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PCR deduction of invasive and colonizing pneumococcal serotypes from Venezuela: a critical appraisal

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Abstract

Introduction: Serotype surveillance of *Streptococcus pneumoniae* is indispensable for evaluating the potential impact of pneumococcal conjugate vaccines. Serotyping by the standard Quellung reaction is technically demanding, time consuming, and expensive. A simple and economical strategy is multiplex PCR-based serotyping. We evaluated the cost effectiveness of a modified serial multiplex PCR (mPCR), resolving 24 serotypes in four PCR reactions and optimally targeting the most prevalent invasive and colonizing pneumococcal serotypes found in Venezuela.

Methodology: A total of 223 pneumococcal isolates, 140 invasive and 83 carriage isolates, previously serotyped by the Quellung reaction and representing the 18 most common serotypes/groups identified in Venezuela, were serotyped with the adapted mPCR.

Results: The mPCR serotyped 76% of all the strains in the first two PCR reactions and 91% after four reactions, correctly identifying 17 serotypes/groups. An isolate could be serotyped with mPCR in less than 2 minutes *versus* 15 minutes for the Quellung reaction, considerably lowering labor costs. A restrictive weakness of mPCR was found for the detection of 19F strains. Most Venezuelan 19F strains were not typeable using the mPCR, and two 19F *cps* serotype variants were identified.

Conclusions: The mPCR assay is an accurate, rapid, and economical method for the identification of the vast majority of the serotypes from Venezuela and can be used in place of the standard Quellung reaction. An exception is the identification of serotype 19F. In this setting, most 19F strains were not detectable with mPCR, demonstrating a need of serology-based quality control for PCR-based serotyping.

Key words: sequential multiplex PCR; Quellung; serotyping; *Streptococcus pneumoniae*; Venezuela; serogroup 19

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Introduction

Streptococcus pneumoniae causes a broad variety of diseases in humans, ranging from upper respiratory tract infections to invasive pneumococcal disease. The major virulence factor of this Gram-positive bacterium is the capsular polysaccharide upon which pneumococcal serotyping is based. Today, 93 different serotypes, including the recently discovered 6C, 6D, and 11E are recognized. Three vaccines are available to protect children and adults against pneumococcal invasive disease: the 10-valent and 13-valent conjugate vaccines and the 23-valent polysaccharide vaccine, which protect against disease caused by the serotypes covered by these vaccines. In Venezuela, the three vaccines are available through private medical practices but are not included in the vaccination

scheme for the general population. No information is available about a future introduction of these vaccines to publicly funded health programs.

To accurately assess the need for and effect of immunization with pneumococcal vaccines, knowledge about serotype distribution in pneumococcal disease and in nasopharyngeal carriage is critical. In addition, knowledge of replacement of important invasive serotypes by other serotypes allows for modifications to the vaccination strategy in order to maximize its effects. The Quellung reaction, the swelling of the capsule surrounding the microorganism after reaction with an antibody, sometimes called the Neufeld test and described originally in 1933 by Neufeld and Etinger-Tulczynska [1], is the gold standard for serotyping of *S. pneumoniae*. However,

this method, which uses anti-pneumococcal capsule serum from the Statens Serum Institute, Denmark, is time consuming, subject to interpretation, and expensive because of the high cost of the antisera. The approximate mean material cost of serotyping by Quellung testing has been calculated at 21.25 USD per isolate [2]. Regarding labor hours, serotyping by Quellung requires pool, group, and type reactions with a minimum of 3 minutes for each reaction and a mean test time of approximately 15 minutes per strain [2].

PCR-based serotyping targeting the *cps* locus has been shown to be effective for the serotyping of pneumococcal specimens [3-6]. Based upon cumulative results from simultaneous serology and PCR-based testing of diverse isolate sets, a sequential PCR assay is considered accurate for the serotyping of *S. pneumoniae* isolates [2,7]. In addition, sequential multiplex PCR assays have been shown to be more economical, with a mean material cost of approximately USD\$7.50 per isolate [2].

