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A useful tool in determination of serotypes among *Streptococcus pneumoniae*: Multiplex-PCR combined with Quellung reaction

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Revised Abstract

Background: *S. pneumoniae* remains an important pathogen despite licensure of a seven-valent pneumococcal protein conjugate vaccine. The appropriate clinical applications of pneumococcal polysaccharide vaccines against recent increases in antimicrobial resistant *S. pneumoniae* urgently require accurate analytical methodologies for determining and characterizing the serotypes. Current immunological determinations of serotypes by Quellung are time-consuming and expensive. We evaluated the multiplex PCR technique for the rapid identification confirmation of pneumococci and simultaneous rapid determinations of their serotypes followed by the traditional Quellung reaction.

Methods: 550 *S. pneumoniae* clinical isolates collected through the Tigecycline Evaluation and Surveillance Trial (T.E.S.T.) were examined. We devised a simple PCR-based assay of five sequential multiplex reactions to reliably deduce 28 specific pneumococcal serotypes. All PCR results were used to focus the Quellung reactions and allowed use of only type- or factor-specific sera for final serotype determination.

Results: The first step (PCR reactions 1, 2 and 3) enables us to determine the serotype for 67 % of the isolates. Overall, this multiplex typing scheme identified the serotype of 90% of the isolates with 100% correlation with the Quellung reaction. 10% of isolates were non-typeable by PCR. This PCR assay allowed the testing of 92 isolates in 1 day versus 5 days by Quellung. Currently, the most promising assay combines multiplex-PCR for determination of the 28 most common serotypes followed by Quellung reaction to determine the serotype for isolates that could not be typed by PCR. The most common serotype was 19A (18.7%) followed by serogroup 6A/B (10%), 22F/A (6.7%) and 3 (5.1%).

Conclusions: This study confirms the accuracy and utility of multiplex-PCR for serotyping of *S. pneumoniae* which could be of valuable use in public health laboratories to monitor sero-epidemiological changes. Additionally, this high-throughput, fast and cost-effective PCR approach, combined with the Quellung reaction, could improve ascertainment of pneumococcal serotype distributions.

Introduction

- S. pneumoniae* is an important pathogen that causes severe life-threatening illnesses in the elderly and children.
- The capsule is a major virulence factor of pneumococci. Indeed, the ability of pneumococci to cause disease is directly related to the production of a capsule, a polysaccharide structure external to the cell wall that provides resistance to phagocytosis and permits evasion of the host immune system by the bacteria.
- The immunochemistry of this capsular polysaccharide helps to differentiate pneumococci into **90 distinct serotypes** (4). However, only about **15 serotypes** cause the majority of invasive pneumococcal disease worldwide (3).
- To optimize the **development of future conjugate vaccines** and to evaluate their efficacy, it is necessary to understand the serogroup specific epidemiology of pneumococci and their associated diseases (2).
- Continuous monitoring of *S. pneumoniae* serotypes is essential** since it has been shown that the incidence of serotypes responsible for invasive disease can change over time (5).
- The high-cost of antisera, subjectivity in interpretation, and technical expertise requirements are serious limits of the system. The development of PCR-based serotyping systems has the potential to overcome some of the difficulties associated with serologic testing (1, 7, 8).
- In this work, we describe a **rapid, simple and cost-effective multiplex-PCR-based method combined with Quellung reaction** to type pneumococci in large scale and reduce the number of strains that may have to be serotyped by using the complete standard capsular reaction test.

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Materials & Methods

Bacterial isolates

- 550 *S. pneumoniae* clinical isolates from 2004-2006 collected through the Tigecycline Evaluation and Surveillance Trial (T.E.S.T.) were tested
- 28 control strains, representing the serotypes and serogroups targeted by multiplex-PCR, were used in this study.

Site Location

*Blood: 76%
*Fluids: 11%
*Respiratory: 11%
*CNS: 2%

Region: North America

*United States: 98.2%
*Canada: 1.8%

Departments

*Medicine: General (41.1%) and ICU (7.5%)
*Emergency Room: 23.5%
*Clinic Office: 10.4%
*Pediatric: General (4.5%) and ICU (1.8%)
*Surgery: General (3.3%) and ICU (1.8%)
*None Given: 6.1%

Molecular capsular typing by multiplex-PCR

DNA extraction (Fast boiling)

- Subculture on blood agar plates (Tryptic Soy Agar base supplement with 5% sheep blood)
- Incubation overnight at 37°C in 5% CO₂
- Bacterial suspension in TE buffer (McFarland 1)
- Suspension heated for 5 min at 95°C and immediately frozen at -20°C at least for 5 min.

