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ORIGINAL ARTICLE

Comparison of Four Methods for *Streptococcus pneumoniae* Capsular Typing

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SUMMARY

Background: **Pneumococcal capsular-type identification is essential for monitoring the epidemiology of pneumococcal infections and for establishing the effectiveness of pneumococcal vaccines. The objective of this work was to compare the accuracy of four methods of *Streptococcus pneumoniae* capsular typing.**
Methods: **A prospective blind study was carried out at Donostia University Hospital (northern Spain) to determine the capsular types of 50 pneumococcal clinical isolates using four techniques:**
a) S. PneumoStrip™: a reverse-hybridization strip-based commercial assay that detects 76 pneumococcal serotypes: 42 individually and 34 in pairs.
b) FAF-mPCR: a single-step multiplex-PCR (polymerase chain reaction) assay combined with fragment analysis using automated fluorescent capillary electrophoresis, which can differentiate 92 serotypes in a single tube: 31 individually, 28 in pairs, and 33 in groups of 3 to 5 serotypes.
c) PCRSeqTyping: which enables the detection of 91 serotypes after sequencing the regions of the *cpsB* gene in two steps: 59 directly and the remaining 32 serotypes in a second step.
d) The Quellung reaction.
Results: **The S. PneumoStrip™, FAF-mPCR and PCRSeqTyping identified the serotypes of all the 50 clinical isolates. With the Quellung reaction 46/50 (92%) isolates were correctly serotyped. The quickest technique was the S. PneumoStrip™, followed by the single-step multiplex PCR assay and PCRSeqTyping. The Quellung reaction was the slowest technique.**
Conclusions: **The S. PneumoStrip™, PCRSeqTyping, and FAF-mPCR were very accurate techniques for pneumococcal serotyping, with S. PneumoStripTM obtaining results more rapidly. The combination of any of these *S. pneumoniae* molecular typing techniques and the Quellung reaction as confirmation reference method is a highly precise and fast strategy for the serotyping of high number of pneumococcal clinical isolates.**
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KEY WORDS

S. PneumoStrip™, FAF-mPCR, PCRSeqTyping, Quellung reaction

INTRODUCTION

Streptococcus pneumoniae is a frequent commensal of the human oropharyngeal microbiota but also a pathogen with important clinical significance. The surfaces of

most pneumococcal clinical isolates are covered by the capsule, which is considered to be the main virulence factor of this pathogen [1]. Depending on the specific antibody response generated by the various capsular polysaccharides, 100 pneumococci capsule types (also called serotypes) have been described to date [2].

From a clinical-epidemiological standpoint, determining *S. pneumoniae* serotypes is one of the keys in fighting pneumococcal infection, as it provides information about the serotypes causing severe invasive diseases, the effectiveness of current vaccines, the theoretical coverage of future vaccines and the prevalence of antibiotic resistance associated with specific serotypes.

Most of the techniques for identifying *S. pneumoniae* serotypes are based on techniques that use antibodies to recognize antigenic differences in the capsule, or based on techniques that detect the sequence diversity of the genes encoding the different capsules. Among antibody-based techniques, the Quellung reaction (or the Neufeld reaction) stands out as the gold standard for *S. pneumoniae* serotyping [3].

From a practical point of view, 92 serotypes can be currently identified with the specific sera provided by the Staten Serum Institute [3] by the Quellung reaction.

The Quellung's time-consuming nature, coupled with the difficulty of obtaining and preserving all of the necessary antisera for the final identification of all serotypes and the impossibility of studying batches of isolates has restricted its use to specialized reference and research laboratories.

The first work using molecular techniques for detecting the genes encoding the *S. pneumoniae* capsular loci appeared in 2003, in which seven multiplex-PCRs (polymerase chain reaction) were used to identify 9 serotypes (1, 3, 4, 6B, 14, 18C, 19F, 19A, and 23F) [4]. However, it was not until 2006 that the sequences of the capsular genes of the 92 serotypes known to that date were completed [5]. This knowledge made the detection of all different *S. pneumoniae* serotypes possible via nucleic acid amplification techniques (PCR) followed by hybridization of membranes [6] or deoxyribonucleic acid (DNA) arrays [7]. In general, PCR-based typing methods are rapid and sensitive to determine the capsular types; however, the high sequence similarity between the capsular genes of some serotypes makes a second method necessary to establish a single serotype [6].

The use of new technologies such as nuclear magnetic resonance along with next-generation sequencing has led to the description of new serotypes, with the last, serotype 7D, being reported in 2018 [8,9].

The main objective of this work was to compare the accuracy of three molecular methods for *S. pneumoniae* serotyping compared with the Quellung reaction.

MATERIALS AND METHODS

Isolates

A double-blind study comparing the effectiveness of four serotyping techniques for *S. pneumoniae* was performed in 2020 at the Microbiology Department of Donostia University Hospital (Donostia-San Sebastian, Spain). Fifty *S. pneumoniae* strains isolated from invasive disease and otitis media during 2019, previously identified via the optochin sensitivity test, bile solubility test, and recA [10] sequencing, were randomly selected for the study.

