Refining PCR-based serotyping for detection of vaccine-preventable Streptococcus pneumoniae

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ABSTRACT

Conventional multiplex PCR (cmPCR) reactions have been developed to monitor the most predominant serotypes of Streptococcus pneumoniae causing invasive pneumococcal disease (IPD). Since cmPCR assigns serotypes based on differences in the capsule biosynthesis (cps) loci, DNA extracted from clinical specimens can be used directly to monitor changes in serotype distribution and assess the impact of pneumococcal vaccines. Given that cmPCR can require up to eight reactions to assign a serotype, testing is often conducted in sequential algorithms. Sequential cmPCR reactions; however, may not be the most cost effective strategy to determine whether a S. pneumoniae serotype is vaccine-preventable. This study used oligonucleotide permutations in a modified set of cmPCR reactions (termed cmPCRmod) to reduce the number of PCR reactions required to identify S. pneumoniae serotypes covered by the 7- and 13-valent pneumococcal conjugate vaccines (PCV7 and PCV13, respectively) and the 23-valent pneumococcal polysaccharide vaccine (PPV23). While oligonucleotide permutations have previously been reported for regional differences in serotype distribution, the impact on assay performance had not been assessed. This study demonstrated that equivalent analytical sensitivity and specificity was seen when comparing cmPCR and cmPCRmod, and 100% concordance was seen when 308 clinical isolates of S. pneumoniae were evaluated. Compared to cmPCR, cmPCRmod reduced the number and reactions required to detect serotypes covered by PCV7, PCV13, and PPV23. This study demonstrated that conventional multiplex reactions can be reformulated for more efficient detection of vaccine-preventable serotypes, without compromising test performance characteristics. As such, cmPCRmod reactions could provide significant cost savings for large surveillance studies.

Key Words: Streptococcus pneumoniae, PCR, Serotyping, Vaccine, Multiplex

1. INTRODUCTION

Streptococcus pneumoniae (or pneumococcus) is a bacterium that normally colonizes the human naso- and oropharynx but can also cause a spectrum of pneumococcal disease including community acquired pneumonia (CAP) and invasive pneumococcal diseases (IPD) such as meningitis and bacteremia.¹⁻⁴ Both CAP and IPD are major causes of morbidity and mortality, and pose a significant burden on our healthcare system.¹⁻⁵ Pneumococcal diseases are responsible for approximately 1.6 million deaths worldwide each year, with incidence rates greatest in children, the elderly, or individuals with risk factors.⁶

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Childhood immunization programs have played an important role in reducing the burden of pneumococcal disease. Prior to introduction of the 7-valent pneumococcal conjugate vaccine (PCV7), most infections caused by *S. pneumoniae* were attributed to serotypes covered by this vaccine.[7–9] While diseases caused by PCV7-serotypes have declined over the years, other serotypes have become predominant. This led to the use of the 13-valent pneumococcal conjugate vaccine (PCV13) in childhood immunization programs.[10] Following the Community Acquired Pneumonia Trial In Adults (CAPITA),[11] recommendations were made for use of PCV13 in adults aged ≥65 years.[12–14] Both PCV13 and the 23-valent pneumococcal polysaccharide vaccines (PPV23) are also recommended for individuals at risk for pneumococcal disease.[12, 13] With the changing epidemiology of pneumococcal disease worldwide, monitoring the serotype distribution of *S. pneumoniae* is crucial to assess the impact of pneumococcal vaccines and help make informed recommendations for their use.

To date, over 90 different *S. pneumoniae* serotypes have been identified using traditional Quellung serotyping, a microscopic method that classifies pneumococci based on capsule-specific antisera.[15] More recently, molecular methods for serotype deduction like conventional multiplex PCRs (cmPCR) have been developed, and are widely used from DNA extracted from *S. pneumoniae* isolates or clinical specimens.[16–28] Since cmPCR have a limited number of serotypes in each PCR reaction, they have been designed to target the most prevalent serotypes causing IPD, often in sequential reactions.[21–26] However, sequential PCR may not be the most cost effective strategy to identify *S. pneumoniae*.

