

## Revised Abstract

**Background:** *S. pneumoniae* remains an important pathogen and a major causative agent of infections among young children and the elderly. As antimicrobial resistance may be associated with specific serotypes, rapid and accurate methodologies for determining serotypes are essential in large epidemiological studies as well as clinical laboratories.

**Methods:** 2,173 *S. pneumoniae* clinical isolates were tested. We designed a 2-step assay for determining serotypes using multiplex PCR combined with the conventional Quellung. The first step was a PCR-based assay of two sequential sets of multiplex reactions to reliably deduce specific pneumococcal serotypes for a large number of isolates. All PCR results were confirmed by the conventional Quellung reaction as a second step. Non-typeable isolates by PCR were subjected to the complete Quellung checkerboard.

**Results:** The sequential PCR reactions enabled us to determine the serotype for 91% of the isolates with 100% correlation with the Quellung reaction. Overall, 9% of isolates were non-typeable by PCR. This PCR assay followed by the Quellung reaction allowed the testing of 92 isolates in no more than 3 working days. 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 different PCR reactions can easily be set up to reflect the current worldwide epidemiology.

**Conclusions:** This study, performed on a large number of isolates, confirms the accuracy and usefulness of multiplex-PCR as a first line assay for serotyping of *S. pneumoniae* which could be of valuable use in clinical microbiology laboratories and for monitoring sero-epidemiological changes. Furthermore, this high-throughput, fast and cost-effective PCR approach along with the Quellung reaction, could improve ascertainment of global pneumococcal serotype distributions.

## Introduction

*Streptococcus pneumoniae* is an important pathogen that causes severe life-threatening illnesses in the elderly and children. The capsule, a polysaccharide structure external to the cell wall that provides resistance to phagocytosis and permits evasion of the host immune system, is a major virulence factor of pneumococci, with the ability to cause disease directly related to the production of a capsule. The immunochimistry of this capsular polysaccharide differentiates pneumococci into 90 distinct serotypes [1]. However, only about 15 serotypes cause the majority of invasive pneumococcal disease worldwide [2]. 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 [3]. 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 [4]. Historically, the Quellung reaction (a swelling of the capsule when combined with serotype-specific antisera) has been the accepted method of serotyping. However, the high-cost of antisera, subjectivity in interpretation, and technical expertise requirements are limitations of this method. The development of PCR-based serotyping systems has the potential to overcome some of the difficulties associated with serologic testing [5, 6, 7] In this work, we describe a rapid, simple and cost-effective multiplex-PCR-based method combined with Quellung reaction to type pneumococci on a large scale and reduce the number of strains that may have to be serotyped by using the complete standard capsular reaction test.

## Materials & Methods

### Bacterial isolates

- 2,173 *S. pneumoniae* clinical isolates from 2004-2005 collected through global surveillance studies were tested.
- 28 control strains, representing the serotypes and serogroups targeted by multiplex-PCR, were used in this study.

### Table 1. Body Sites

Region	Number of isolates (%)
Blood	1,672 (76.9)
Fluids	241 (11.1)
Respiratory	219 (10.1)
Other	41 (1.9)
<b>Total</b>	<b>2,173</b>

### Table 2. Regions

Region	Number of isolates (%)
North America	1,279 (58.9)
Europe	480 (22.1)
Latin America	132 (6.07)
Middle East	84 (3.9)
South Pacific	83 (3.8)
Asia	82 (3.8)
Africa	42 (1.9)
<b>Total</b>	<b>2,173</b>

### Table 3. Departments

Departments	Number of isolates (%)
Medicine General/ICU	803/196 (37/9)
Emergency Room	520 (23.9)
Pediatric General/ICU	184/49 (8.5/2.2)
Surgery General/ICU	66/35 (3/1.6)
Clinic/Office	153 (7.1)
None Given	167 (7.7)
<b>Total</b>	<b>2,173</b>

### Capsular typing by multiplex-PCR

**DNA extraction:** a small amount of bacteria from an overnight culture plates was suspended to a 1 McFarland standard in TE buffer. The suspension was heated at 95°C and immediately frozen at -20°C at least for 5 min.

**Oligonucleotides primers:** the 28 oligonucleotides pairs [7] 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 [8].

**Multiplex PCR:** the QIAGEN Multiplex PCR kit (15 µL-volume reaction) was used to amplify the target DNA. Five different multiplex reactions were set up as described in Table 4 (55°C, 30 cycles).

