possibility of their emergence as the dominant serotypes post-PCV. Moreover, in recent years, 45% of all serogroup 18 meningitis cases were culture negative, similar to results for other serotypes (7). The lack of isolates from these cases has prevented us from determining the prevalence of specific serotypes and hence limited assessments of the effectiveness of PCVs. The new primers described here will be of paramount significance for comprehensive surveillance on invasive and carriage pneumococcus and PCV effectiveness studies, specifically in South Asian countries, where disease burden is high and prior use of antibiotics is common.

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A.M.T. designed the primers; A.M.T. and S.K.S. designed the study; A.M.T., S.S., G.L.D., C.G.W., and S.K.S. interpreted the data; A.M.T., S.S., and S.K.S. wrote the manuscript; A.M.T. and S.S. constructed the figures and tables and G.L.D., C.G.W., and S.K.S. reviewed the manuscript.

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