In Venezuela, serotyping is only available for invasive pneumococcal isolates and is carried out in the reference laboratory in the National Health Institute in Caracas. The institute uses the Quellung reaction. Ongoing research, especially nasopharynx carriage studies, carried out in the last 10 years in our country to increase the knowledge of pneumococcal epidemiology, has demonstrated a need for a faster and cheaper technique for serotyping. Here, we evaluate for future investigational use a serial multiplex PCR (mPCR) approach, based on one originally developed by Pai *et al.* [7] for detecting 29 serotypes/groups. Over time, this protocol has been adapted and extended several times and actually resolves 40 serospecificities in eight PCR reactions (see Centers of Disease Control and Prevention [CDC]:

<http://www.cdc.gov/ncidod/biotech/strep/pcr.htm> [June 2011] and reference [8]). For the PCR detection of strains from Venezuela, we adapted this multiplex PCR scheme by modifying the order of the reactions and moving serotypes into different reaction mixtures from where they were previously, based on the prevalence data of invasive pneumococcal serotypes/groups in Venezuela. We developed a four-step sequential multiplex PCR assay that is capable of distinguishing 24 pneumococcal serotypes, increasing the number serotypes in each reaction from 5 to 6, and focusing on detection of the most common invasive serotypes in Venezuela in the first two PCR reactions. The PCR assay was limited to 24 serotypes, as approximately 96% of the isolates recovered in

Venezuela from carriers of invasive disease belonged to these 24 serotypes/groups. The CDC protocol detects an additional 16 serotypes/groups, never or rarely isolated and reported from patients with invasive pneumococcal disease or in carriage studies in Venezuela; for this reason, these serotypes were not included in the typing scheme.

For method evaluation, 140 invasive isolates, representing the 16 most important invasive serotypes/groups as previously determined in the SIREVA study from Venezuela [9], were used. In addition, 83 isolates representing the 15 most common serotypes/groups isolated from the nasopharynx of the Warao people [10] were tested. These isolates, representing 19 different serotypes/groups, were previously serotyped by the Quellung reaction. Sensitivity and the economic costs of the sequential multiplex PCR were determined and compared to the costs of the Quellung reaction.

Methodology

Bacterial isolates

A total of 140 clinical pneumococcal isolates were used, representing the 16 most important invasive serotypes/groups in Venezuela [9]: 1 (n = 7), 3 (n = 3), 4 (n = 1), 5 (n = 10), 6A (n = 19), 6B (n = 21), 7F (n = 3), 9V (n = 2), 14 (n = 41), 15 B/C (n = 1), 18A (n = 1), 18C (n = 2), 19A (n = 14), 19F (n = 5), 23F (n = 9), and 33F (n = 1). The isolates were recovered from sterile fluids, including blood culture (41 isolates), cerebrospinal fluid (39 isolates), pleural fluid (51 isolates), and ocular secretion (6 isolates); 3 isolates had no patient information. A total of 83 nasopharynx carriage isolates representing 15 different serotypes/groups and isolated from Warao Amerindian children [10] were used: serotype/group 4 (n = 2), 6A (n = 11), 6B (n = 9), 7F (n = 2), 9V (n = 6), 10 (n = 2), 11 (n = 2), 12F (n = 1), 14 (n = 6), 15B (n = 2), 18C (n = 3), 19A (n = 5), 19F (n = 20), 23A (n = 1), and 23F (n = 11). The number of isolates tested for each serotype/group followed the relative prevalence of these serotypes in invasive pneumococcal disease [9] and carriage in Venezuela [10]. All bacteria were cultured on blood agar (BD) with 5% CO₂ at 37°C.

Quellung serotype reaction

Isolates were serotyped by the capsular swelling procedure (Quellung reaction) and observed microscopically using commercially available antisera (Statens Serum Institute, Copenhagen, Denmark).

Multiplex PCR assay

Genomic DNA was isolated using the boiling method starting from three to four colonies grown on blood agar and suspended in 100 µL of TE buffer 1X (10 mM Tris-HCl [pH 8.0], 1mM EDTA [pH 8.0]). After boiling for 10 minutes, cell debris was pelleted by centrifugation (13.000 x g for 2 minutes) and the supernatant was used for the PCR. The multiplex PCR was performed using the primers of the published protocol of Pai *et al.* [7], adapting the primer set as proposed by Carvalho *et al.* [8]. Because the CDC protocol detects 16 serotypes/groups which have never been reported from patients with invasive pneumococcal disease or in carriage studies in Venezuela, these serotypes were excluded from the typing scheme. Primer sets were combined in such a way that approximately 77% of the most important invasive serotypes would be detected in the first two reactions. Each of the four reactions included six primer pairs targeting different serotype-specific sequences as well as primers for the internal positive control, amplifying a 160 bp conserved region in the pneumococcal *cps* locus (Table 1). Reference strains of known serotypes, as determined with the Quellung reaction, were used as controls in the PCR reactions.