### Table 1. Serotypes detected by the traditional and modified cmPCR reactions

| Vaccine Coverage | Reaction | Serotype (Expected size in bp) |
|------------------|----------|-------------------------------|
| **PCV7**         |          |                               |
| 6B               | cmPCR 1  | 3A/6B/6C/6D                   |
|                  |          | 3 (371)                       |
|                  |          | 19A (566)                     |
|                  |          | 22F/22A (643)                 |
|                  |          | 16F (988)                     |
|                  | cmPCR 2  | 8 (201)                       |
|                  |          | 33F/33A/37 (338)              |
|                  |          | 15A/15F (434)                 |
|                  |          | 23A (722)                     |
|                  |          | 7F/7A (826)                   |
|                  | cmPCR 3  | 19F (304)                     |
|                  |          | 12F/12A/12B/44/46 (376)       |
|                  |          | 11A/11D (463)                 |
|                  |          | 38/25F/25A (574)              |
|                  |          | 35B (677)                     |
|                  | cmPCR 4  | 24A/24B/24F (99)              |
|                  |          | 7C/7B/40 (260)                |
|                  |          | 4 (430)                       |
|                  |          | 18C/18F/18B/18A/573 (816)     |
|                  | cmPCR 5  | 14 (189)                      |
|                  |          | 1 (280)                       |
|                  |          | 23F (384)                     |
|                  |          | 15B/15C (496)                 |
|                  |          | 10A (628)                     |
|                  | cmPCR 6  | 39 (98)                       |
|                  |          | cpsA (160)                    |
|                  |          | 5 (362)                       |
|                  |          | 35F/47F (517)                 |
|                  |          | 17F (693)                     |
|                  | cmPCR 7  | 23B (190)                     |
|                  |          | 35A/35C/42 (280)              |
|                  |          | 34 (408)                      |
|                  |          | 9N/9L (516)                   |
|                  |          | 31 (701)                      |
|                  | cmPCR 8  | 21 (192)                      |
|                  |          | 2 (290)                       |
|                  |          | 20 (514)                      |
|                  |          | 13 (655)                      |
|                  | cmPCR 6CD* | 14 (189)                    |
|                  |          | 19F (304)                     |
|                  |          | 3 (371)                       |
|                  |          | 19A (566)                     |
|                  |          | 7F/7A (826)                   |
|                  | cmPCRmod A | 6A/6B/6C/6D                   |
|                  |          | (250)                         |
|                  |          | 6C/6D (727)                   |
|                  | cmPCRmod B | 6A/6B/6C/6D                   |
|                  |          | (250)                         |
|                  |          | 23F (384)                     |
|                  |          | 4 (430)                       |
|                  |          | 18C/18F/18B/18A/573 (816)     |
|                  | cmPCRmod C | 8 (201)                      |
|                  |          | 33F/33A/37 (338)              |
|                  |          | 11A/11D (463)                 |
|                  |          | 9N/9L (516)                   |
|                  |          | 10A (628)                     |
|                  | cmPCRmod D | 23B (190)                    |
|                  |          | 2 (290)                       |
|                  |          | 12F/12A/12B/44/46 (376)       |
|                  |          | 20 (514)                      |
|                  |          | 17F (693)                     |
|                  | cmPCRmod F | 7C/7B/40 (260)               |
|                  |          | 15A/15F (434)                 |
|                  |          | 13 (655)                      |
|                  |          | 23A (722)                     |
|                  |          | 16F (988)                     |
|                  | cmPCRmod G | 24A/24B/24F (99)             |
|                  |          | cpsA (160)                    |
|                  |          | 34 (408)                      |
|                  |          | 35F/47F (517)                 |
|                  |          | 35B (677)                     |
|                  | cmPCRmod H | 39 (98)                     |
|                  |          | cpsA (160)                    |
|                  |          | 21 (192)                      |
|                  |          | 35A/35C/42 (280)              |

*When a positive reaction is obtained in cmPCR1 for serotype 6A/6B/6C/6D, and additional reaction is performed to distinguish 6A/6B for 6C/D using cmPCR 6CD. In the modified reactions, serotype 6A/6B/6C/6D is found in cmPCRmodB and the 6CD reaction is incorporated into cmPCRmod C.*
Serotypes that are vaccine-preventable. This study used oligonucleotide permutations in a modified set of cmPCR reactions (termed cmPCRmod) to reduce the amount of testing required to identify S. pneumoniae serotypes covered by the PCV7, PCV13, or PPV23 vaccines. By redistributing the primer pairs for vaccine-preventable serotypes, the number of cmPCRmod reactions required to span the coverage of PCV7, PCV13, and PPV23 was 2, 3, and 5, compared to the traditional cmPCR which require 4, 6, and 8 reactions, respectively (see Table 1).