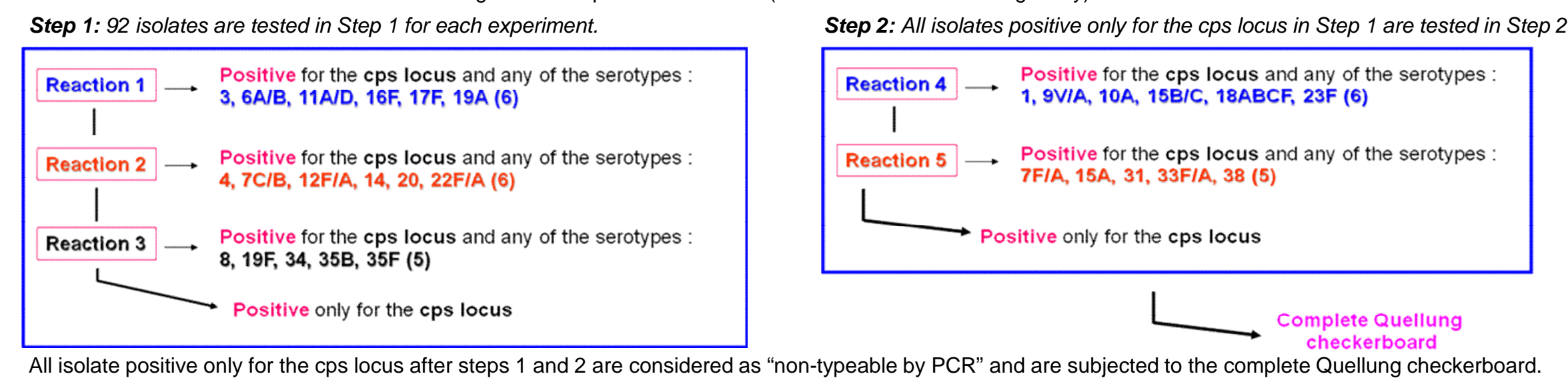
### Table 4.

Components	Description of components	Final Concentration
2X QIAGEN Multiplex PCR Master Mix (6 mM MgCl <sub>2</sub> )	-Hot Start Taq® DNA Polymerase -Multiplex PCR buffer -dNTP Mix	1X (3 mM MgCl <sub>2</sub> )
10X Primer Mix (2 µM each primer)	-Primers Mix (5 or 6 primers + cps)	0.2 µM
RNase-free water	-	-
Template DNA	-bacterial suspension	-

### Table 5: Expected PCR products size for the five multiplex reactions

REACTION 1		REACTION 2		REACTION 3		REACTION 4		REACTION 5	
Serotype	Product size (bp)	Serotype	Product size (bp)	Serotype	Product size (bp)	Serotype	Product size (bp)	Serotype	Product size (bp)
cps	160	cps	160	cps	160	cps	160	cps	160
6A/B	250	14	189	8	201	1	280	33F/A	338
3	371	7C/B	260	19F	304	23F	384	15A	434
11A/D	463	12F/A	376	34	408	15B/C	496	38	574
19A	566	4	430	35F	517	18ABCF	573	31	701
17F	693	20	514	10A	628	10A	628	7F/A	826
16F	988	22F/A	643	35B	677	9V/A	816		

Figure 1: Multiplex PCR scheme (92 isolates tested in a single day)

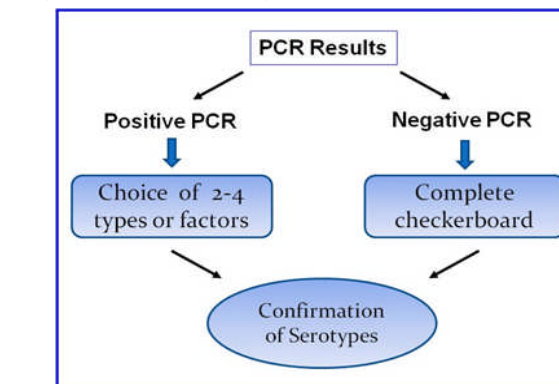


### Conventional serotyping: capsular or Quellung reaction

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 2: Quellung scheme from PCR data



## References

- Henrichsen, J. 1995. Six newly recognized types of *Streptococcus pneumoniae*. J. Clin. Microbiol. 33:2759-62.
- Hausdorff, W. P., J. Bryant, P. R. Paradiso, and G. R. Siber. 2000. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clin. Infect. Dis. 30:100-21.
- Ghaffar, F., T. Barton, J. Lozano, L. S. Muniz, P. Hicks, V. Gan, N. Ahmad, and G. H. McCracken, Jr. 2004. Effect of the 7-valent pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae* in the first 2 years of life. Clin. Infect. Dis. 39:930-8.
- Kellner, J. D., O. G. Vanderkooi, J. MacDonald, D. L. Church, G. J. Tyrrell, and D. W. Scheifele. 2009. Changing epidemiology of invasive pneumococcal disease in Canada, 1998-2007: update from the Calgary-area *Streptococcus pneumoniae* research (CASPER) study. Clin. Infect. Dis. 49:205-12.
- Dias, C. A., L. M. Teixeira, G. Carvalho Mda, and B. Beall. 2007. Sequential multiplex PCR for determining capsular serotypes of pneumococci recovered from Brazilian children. J. Med. Microbiol. 56:1185-8.
- Morais, L., G. Carvalho Mda, A. Roca, B. Flannery, I. Mandomando, M. Soriano-Gabarro, B. Sigauque, P. Alonso, and B. Beall. 2007. Sequential multiplex PCR for identifying pneumococcal capsular serotypes from South-Saharan African clinical isolates. J. Med. Microbiol. 56:1181-4.
- Pai, R., R. E. Gertz, and B. Beall. 2006. Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. J. Clin. Microbiol. 44:124-31.
- Mavroidi, A., D. Godoy, D. M. Aanensen, D. A. Robinson, S. K. Hollingshead, and B. G. Spratt. 2004. Evolutionary genetics of the capsular locus of serogroup 6 pneumococci. J. Bacteriol. 186:8181-92.