PCRs were performed in 25 µL volumes, with each reaction mixture containing the following: 1x PCR buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl), 200 µM of each deoxynucleoside triphosphate, 1 M betaine, 2.5 mM of MgCl₂, 2.0 U of *Taq* DNA polymerase (FUNDAIM, Instituto de Biomedicina, Caracas, Venezuela), and primers with concentrations of 0.1 µM (*cpsA*) and 0.5 µM serotype-specific primers, (Bioneer, Inc, Alameda, USA). Crude, boiled bacterial extract (5 µL) was used as the DNA template for each PCR. Cycling conditions were 94°C for 4 minutes, followed by 30 amplification cycles of 94°C for 45 seconds, 54°C for 45 seconds, and 65°C for 150

seconds. Ten µL of each reaction mixture was separated through 2% agarose by electrophoresis, and PCR amplification bands were visualized after ethidium bromide staining and UV illumination. The sizes of the PCR products were determined by comparison with two molecular size standards: 50 bp and 100 bp ladders (Promega, USA). Personnel carrying out the multiplex PCR and reading the agarose gels were blinded for the results of each isolate’s Quellung reaction.

Results

From the invasive pneumococcal isolates, 138 of 140 isolates (98%) showed a serospecific PCR product in one of the four multiplex PCR reactions, and the reader of the agarose gels correctly matched the PCR results to the results of the Quellung reaction 100% of the time. Two of the five isolates classified by the Quellung reaction as 19F were not detected by PCR but showed the positive pneumococcal control band of 160 bp for the *cpsA* gene.

Of the 83 pneumococcal carriage isolates recovered from the nasopharynx of Amerindian Warao children [10], only 63 showed a serospecific PCR product in one of the four multiplex PCR reactions. The reader of the agarose gels predicted the serotypes of these 63 strains in agreement with the results obtained by the Quellung reaction. Twenty *S. pneumoniae* isolates, serotyped as 19F using the serology-based reaction, yielded a false negative PCR result with the primers targeting the 19F *wzy* gene, but all 20 showed the positive pneumococcal control band of 160 bp for the *cpsA* gene. Overall, the sequential multiplex PCR quickly and accurately identified 76% of the pneumococcal serotypes in only two PCR reactions and approximately 90% of the isolates, covering the range of 19 serotypes/groups, were detected using four PCR assays. Misinterpretation of the PCR results, the main source of discordance

Table 1: Sequential multiplex PCR approach for determining capsular serotypes/groups of *Streptococcus pneumoniae* isolates in Venezuela

Reaction	Serotypes and serogroups (Sg)	Size of the PCR products (bp)
1	9V, 19A, 15B/C, 3, 19F, and Sg 6	753, 566, 496, 371, 304, 250
2	23A, Sg 18, 4, 23F, 33F, and 14	712, 573, 430, 384, 338, 198
3	7F, 22F/A, 9N/L, 15A/F, 5, and 1	826, 643, 516, 434, 362, 280
4	17F, 10A, 20, 11A/D, 12F, and 8	693, 628, 514, 463, 376, 294

The table shows the four reactions, the serotypes/groups detected in each reaction, and the size of the corresponding PCR products. Serotypes of group 6 and 18 were not individually targeted in our multiplex PCR. The oligonucleotide primer pairs used in this study have been described in the protocol of Pai *et al.* [7]. This protocol, which detects 29 serotypes/groups, has been adapted and extended several times and actually resolves 40 serospecificities. Our protocol follows this CDC protocol, including the last modification in the year 2009, but 16 serotypes/groups are left out from this typing scheme because they have never been isolated from patients with invasive pneumococcal disease or in carriage studies in Venezuela. See also the webpage of the Centers of Disease Control and Prevention (CDC): <http://www.cdc.gov/ncidod/biotech/strep/pcr.htm> (June 2011).

previously observed [12] did not appear to raise a problem in this setting.