2. METHODS

2.1 S. pneumoniae source and culture

For the specificity analysis, each PCR-based serotyping method was tested against a panel of pre-characterized S. pneumoniae isolates and non-pneumococcal streptococci that were obtained from one of five sources: the American Type Culture Collection (ATCC), the Centers for Disease Control and Prevention Global Pneumococcal Strain Bank (http://www.cdc.gov/streplab/global-pneum-o-strain-bank.html), the National Microbiology Laboratory (NML) in Winnipeg MB, Canada, the Serious Outcomes Surveillance (SOS) Network of the Canadian Immunization Research Network (CIRN) in Halifax, NS, Canada[29] or the biorepository in the Division of Microbiology at Nova Scotia Health Authority (NSHA), Halifax, NS, Canada.

S. pneumoniae isolates for the clinical validation (n = 308) were obtained from two different sources: 87 S. pneumoniae specimens were collected as part of a national surveillance program for CAP and IPD by the CIRN SOS Network between Dec. 01, 2010 and Dec. 31, 2012. The other 221 S. pneumoniae isolates (206 blood and 15 fluids: 8 cerebral spinal, 3 vitreous, 2 peritoneal, and 1 synovial) were collected as standard practice in the Division of Microbiology at NSHA (Halifax, NS) between June 2009 and December 2013. S. pneumoniae were characterized by Quellung serotyping using commercial pool, group, type and factor antisera (SSI Diagnostica; Statens Serum Institute, Copenhagen, Denmark) at the Streptococcus and STI Unit at the NML (Winnipeg, MB).[15,30] All isolates were stored in skim milk at -80°C.

All specimens were cultured according to standard laboratory techniques. Pneumococcal isolates were confirmed by optochin disc susceptibility (Oxoid, Basingstoke, Hampshire, UK) and tube bile solubility analyses.[30,31] All streptococci were cultured at 35°C in 5% CO2 on trypticase soy agar (TSA) with 5% sheep blood (Becton Dickinson, Mississauga, ON). Bacterial growth was harvested from overnight cultures and suspended in phosphate-buffered saline (PBS) to a McFarland value of approximately 1.0 prior to nucleic acid extraction. The limit of detection (LoD) or analytical sensitivity of cmPCR and cmPCRmod was performed using three independent 10-fold serial dilutions (in PBS) of each identifiable S. pneumoniae serotype prior to nucleic acid extraction. The template nucleic acids extracted from each S. pneumoniae serotypes dilution were tested in triplicate with the respective cmPCR and cmPCRmod reactions used to identify each serotypes. Dilutions yielding 100% reproducibility (n = 9) for cmPCR or cmPCRmod reactions were assigned a representation of 1 × LoD, thus representing the analytical sensitivity.

2.2 Nucleic acid extraction and PCR-based serotyping

Nucleic acids and cmPCR reactions were performed under conditions previously described (Lang et al., 2015). Nucleic acids were isolated from 200 µl bacterial suspension using a MagNA Pure Total Nucleic Acid Isolation kit (Roche, Laval, QC) on a MagNA Pure LC instrument, as recommended by the manufacturer. Elution volume was set at 100 µl, and 5 µl served as template for all PCR reactions. Both cmPCR and cmPCRmod reactions were performed in 25 µl volumes that consisted of 1 × enzyme mix from the Multiplex PCR kit (Qiagen Inc, Toronto, ON) with primer combinations and concentrations listed in Tables 1 and 2, respectively. For cmPCRmod, different primer combinations were used but the concentration of each remained the same as cmPCR reactions (see Tables 1, 2). All cmPCR and cmPCRmod reactions contained primers cmCpsA-F and cmCpsA-R which target the capsule biosynthesis gene a (cpsA) that is used as an internal control (see Tables 1, 2). Amplification for cmPCR and cmPCRmod were performed in 96-well plates using a C1000 thermocycler (Biorad Laboratories, Mississauga, ON) as follows: 95°C for 90s, 35 cycles of 95°C for 30s, 54°C for 90s, and 72°C for 60s, followed by 72°C for 10 min. Amplicons were resolved using 1.2% agarose gel electrophoresis with 10 µg/ml ethidium bromide staining and visualized using a GelDoc XR + with ImageLab software (version 5.1) (Biorad Laboratories). Expected amplicon sizes in base pairs (bp) for cmPCR and cmPCRmod are denoted in Table 1. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

