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Serotyping of pneumococci: evaluation of the genetic approach and performance with clinical samples

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ABSTRACT

Determination of pneumococcal serotypes depends on a successful culture and the Quellung's reaction. However, in 2006, the capsular sequences of 90 different pneumococcal capsular loci were published, thus making "genetic" serotyping via PCR possible. We wanted to determine the reliability of the published primers for the 13 serotypes included in pneumococcal conjugated vaccine 13 (PCV13) with pneumococcal isolates from Germany. We used a multiplex PCR approach and agarose gel detection of amplicons. Three hundred ninety well-characterized strains of *Streptococcus pneumoniae* and 46 clinical samples were used in the study. A 100% concordance was achieved between PCR and Quellung's reaction. In 7 clinical samples with a PCR positive for *S. pneumoniae*, we could determine a serotype included in PCV13.

1. Introduction

Pneumococcal vaccines are powerful tools to protect patients from disease. This was initially documented by Black et al. (2000), who first evaluated pneumococcal conjugated vaccine 7 (PCV7) vaccination in young children in California in 2000. The protection offered by polysaccharide and conjugated vaccines is serotype specific. The initial serotype composition of the respective vaccines was guided by the frequency of the serotypes causing invasive disease. Post-marketing surveillance looks into the development of the absolute and relative frequencies of vaccine and non-vaccine serotypes. The existence of different pneumococcal serotypes in human disease was recognized as early as the beginning of the 20th century. In 1915, Avery (1915) and Dochez and Avery (1915) described 4 different serotypes in strains from patients with lobar pneumonia. Currently, the most widely used method for serotyping is based on the method described by Neufeld in 1902, which was successfully used in a public health laboratory as early as 1934. At that time, in Boston, Beckler and Macleod (1934) determined 32 different pneumococcal serotypes directly from sputum. Currently, the Statens Serum Institute in Copenhagen (Denmark, www.ssi.dk) provides sera to differentiate 91 different serotypes. However, to use these sera, a pneumococcal culture is needed. Therefore, the ongoing surveillance relies on grown strains only.

As a consequence, culture-negative samples are lost to surveillance. In 2006, Bentley et al. (2006) published the capsular sequences of 90 pneumococcal serotypes, thus enabling the development of serotype-specific primers. This task was first undertaken by Pai et al. (2006) who published the sequences of 29 different primer pairs in 2006. We wanted to explore the possibility to use these primers to "genetically" serotype pneumococci in general and their capacity to type patient samples, which were culture negative.

We decided to establish and validate a multiplex PCR method based on the primers previously published and limited our approach to the 13 serotypes included in the 13-valent conjugated vaccine because this is the vaccine mainly used for routine childhood vaccination in Germany since its release in 2009. To distinguish the members of the serogroup 6, we combined the data from Pai et al. (2006) with Jin et al. (2009).

2. Materials and methods

Primer sequences are as described in Pai et al. (2006). Contrary to the original description, primers for serogroup 6 were used unbiotinylated. Primers were divided into 3 different groups, group 1 (serotype 1, 9 V, 14, 23 F), group 2 (serotype 5, 6 [serogroup], 7 F, 19A), and group 3 (serotype 3, 4, 18 [serogroup], 19 F). Primers for the detection of the *cpsA* gene (present in all serotypes studied) were included in each group and used as an internal control (to check for the presence of a pneumococcus DNA and to detect PCR inhibition.). For further dividing serogroup 6 into 6A, 6B, 6C, and 6D, primers and protocols published by Jin et al. (2009) were used. For a complete list of all primers, see Table 1.

We validated our primer groups with DNA from clinical strains with known serotype. All strains used belonged to the pneumococcal conjugated vaccine 13 (PCV13) serotypes/serogroups. Non-PCV13 serotypes/serogroups were not used. All strains had been isolated from patients with invasive disease, i.e., meningitis or septicemia.

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Table 1
Primer sequences with sources.