Oligonucleotides primers

The 28 oligonucleotides pairs (8) used for this study were designed to target the following serotypes: 1, 3, 4, 6A/B, 7C/B, 7F/A, 8, 9V/A, 10A, 11A/D, 12F/A, 14, 15A, 15B/C, 16F, 17F, 18ABCF, 19A, 19F, 20, 22F/A, 23F, 31, 33F/A, 34, 35B, 35F, 38. A primer pair (cpsA-1/cpsA-r) was also included as an internal control targeting the cpsA locus found in all pneumococci (6)

Multiplex PCRs

QIAGEN Multiplex PCR kit (15 µL-volume reaction)

Components	Description of components	Final Concentration
2X QIAGEN Multiplex PCR Master Mix	Hot Start Taq [®] DNA Polymerase	1X (3 mM MgCl ₂)
100 Primer Mix	Multiplex PCR buffer	100 µM
100 Primer Mix	Primer Mix	0.2 µM
100 Primer Mix	Primer Mix (5 or 6 primers = cps)	0.2 µM
100 Primer Mix	Primer Mix	0.2 µM
Template DNA	Bacterial suspension	-

REACTION 1	REACTION 2	REACTION 3	REACTION 4	REACTION 5
Serotype	Serotype	Serotype	Serotype	Serotype
Product size (bp)	Product size (bp)	Product size (bp)	Product size (bp)	Product size (bp)
6A/B 350	14 389	8 300	1 380	3 360
3 250	7C/B 200	19F 304	23F 384	33F/A 338
11A/D 463	12F/A 376	15B/C 376	18ABCF 496	15A 434
19A 566	4 430	34 408	18ABCF 573	38 574
17F 693	20 514	35F 517	30A 628	31 701
16F 988	22F/A 643	35B 677	9V/A 816	7F/A 826

Figure 1: Expected PCR products size for the five multiplex reactions

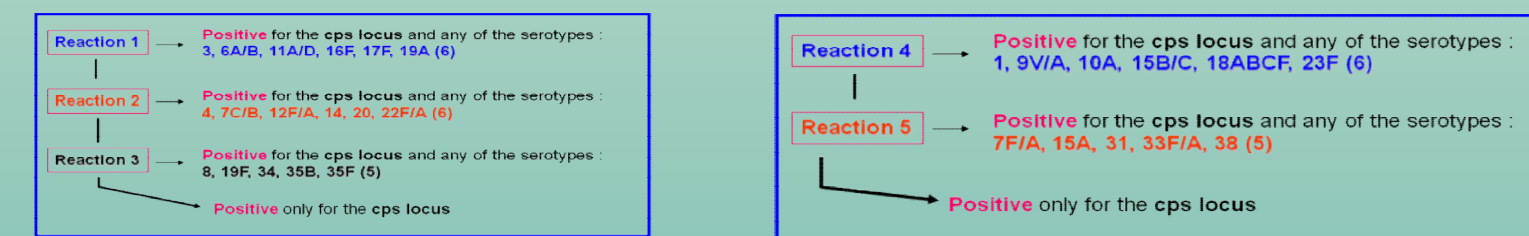


Figure 2: Multiplex PCR scheme (92 isolates tested in a single day)

Step 1: 92 isolates are tested in Step 1 for each experiment. Step 2: All isolates positive only for the cps locus in Step 1 are tested in Step 2.

All isolates positive only for the cps locus after the steps 1 and 2 are considered as "non-typeable by PCR" and are subjected to the complete Quellung checkerboard.

Conventional serotyping: capsular or Quellung reaction

A Quellung reaction is the result of the binding of the pneumococcal capsular polysaccharide with type specific antibody contained in the antiserum:

- An antigen-antibody reaction causes a change in the refractive index of the capsule and it appears "swollen" and more visible.
- The pneumococcal cell stains dark blue and is surrounded by a sharply demarcated halo, which represents the outer edge of the capsule. The light transmitted through the capsule appears brighter than either the pneumococcal cell or the background.
- Single cells, pairs, chains, and even clumps of cells may have Quellung reactions. Microscopic agglutination of the bacterial cells may also be visible.