2.3 Cost analysis

The cost account for nucleic acid extraction and reagent and consumable costs for the number of cmPCR or cmPCRmod reactions required, when processed sequentially, to identify S. pneumoniae serotypes covered by PCV7, PCV13, and PPV23. The overall cost savings of cmPCRmod was expressed as a percentage of the cost of cmPCR.
### Table 2. Oligonucleotides used in this study

| Name               | Primer Sequence (5’ to 3’)                                                                 | Concentration (nM) | Serotypes detected                  | Reference |
|--------------------|-------------------------------------------------------------------------------------------|--------------------|-------------------------------------|-----------|
| cmCpsA-F           | GCA GTA CAG CAG TGT TGT GGA CTA CGT ACC                                                   | 100                | All but 25A, 25F, and 38            | [25]      |
| cmCpsA-R           | GAA TAT TTT CAT CAT TAC CAG TCC CAG TC                                                   | 100                |                                     |           |
| cm1-F              | CTC TAT AGA ATG GAT TAT ATA AAC TAT GGT TA                                               | 300                |                                     |           |
| cm1-R              | CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C                                            | 300                | 1                                   | [25]      |
| cm2-F              | TAM CCC AGT TCA ATA TTT CTC CAC TAC ACC                                                   | 300                |                                     |           |
| cm2-R              | ACA CAA AAT ATA GGC AGA GAG AGA CTA CT                                                   | 300                | 2                                   | [24]      |
| cm3-F              | ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G                                                | 300                | 3                                   | [25]      |
| cm3-R              | CTC CTA CAA TTT TTT ACC AAG TGC AAT AAC G                                               | 300                |                                     |           |
| cm4-F              | CTT GAC TTA CTT CTG GAC TCC CGA TAA TCT G                                                | 300                | 4                                   | [25]      |
| cm4-R              | GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G                                                | 300                |                                     |           |
| cm5-F              | ATA CCT ACA CAA CTT CTG ATT ATG CCT TGG                                                  | 300                | 5                                   | [25]      |
| cm5-R              | GCT GAA TAA ACA TCA TCA TTA ATT TTA GAA AAA GTA TG RT                                    | 300                |                                     |           |
| cm6A/6B/6C/6D-F    | AAT TTT TAT TTT ATT CAT GGC TAT TAC GTG                                                  | 300                | 6A, 6B, 6C, 6D                      | [25]      |
| cm6A/6B/6C/6D-R    | TTA GCG GAG ATG TTA AAT AAT AAC GCC TA                                                   | 300                |                                     |           |
| cm6C/6D-F          | CAY TTT TTT ATT AGA AGA GAG TAT TCA AAT GTG                                              | 300                |                                     |           |
| cm6C/6D-R          | CAA ATG TAA CAC AAC ATG GTC GAC GAA TCA                                                   | 300                |                                     |           |
| cm7C/7B-F          | CTA TCT TCG TCA TCT ATT GTC AAA GTC GAC GAC GAA                                         | 300                | 7B, 7C, 40                          | [25]      |
| cm7C/7B-R          | GAA CAT AGA TGT TGA GAC TCT TGG TGT AAT TCC                                             | 300                |                                     |           |
| cm7F/7A-F          | CCT ACG GGA GGA TAT AAA ATT ATG G                                                        | 400                | 7A, 7F                              | [25]      |
| cm7F/7A-R          | CAA ATA CAT AAC ATT AGG TCG ATG AG ACAA CTA                                             | 400                |                                     |           |
| cm8-F              | GAA GAC AAC AAA GTC CTA GAG CTA GAT TCA                                                  | 200                |                                     |           |
| cm8-R              | CTA CAT AGA TCA GAT GGC TTT ATT TGC G                                                    | 200                | 8                                   | [24]      |
| cm9N/9L-F          | GAA GCT ATG AAT AAC CTA CAT GTG TGG ATG TCA                                              | 500                | 9L, 9N                              | [21]      |
| cm9N/9L-R          | ACC AAG ATC TGA CGG GCT AAT CAA T                                                        | 500                |                                     |           |
| cm9V/9A-F          | GGAC TCG TAT AAA GTC AGA GAG ATC TTA A                                                    | 500                | 9A, 9V                              | [24]      |