## Results

### Optimization of PCRs:

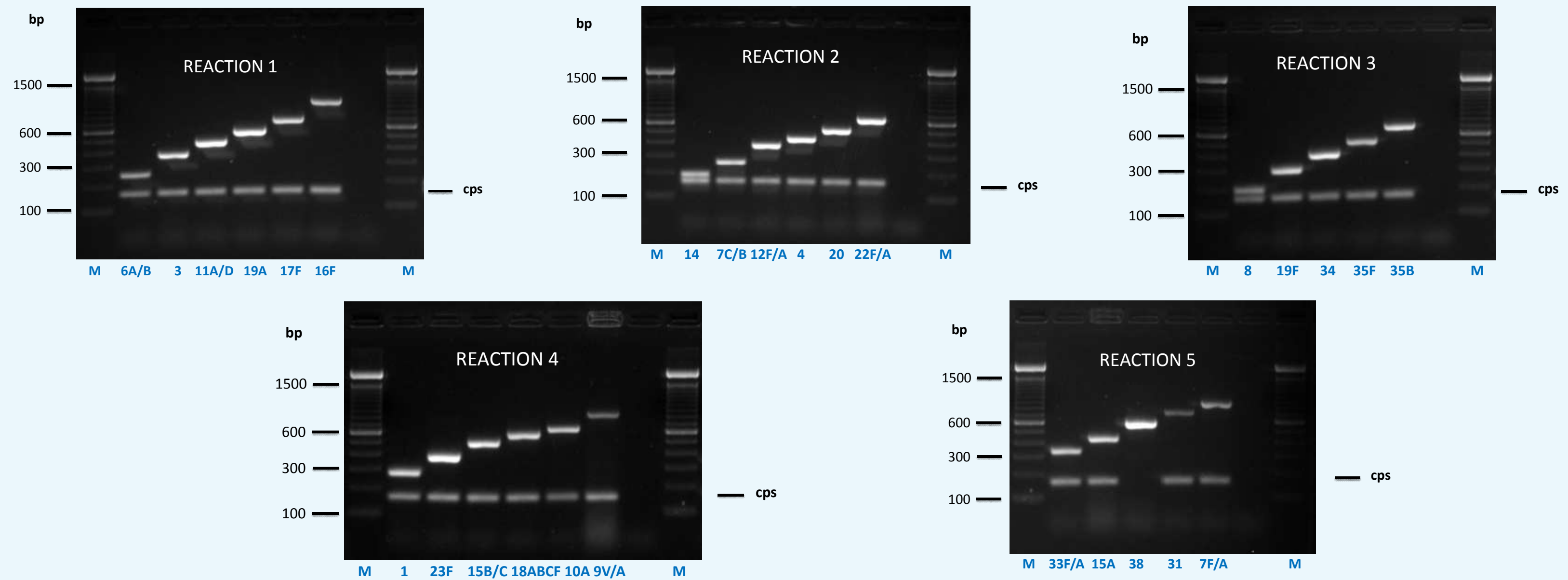


Figure 3: Representative multiplex reactions-28 control strains.

Steps 1 and 2 enabled us to determine 91% of the serotypes; the remaining (9%) were determined by the complete Quellung checkerboard.

Advantages of the combination of multiplex PCR-Quellung:

- Simple and fast DNA template preparation
- Minimal number of PCRs (5 reactions)
- Faster, more reliable and more cost-effective method than the conventional method
- Orients the Quellung reaction scheme by limiting the sera to be tested
- Only the non-typeable isolates must be tested using the complete Quellung checkerboard
- Very useful way of analyzing large numbers of isolates

Table 6: Confirmation of pneumococcal serotypes using Quellung reaction

Serotypes	Number of isolates
19A	338
3/7F/6A	132/142/150
14/19F/22F	94/96/117
23F/4/1/11A/6B	67/72/80/81/82
35B/9V/12F/23A	45/61/62/63
17F/38/31/33F/10A/15B/16F/8/18C/9N/15A	24/24/24/27/28/29/31/35/37/38/41
20/35F/15C/5/23B	13/14/17/18/19
33A/18B/7C/34/13	5/6/7/8/9
9L/10F/35A/18F/18A/21/24F/12A	3/3/3/3/3/4/4
28F/33B/28A/19B/25F/9A/7B/7A/29/11D	1/1/1/1/1/1/1/1/2

## Conclusions

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

- 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 and concurrent antibiotic susceptibility patterns.

## Acknowledgements

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