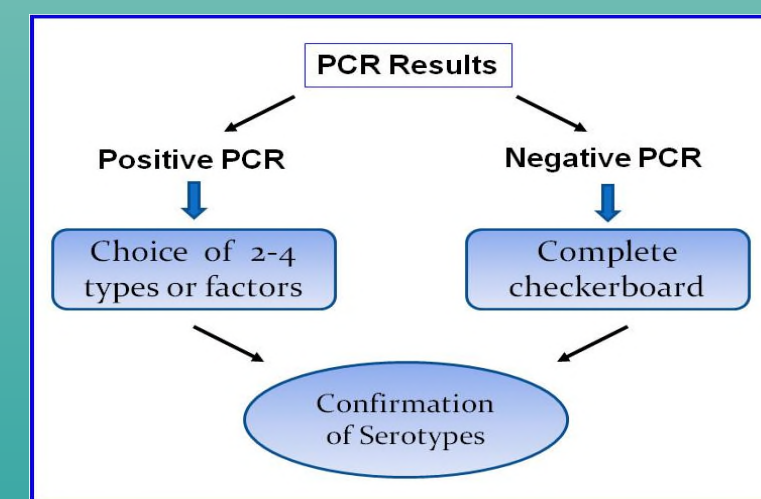


Figure 3: Quellung scheme from PCR data

Acknowledgements

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Results

Optimization of PCRs:

Figure 4: Representative multiplex reactions-28 control strains

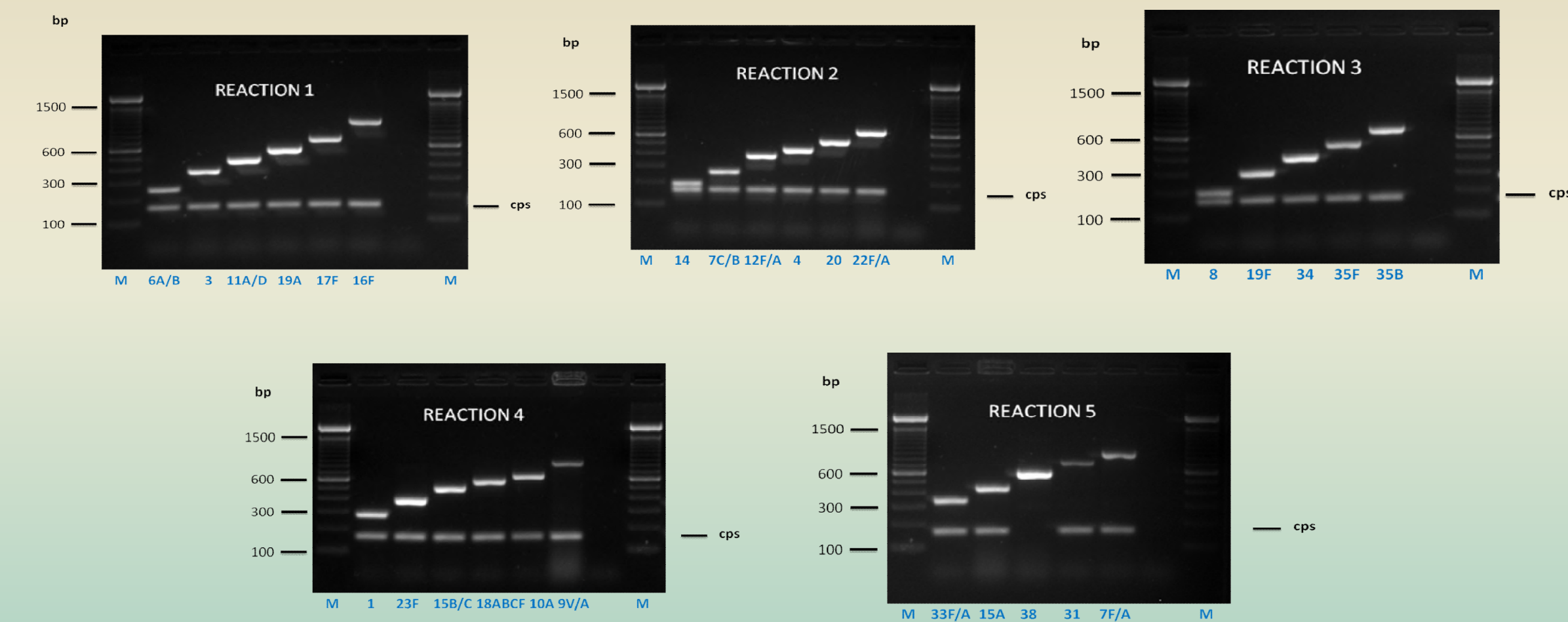


Figure 5: Deduction of pneumococcal serotypes and serogroups using multiplex PCR

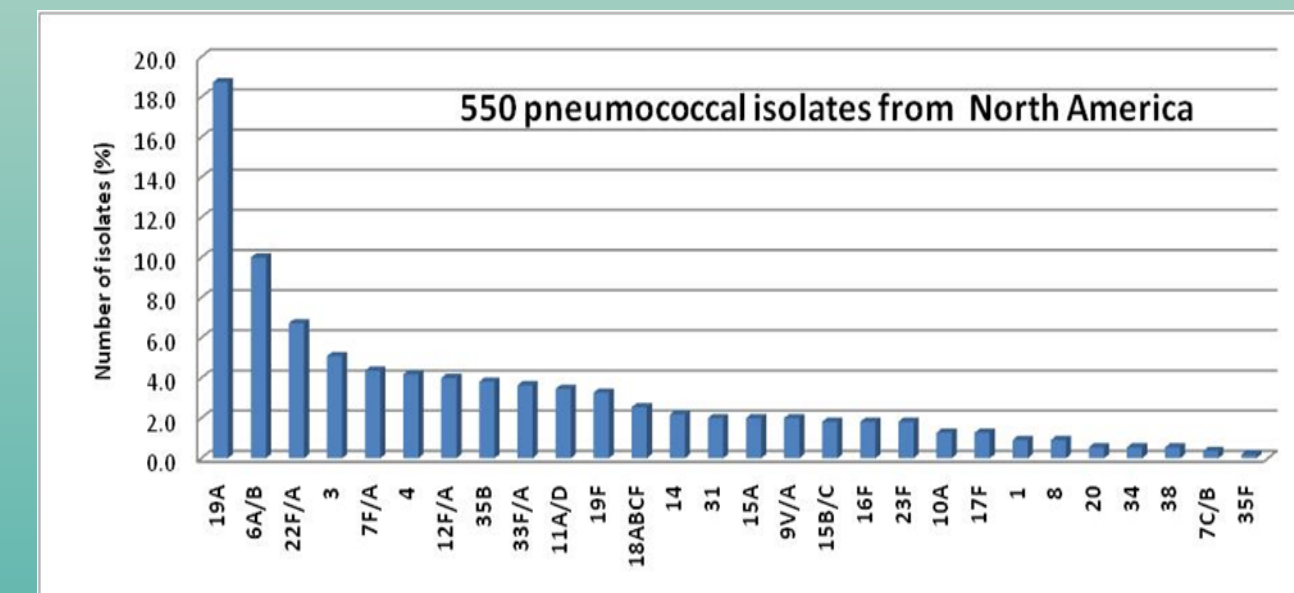


Table 1: Confirmation of pneumococcal serotypes and serogroups using Quellung reaction

Serotypes	Number of isolates	%
19A	103	18.7
6A/B	55	10.0
22F/A	37	6.7
3	28	5.1
7F/A	24	4.4
4	23	4.2
12F/A	22	4.0
35B	21	3.8
33F/A	20	3.6
11A/D	19	3.5
19F	18	3.3
18ABCF	14	2.5
14	12	2.2
31-15A-9V/A	11 of each	2.0
15B/C-16F-23F	10 of each	1.8
10A-17F	7 of each	1.3
1-8	5 of each	0.9
20-34-38	3 of each	0.5
7C/B	2	0.4
35F	1	0.2

65 non typeable isolates to be tested by Quellung (10%)

Conclusions

This study, performed on a large number of isolates, confirms that:

- The most common *Streptococcus pneumoniae* serotypes in the United States are:
 - Serotype 19A: 18.7%
 - Serotype 6 A/B: 10%
- Multiplex-PCR is an accurate, fast and cost effective method that is very useful in large scale studies.
- This multiplex-PCR and Quellung combination assay, which could be implemented in many microbiological laboratories, is very useful for monitoring serotype prevalence after the introduction of conjugate vaccines.