| cm9V/9A-R          | CCA TGA ATG A AA TGA TCA ACA TTA GTC GCA TCA GAC                                         | 500                |                                     |           |
| cm10A-F            | GGT GTA GTT CAA TTA GTG TCG GCT GCA AGC                                                  | 500                | 10A                                 | [25]      |
| cm10A-R            | GAA TTT CTT CTG TAA AAT GAT TCG GAT ATT TCT                                             | 500                |                                     |           |
| cm10F/10C/33C-F    | GGA GCT TAT CGG TAG TGC TCG TCA TTT TAK                                                | 300                | 10C, 10F, 33C                       | [24]      |
| cm10F/10C/33C-R    | CTA ACA AAT TCG CAA CAC GAG GCA ACA                                                     | 300                |                                     |           |
| cm11A/11D-F        | GGA CAT GTG CAG GTG ATT TCT CAA TAT AGT G                                                | 300                | 11A, 11D                            | [25]      |
| cm11A/11D-R        | GAT TAT GAG TGG TAT TTA TTA TCA CAA TTT TCT C                                           | 300                |                                     |           |
| cm12F/12A/44/46-F  | GCA ACA AAC GGC GTG AAA GGA GTT G                                                        | 500                | 12A, 12B, 12F, 44, 46              | [25]      |
| cm12F/12A/44/46-R  | CAA GAT GGA TAT CAC TAA CAA AAA AAC                                                       | 500                |                                     |           |
| cm13-F             | TAC TAA GGT AAT CTC GCG AAA TCG AAA                                                        | 400                | 13                                  | [24]      |
| cm13-R             | CTC ATG CTT ATT AAT AAC CGC TCT GTC G                                                  | 400                |                                     |           |
| cm14-F             | GAA ATG TTA CTT GCG GCA GGT GTC AGA ATT                                                   | 300                | 14                                  | [21]      |
| cm14-R             | GCC AAC ATC TCT TCT CTA AGA TGA AT                                                       | 300                |                                     |           |
| cm15A/15F-F        | ATT AGT ACA GCT GCT GCA ATG CTA TTT TCC                                                 | 300                | 15A, 15F                            | [25]      |
| cm15A/15F-R        | GAT CTA GTG AAT GCA TTA TTA TCA CAA CAC                                                  | 300                |                                     |           |
| cm15B/15C-F        | TTG GAA TTT TTT ATT TAG TAGG TGG TCT ACC TCA                                              | 300                | 15B, 15C                            | [25]      |
| cm15B/15C-R        | CAT CCG CTT ATT AAT AGT AAT CTC AAC                                                       | 300                |                                     |           |
| cm16-F             | CTG TTC AGA TAG GCC ATT TAC AGC TCT AAT G                                               | 400                | 16F                                 | [25]      |
| cm16-R             | CAT TCC TTTGTTATA TAG TGC TAG TGT TAC TCC                                                | 400                |                                     |           |
| cm17-F             | TTC GTC ATG ATT TTA ATG CGA TTA CAA ATG CAC                                              | 500                | 17F                                 | [25]      |
| cm17-R             | GAT GTA ACA AAT TGA CGA CTA AGG TCC TGC GC                                                | 500                |                                     |           |
| cm18C/18F/18B/18A-F| CTT AAT AGC TCT CAT TAT TCT TTT AAT GGC                                                 | 300                | 18A, 18B, 18C, 18F                 | [25]      |
| cm18C/18F/18B/18A-R| TTA TCT GTA AAC ATC ATG AGC ATG TCA AAC                                                   | 300                |                                     |           |
| cm19A-F            | GAG AGA TTA ATT ATG GTC AAG GAC GCA C                                                  | 300                | 19A                                 | [33]      |
| cm19A-R            | CAT AAT AGC TAA AAA TGA CTC ATC GCC                                                      | 300                |                                     |           |
| cm19F-F            | GAT AAG ATT GCT GAG CTA ATT ATG GTC TTT AGT                                              | 500                | 19F                                 | [25]      |
| cm19F-R            | GTA ATA TCT TTT TGG GGC GAT TGG ATG G                                                    | 500                |                                     |           |
| cm20-F             | GAG CAA GAG TTT TTT ACC TGC TGG CAG CAG GGA                                              | 300                | 20                                  | [25]      |
| cm20-R             | CTA AAT TCC TGT ATT TTA GCT AAA ATC CTT TGC                                              | 300                |                                     |           |