Included in primer group	Serotype	Primer sequences (5′→3′)	Fragment length (bp)	Final concentration (μmol/L)	Source
Group 1	1	Forward: CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA Reverse: CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C	280	1.5	Pai et al. (2006)
Group 1	9 V	Forward: CTT CGT TAG TTA AAA TTC TAA ATT TTT CTA AG Reverse: GTC CCA ATA CCA GTC CTT GCA ACA CAA G	753	1.5	Pai et al. (2006)
Group 1	14	Forward: CTT GGC GCA GGT GTC AGA ATT CCC TCT AC Reverse: GCC AAA ATA CTG ACA AAG CTA GAA TAT AGC C	208	1.0	Pai et al. (2006)
Group 1	23 F	Forward: GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC Reverse: CAC AAC ACC TAA CAC ACG ATG GCT ATA TGA TTC	384	1.5	Pai et al. (2006)
Group 2	5	Forward: ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG Reverse: GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG	362	0.5	Pai et al. (2006)
Group 2	6 (serogroup)	Forward: AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG Reverse: TTA GCG GAG ATA ATT TAA AAT GAT GAC TA	250	0.5	Pai et al. (2006)
Group 2	7 F	Forward: CCT ACG GGA GGA TAT AAA ATT ATT TTT GAG Reverse: CAA ATA CAC CAC TAT AGG CTG TTG AGA CTA AC	826	2.0	Pai et al. (2006)
Group 2	19A	Forward: GTT AGT CCT GTT TTA GAT TTA TTT GGT GAT GT Reverse: GAG CAG TCA ATA AGA TGA GAC GAT AGT TAG	478	1.0	Pai et al. (2006)
Group 3	3	Forward: ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G Reverse: CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G	371	1.5	Pai et al. (2006)
Group 3	4	Forward: CTG TTA CTT GTT CTG GAC TCT CGA TAA TTG G Reverse: GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G	430	1.5	Pai et al. (2006)
Group 3	18 (serogroup)	Forward: CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC Reverse: TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC	573	1.25	Pai et al. (2006)
Group 3	19 F	Forward: GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C Reverse: GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG	304	1.5	Pai et al. (2006)
Group 1, 2, 3	cpsA	Forward: GCA GTA CAG CAG TTT GTT GGA CTG ACC Reverse: GAA TAT TTT CAT TAT CAG TCC CAG TC	160	0.5	Pai et al. (2006)
n.a.	6A	Forward: ATTTATATATAGAAAAACTGGCTCATGATAG Reverse: GCGGAGATAATTTAAAATGATGACTAGTTG	149	1.0	Jin et al. (2009)
n.a.	6B	Forward: AAGATTATTTATATATAGAAAAACTGTCTCATGATAA Reverse: GCGGAGATAATTTAAAATGATGACTAGTTG	155	1.0	Jin et al. (2009)
n.a.	6C	Forward: ATCTCTAAATCTGAATATGAAGCGGCTCAATC Reverse: GAACTGAGCTAAATAATCCTCTGGATTATCCACC	359	1.0	Jin et al. (2009)
n.a.	6D	Forward: ATCTCTAAATCTGAATATGAAGCGGCTCAATC Reverse: GAACTGAGCTAAATAATCCTCTGGATTATCCACC	359	1.0	Jin et al. (2009)

n.a. = not applicable; primers for determination of serotype 6C and 6D are identical, a "serotype" 6A with a positive PCR with primers for serotype 6C/D is serotype 6C, and a "serotype" 6B with a positive PCR with primers for serotype 6C/D is serotype 6D.

Serotyping had been done by the National Reference Center for Streptococci in Aachen, Germany. From each serotype, DNA from a minimum of 20 different strains was used except serotype 5 (only 7 strains available; see Table 2). Strains for evaluation of the primer groups were grown on Columbia agar 5% sheep blood (BD, Heidelberg, Germany) overnight at 36 °C with 5% CO_2. DNA preparation was done as follows: 3–5 colonies were suspended into TRIS-Acetate-EDTA (TAE) buffer with glass beads in a 1.5-mL Eppendorf tube, heated to 95 °C for 5 minutes, shaken for 10 minutes, centrifuged for 3 minutes at 13,000 rpm, and frozen at −80 °C until usage.

PCR was performed in a volume of 25 μL (12.5 μL MMX Fast start [Roche, Berlin, Germany], 2.5 μL DNA, 1 μL of each primer, and water up to 25 μL). The following final primer concentrations were used: 0.5 μmol/L: cpsA, serotype 5, serogroup 6; 1.0 μmol/L: serotype 19A, 14; 1.25 μmol/L: serogroup 18; 1.5 μmol/L: serotype 1, 3, 4, 9 V, 19 F, 23 F; 2.0 μmol/L: serotype 7 F. PCR programs (30 cycles) were as follows: 94 °C 45 seconds, 54 °C 45 seconds, 65 °C Xmin. Primer group 1: X = 2.5 min; Primer group 2: X = 2 min; Primer group 3: X = 1.5 min. Each PCR was performed once for each strain.

Table 2
Number and serotypes of pneumococcal strains used for validation.

Serotype	No. of strains tested
1	70
3	23
4	25
5	7
6A	26
6B	23
7 F	26
9 V	23
14	32
18C	22
19A	24
19 F	24
23 F	65
Total	390

PCR samples were analyzed using 2% agarose gels with a 100-bp ladder and run for 45 minutes at 120 V. Amplicon sizes were estimated using a 100-bp ladder. Sizes were compared to the expected sizes of the different serotypes and to the positive controls included in each run. Inhibition was excluded by a positive cpsA PCR in each sample. Agreement of both methods was statistically tested by Cohen's kappa test using SPSS (version 19) by IBM, Ehningen, Germany.

Clinical samples were acquired between November 2011 and March 2013. DNA had been prepared using Qiagen extraction kit (QIAamp DNA minikit) and DNA stored at −20 °C. DNA from 46 materials was available. All clinical material with a negative cpsA PCR was checked for PCR inhibition by spiking with 1 μL of pneumococcal DNA. Pneumococcal DNA was prepared as described above. 95% confidence intervals (CIs) for the proportion of "PCR-serotyped" samples of all cpsA-positive samples were calculated using binomial exact methods with SPSS (version 19).

3. Results

Evaluation of our primer groups was done with DNA from 390 different serotyped strains. We did not find any cross-reaction between serotypes. Each strain produced a single amplicon. Initially, 362 of 390 (93%) of the PCR results matched with the serotype determined with the Quellung's reaction (kappa = 0.92).

However, for 28 strains (primer group 1, 2, and 3: 16, 3, and 9 strains, respectively), the initial results were different between Quellung's reaction and the PCR approach.