The 2 invasive and 20 carrier isolates, undetectable with primers targeting the 19F *wzy* gene, were further characterized with a primer set targeting the 19A *mnaA* gene, used previously in the original typing scheme by Pai *et al.* [7] for the detection of serotype 19A. In 2009, this primer set was replaced by Pimenta *et al.* [11] because it yielded a false positive PCR product in concordance with the size for the serotype 19A with one 19F isolate (as determined by Quellung) from Canada (strain 2584-08 [19F]). In agreement with this study's 19F strains, the Canadian 19F strain was undetectable with PCR using the primers targeting the 19F *wzy* gene. Using this 19A primer set in a PCR reaction, both invasive Venezuelan 19F isolates showed a PCR product with the size for a 19A serotype, suggesting a variant 19F genotype as has been described by Pimenta *et al.* [11]. Surprisingly, this 19A primer set yielded no PCR product with the twenty 19F carrier isolates, showing the existence of at least two different 19F variants in Venezuela. All 22 isolates with conflicting PCR results were retyped with the Quellung reaction, confirming the 19F serotype.

Conclusion

Our results demonstrate that most pneumococcal serotypes from Venezuela can be identified with sequential multiplex PCR and that the molecular assay is accurate for the vast majority of the isolates recovered in Venezuela. The mPCR assay correctly identified 19 serotypes/groups and 138/140 (98.6%) of the tested invasive isolates and 64/83 (77%) of the tested carriage isolates. Using only two reactions, the mPCR method detected the nine most important invasive serotypes, representing 77% of the total invasive isolates reported in Venezuela [9]. We demonstrated the adaptability of the PCR method for grouping alternative combinations of serotype-specific primer sets. This is an important finding for other settings or geographic regions where the pneumococcal serotype prevalence does not follow the normal distribution. In addition, the assay probably permits adjustments and introduction of other serotypes in the typing scheme when a change in pneumococcal serotype distribution will leave more isolates unresolved in this four reaction PCR serotype assay – for example after a future introduction of pneumococcal vaccines in our country.

A comparison of the costs of the Quellung and the PCR method favors the molecular technique. Material

costs of the sequential PCR assay have been estimated in another publication, demonstrating that a PCR method is more economical than the Quellung reaction, with a mean material cost of approximately \$7.50 per isolate, approximately 30% of the cost of the Quellung reaction [2]. This is mainly because of the costs of the expensive typing sera needed for conventional serotyping. Concerning labor costs, typing by Quellung can be labor intensive and time consuming. In this setting, conventional typing of the 221 isolates with Quellung required 1,041 reactions, divided in 620 for pools and 421 for factors, consuming 1041 μ L of the typing sera. As one Quellung reaction takes about 3 minutes for an experienced technician, 52 working hours were required for the serotyping of this set of strains, or a median time of about 15 minutes per isolate. In contrast, PCR-based typing for the same set of isolates required 357 PCR reactions for the typing of 199 isolates. Our laboratory uses 96-well PCR microplates which, using a multichannel pipette, facilitate the preparation of the PCR reaction mixtures and the loading of agarose gels with the PCR product. The preparation of one microplate for 96 reactions, including loading of the PCR product in an agarose gel and the reading and interpretation of the gel, takes about three working hours. This brings the labor time for the typing with PCR of one isolate down from 15 minutes to about 2 minutes.

Although the vast majority of our isolates could be serotyped with PCR, there is still a need for the Quellung reaction. The Quellung reaction was required to resolve the serotype of non-typeable isolates, *cps* positive in the PCR reaction, but not detected by our four PCR reactions. Moreover, the Quellung reaction will be needed to resolve serotypes of the serogroups 6 and 18 and the serotypes 15B and 15C, as these serotypes are not individually targeted with our multiplex PCR. Concerning serotype 19F, until a discriminative sequence is identified that permits the development of a specific primer set for the PCR detection of these variants, Quellung serotyping will be required for the detection of most of our 19F isolates.

Our mPCR revealed disagreement for 22 of the 25 isolates of serotype 19F used in this study that could not be detected with the PCR method because they yielded no PCR product with the existing primer set. The presence of at least two 19F sequence variants was found: one variant with the same characteristics as described by Pimenta *et al.* with a divergent *mnaA* and *wzyZ* gene [11], and the other variant with only an

altered *wzy* gene. The presence in our setting of the two 19F variants needs further investigation. The *wzy* gene codes for the polymerase activity responsible for the synthesis of the polysaccharides, and this gene is thought to account for the difference between serotypes 19A and 19F [13,14]. Changes within this capsular gene may result in altered polysaccharides or in increased production of the capsule, making this strain less sensitive for the vaccine-induced immunity. Studies are in progress to sequence the *cps* locus of our 19F strains and to study their vaccine-induced immunity.

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