(Table 2 continued on page 32)
Table 2. (Continued.)

| Name           | Primer Sequence (5' to 3')                     | Concentration (nM) | Serotypes detected | Reference |
|----------------|------------------------------------------------|--------------------|--------------------|-----------|
| cm21-F         | CTA TGG TTA TTT CAA CTC AAT CGT CAC C          | 200                | 21                 | [24]      |
| cm21-R         | GGC AAA CTC AGA CAT AGT ATA GCA TAG             | 200                |                    |           |
| cm22F/22A-F    | GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC     | 500                | 22A, 22F           | [25]      |
| cm22F/22A-R    | CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC     | 500                |                    |           |
| cm23A-F        | TAT TCT AGC AAG TGA CGA AGA TGC G              | 500                | 23A                | [24]      |
| cm23A-R        | CCA ACA TGC TTA AAA ACG CTG TTG TAC            | 500                |                    |           |
| cm22F/22A-F    | GAG TAT AGC CAG ATT ATG GCA GTT TTA TTG TC     | 500                | 22A, 22F           | [25]      |
| cm22F/22A-R    | CTC CAG CAC TTG CGC TGG AAA CAA CAG ACA AC     | 500                |                    |           |
| cm23F-F        | GTA ACG TGT GTC GTA GAG GGA ATT GCC TTT TC     | 500                | 23F                | [25]      |
| cm23F-R        | CAC ACC ATG TAA CAC TCG ATG GCT ATA TGA TGC   | 500                |                    |           |
| cm24F/24A/24B-F| GCT CCC TGC TAT TGT AAT CTT TAA AGA G          | 200                |                    |           |
| cm24F/24A/24B-R| GTG CCT TTT ATT GAC TTT ATC ATA GGT CC         | 200                | 24A, 24B, 24F      | [24]      |
| cm31-F         | GGA AGT TTT CAA GGA TAT GAT GGT GGT GC         | 500                |                    |           |
| cm31-R         | CCG AAT AAT ATA TTC AAT AAT TTC CTA CTC        | 500                | 31                 | [25]      |
| cm33F/33A/37-F | GAA GCC AAT CAA TGT GAT TGT GTC GGC            | 300                |                    |           |
| cm33F/33A/37-R | CTT CAA AAT GAA GAT ATT AGT ACC CTT CTA C      | 300                | 33A, 33F, 37       | [25]      |
| cm34-F         | GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC     | 300                |                    |           |
| cm34-R         | CAA TCC GAC TAA GTC TGC TTT AGT AAA AAA CTT TAC| 300                | 34                 | [25]      |
| cm35A/35C/42-F | ATT ACG ACT CCT TAT GTG ACG GGC ATA             | 300                |                    |           |
| cm35A/35C/42-R | CCA ATC CCA AGA TAT ATG CAA CTA GGT T          | 300                | 35A, 33C, 42       | [24]      |
| cm35B-F        | GAT AAG TCT GTG GTG GAC ACT TAA AAA GAA TG     | 500                | 35B                | [25]      |
| cm35B-R        | CTT TCC AGA TAA TTA CAG GTA TTT TGC CTA AAG CAA | 500                |                    |           |
| cm35F/47F-F    | GAA CAT ATG CCG TAT TGT ATT TTA TTA AAT GCA A  | 300                | 35F, 47F           | [25]      |
| cm35F/47F-R    | GAC TAG GAG CAT TAT TCC TAG AGC GAG TAA ACC    | 300                |                    |           |
| cm38/25F/25A-F | CGT TCT TTT ATC TCA TCT CGT ATG ATC TTT ATG    | 300                |                    |           |
| cm38/25F/25A-R | ATG TTT GAA TTA AAG CTA ACG TAA CCA ATG TTT C  | 300                | 25A, 25F, 38       | [25]      |
| cm39-F         | TCA TTG TAT TAA CCC TAT GCT TTA TTG GTG        | 200                |                    |           |
| cm39-R         | GAG TAT CTC CAT TGT ATT GAA ATC TAC CAA        | 200                | 39                 | [24]      |

Table 3. Bacterial strains used in the specificity analysis

| Strains                          | Results                     |
|----------------------------------|-----------------------------|
| **S. pneumoniae** ATCC 49619, serotype 19F | Pos. Pos.          |
| **S. pneumoniae** (clinical isolates), serotype: 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 7A, 7F, 9A, 9V, 11A, 11D, 12A, 12F, 14, 15A, 15F, 16F, 18A, 18B, 18C, 18F, 19A, 19F, 22A, 22F, 23A, 23F, 33A, 33F, 37, 44, and 46 | Pos. Pos. |
| **S. pneumoniae** (clinical isolates), serotype: 7B, 7C, 8, 9L, 9N, 10A, 10C, 10F, 12B, 13, 15B, 15C, 17F, 20, 21, 23B, 24A, 24B, 24F, 25A, 25F, 31, 33C, 34, 35A, 35B, 35C, 35F, 38, 40, 42, 47F | Pos. Pos. |
| **S. pneumoniae** (clinical isolates), serotype: 10B, 11B, 11C, 11F, 16A, 17A, 19B, 19C, 27, 28A, 28F, 29, 32F, 33B, 36, 41A, 41F, 43, 45, 47A, 48 | NT NT          |
| **S. agalactiae** ATCC 12386 | Neg. Neg.           |
| **S. dysgalactiae subsp. equisimilis** ATCC 12388 | Neg. Neg.          |
| **S. equi subsp. zooepidemicus** ATCC 700400 and 43079 | Neg. Neg.          |
| **S. gallolyticus** ATCC 9809 | Neg. Neg.          |
| **S. mitis** ATCC 33399 | Neg. Neg.          |
| **S. mitis** ATCC 49456 | Neg. Neg.          |
| **S. mutans** ATCC 25175 | Neg. Neg.          |
| **S. oralis** ATCC 35037 | Neg. Neg.          |
| **S. pseudopneumoniae** ATCC BAA-960 | Neg. Neg.          |
| **S. pyogenes** ATCC 19615 | Neg. Neg.          |
| **S. salivarius subsp. thermophilus** ATCC 19258 | Neg. Neg.          |
| **S. sanguinis** ATCC 10556 | Neg. Neg.          |
| **S. uberis** ATCC 700407 | Neg. Neg.          |
3. Results

3.1 Analytical specificity and sensitivity

Each of the PCR-based serotyping assays (cmPCR and cmPCRmod) was highly specific, and no cross reactivity occurred between the various serotypes or with non-pneumococcal streptococci (see Table 3). As previously reported, confounding amplicons (as a result of non-specific amplification) sometimes occurred with cmPCR and cmPCRmod reactions.\(^\text{[19]}\) If present, these could readily be resolved with repeat reactions using individual primer pairs targeting the suspected serotype. Normalization of the \textit{S. pneumoniae} culture to a McFarland value of 1.0 prior to extraction provided sufficient template that gave strong and reliable amplicons for all detectable serotypes. Based on end-point titers at \(1 \times \text{LoD}\) for each detectable serotype, no differences in analytical sensitivity were seen between cmPCR and cmPCRmod.

As shown in Table 3, cmPCR and cmPCRmod reactions were equivalent in their abilities to detect \textit{S. pneumoniae} serotypes, and did not cross-react with any other Streptococcus species. Abbreviations: conventional multiplex PCR (cmPCR), modified conventional multiplex PCR (cmPCRmod), and non-typeable (NT).

3.2 Clinical isolate testing

No discordant results were observed between cmPCR and cmPCRmod, and a serotype could be assigned for 99.7\% (307/308) of \textit{S. pneumoniae} isolates. A single non-typeable (NT) isolate was detected as cpsA-positive by the internal control, and was identified as serotype 28A by Quellung serotyping (see Figure 1). The overall trend in serotype distribution obtained by cmPCRmod mirrored Quellung serotyping, showing a predominance of serotypes 3, 7F, and 19A (see Figure 1). Quellung serotyping showed that 5\% of \textit{S. pneumoniae} were PCV7 serotypes, 54\% were PCV13 serotypes, and 74\% were PPV23 serotypes (see Figure 1A). Similarly, cmPCR and cmPCRmod demonstrated 7\%, 54\%, and 77\% for serotypes covered by PCV7, PCV13, and PPV23, respectively (see Figure 1B). Focusing on select reactions containing vaccine-preventable serotypes, the number of cmPCR reactions required to span the coverage of PCV7, PCV13, and PPV23 was 4, 6, and 8, compared to 2, 3, and 5 with cmPCRmod, respectively (see Table 1). When applied to the 308 \textit{S. pneumoniae} isolates used in this study, cmPCRmod reduced the total number of reactions required to identify vaccine-preventable serotypes, leading to significant cost savings (see Table 4).

![Figure 1. S. pneumoniae serotypes distribution using Quellung and PCR-based serotyping. Arrows highlight differences between results for: A) Quellung serotyping, and B) cmPCR or cmPCRmod. The pie charts on the top of the inset show the proportions of PCV7 (pale blue), PCV13 (dark blue), and PPV23 (magenta) serotypes. The same colors are used in the histogram, and non-vaccine serotypes are in red. The pie charts on the bottom of the inset show the proportion of serotypes that are fully differentiated (green) or lack discrimination (red)](image)

| Vaccine coverage | Number of PCR reactions (cost in SCAD) | cmPCR | cmPCRmod | Cost reduction (%) |
|------------------|----------------------------------------|-------|----------|-------------------|
| PCV7             | 1232 (5,904.36)                        | 616   | 4,338.18 | 26.5              |
| PCV13            | 1848 (7,470.54)                        | 924   | 5,121.27 | 31.4              |
| PPV23            | 2464 (9,376.72)                        | 1540  | 6,687.45 | 36.0              |

As shown in Table 4, the number of reactions required to identify vaccine-preventable serotypes is lower in cmPCR-
mod compared to cmPCR, leading to significant cost savings.

4. DISCUSSION
Accurate identification of vaccine-preventable serotypes of *S. pneumoniae* can help determine the burden of disease caused by these serotypes, identify high-risk populations that could benefit from pneumococcal vaccination, and be used to evaluate vaccine effectiveness over time. In contrast to traditional testing algorithms based solely on serotype prevalence, this study used modified PCR reactions that focused on the identification of *S. pneumoniae* serotypes covered by PCV7, PCV13, and PPV23. When applied to 308 clinical isolates of *S. pneumoniae*, cmPCRmod reduced the total number of reactions required for the identification of vaccine-preventable serotypes.

It should be noted that molecular serotyping of *S. pneumoniae* based on differences in the capsular biosynthesis genes like cmPCR, real-time PCR, microarrays, hybridization assays, and sequencing, all suffer from the same limitation: failure to discriminate between some pneumococcal serotypes. For example, while serotype 7F is covered in all three pneumococcal vaccines, 7F cannot be discriminated from serotype 7A using these molecular methods, and therefore the serotype is assigned 7F/7A. Other vaccine-preventable serotypes identified by PCR-based serotyping cannot discriminate: 6A from 6B; 9V from 9A; 9N from 9L; 11A from 11D; 12F from serotypes 12A, 12B, 44, and 46; 15B from 15C; 18C from serotypes 18F, 18B, and 18A; 22F from 22A; 33F from serotypes 33A and 37. For accurate discrimination of vaccine-preventable serotypes, these PCR groups would have to be resolved.

In this study, all vaccine-preventable serotypes identified by Quellung serotyping were also detected with cmPCRmod and cmPCR (see Figure 1). On the other hand, the proportion of serotypes covered by PCV7, PCV13, and PPV23 that could be fully discriminated was 41%, 57%, and 18%, respectively (see Figure 1B). The remaining proportions contained serotypes that were termed “possibly” vaccine-preventable. To overcome this limitation, some laboratories assign serotypes based on assumptions from serotype prevalence (where serotype 7F/7A identified by PCR would be considered 7F, the most prevalent serotype). Using all eight cmPCR and cmPCRmod reactions, the serotype distribution of pneumococcal isolates was identical and mirrored results obtained with Quellung serotyping, with only subtle differences (see Figure 1). A high level of accuracy for the detection of serotypes covered by PCV7 (98.4%), PCV13 (99.4%), and PPV23 (96.1%) was observed; however, nine serotypes including 6B (n = 2), 18F (n = 2), 33A (n = 3), and 37 (n = 2) were misclassified as possibly vaccine-preventable if assumptions were made based on prevalence. The lack of discrimination of serotypes 6A and 6B would not be problematic for PCV13, since this vaccine includes coverage for both serotypes; however, serotype 6A is not covered by PCV7 or PPV23. Investigations are currently underway to identify novel targets for detection and differentiate of serotypes found in all current pneumococcal vaccines.

While other studies have used oligonucleotide permutations to account for geographical differences in *S. pneumoniae* serotype distribution (USA, Latin America, Africa, and Asia), this study showed that oligonucleotides permutations did not affect the performance characteristics of PCR-based serotyping. This study does not preclude additional validation if different permutations are used, or use of ongoing quality assurance controls. Both cmPCR and cmPCRmod suffer from the same limitations where further optimization would be required for accurate discrimination of certain serotypes. On the other hand, cmPCRmod was more cost-effective than cmPCR and reduced the number of PCR reactions were required to identify vaccine-preventable serotypes of *S. pneumoniae*. Overall, serotyping of *S. pneumoniae* isolates using the cmPCRmod reactions could provide significant cost savings for large epidemiological studies such as the active CAP and IPD surveillance conducted by the CIRN SOS Network.

CONFLICTS OF INTEREST DISCLOSURE
Authors declare that they have no competing interests.

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