Typing techniques

The strains were serotyped using four typing techniques:

a) The S. PneumoStrip™, a reverse-hybridization strip - based commercial assay (Operon SL, Zaragoza, Spain) that detects 76 pneumococcal serotypes: 42 individually, and 34 in pairs [11,12]. The S. PneumoStrip™ hybridization was automatically performed using an Auto-LiPA 48 device (Innogenetics).

b) An "in house" single-step multiplex-PCR assay combined with fragment analysis using automated fluorescent capillary electrophoresis (FAF-mPCR), which can differentiate 92 serotypes in a single tube: 31 individually, 28 in pairs, and 33 in groups of 3 - 5 serotypes [13]. Fluorescent fragment size analysis was performed in an ABI Prism 3130xl/Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

c) PCRSeqTyping, which enables the detection of 91 serotypes by sequencing regions of the cpsB gene in two steps: 59 directly and the remaining 32 serotypes in a second step [14]. SANGER sequencing of amplified products was performed using the ABI Prism 3130xl/ Genetic Analyzer.

d) The Quellung reaction, using the sera available at the Staten's Serum Institute (Copenhagen, Denmark) [3].

Statistical analysis

Fisher's test was used to compare the accuracy of each technique to serotype an isolate at the serotype level using the GraphPad InStat version 3.0 for Windows, GraphPad Software (La Jolla, CA, USA) [15]. Statistical significance was established at $p < 0.05$ (two-tailed).

RESULTS

Twenty-five different serotypes were detected in the 50 isolates analyzed: serotype 3 (n = 10), 4, 5, 6A (n = 2), 6B, 6C (n = 2), 7F, 7C (n = 3), 8 (n = 4), 9V, 9N, 10A (n = 2), 11A (n = 3), 14, 15A, 17F, 19F, 19A (n = 2), 23F, 23A (n = 2), 24F (n = 2), 29, 33A, 35F, 38 (n = 4). The three techniques based on molecular typing showed 100% concordance in the results obtained, but the steps to obtain the final identification were different.

Table 1. Comparison of the *S. pneumoniae* molecular typing techniques for detecting at the serotype level the capsular type of 92 of the 100 serotypes described to date.

Serotype	Isolates in the study	Additional technique [1]		
		S. PneumoStrip™	FAF-mPCR	PCRSeqTyping
Serotypes included in the study				
3	10	-	-	-
4	1	-	-	-
5	1	-	-	-
6A	2	-	quellung	-
6B	1	-	quellung	-
6C	2	-	quellung	-
7F	1	quellung	quellung	-
7C	3	quellung	quellung	-
8	4	-	-	-
9V	1	quellung	quellung	additional PCR
9N	1	quellung	quellung	-
10A	2	-	quellung	-
11A	3	quellung	quellung	additional PCR
14	1	-	-	-
15A	1	-	quellung	-
17F	1	-	-	-
19F	1	-	-	-
19A	2	-	-	-
23F	1	-	-	-
23A	2	-	-	-
24F	2	quellung	quellung	-
29	1	ND	-	-
33A	1	quellung	quellung	additional PCR
35F	1	-	quellung	additional PCR
38	4	-	quellung	-
Serotypes not included in the study				
1, 16F, 21, 23B, 31, 33C		-	-	-
2, 20		-	-	additional PCR
6D, 9L, 10B, 11F, 15F, 18A, 24A, 35A, 37, 41F, 47F		-	quellung	-
7B, 11B, 11C, 12F, 18F, 35C		-	quellung	additional PCR
36		quellung	-	-
44		quellung	-	additional PCR
7A, 12A, 12B, 15B, 15C, 18B, 18C, 19B, 19C, 24B, 33B, 33D		quellung	quellung	-
9A, 10F, 10C, 11D, 22F, 22A, 25F, 25A, 32F, 32A, 33F, 46		quellung	quellung	additional PCR
16A, 27, 39, 45, 47A, 48		ND [2]	-	-
17A, 34, 35B		ND	-	additional PCR
28F, 28A, 42, 43		ND	quellung	-
40		ND	quellung	additional PCR

[1]. Additional technique required to achieve the result at the serotype level.
[2]. ND - Not detected with this method.

DISCUSSION

Three PCR-based methods were analyzed in this study. Serotyping *S. pneumoniae* via multiplex-PCR has been developed over time, with the first one described in 2003, amplifying the serotypes that caused the majority of invasive disease at that time [16]. The development of other tools, such as massive DNA sequencing or nanotechnology, has enabled the description and detection of the 100 serotypes described to date [8,9].

The S. PneumoStrip™ test is a commercially available serotyping technique that allows for the identification of 76 pneumococcal serotypes by reverse-hybridization and does not require sequencing. Among the advantages of the S. PneumoStrip™ test is the use of common laboratory equipment (a conventional PCR thermocycler and a hybridization device). The need for an automated hybridization device could be regarded as a problem, but reverse hybridization can also be performed in manually operated incubators. The S. PneumoStrip™ test proved to be a useful technique for identifying most *S. pneumoniae* serotypes. In fact, the results were comparable with those obtained with the FAF-mPCR and the PCRSeqTyping. Although most circulating serotypes can be determined by this technique, some infrequent ones, such as serotype 29 in our series, will not be detected.

The second technique studied was an in-house developed FAF-mPCR. The advantages of this method include a lower cost compared with the Quellung reaction (~4.50 euro vs. ~17 euros) [16] and the operating speed (92 serotypes are detected in only one reaction at the serotype or serogroup level). The main limitation of this method is the requirement of an automated fluorescent fragment-size analyzer and the inability to distinguish between serotypes with similar *wzy* genes, although it greatly guides and reduces the number of Quellung reactions needed to determine the specific serotypes [12].

The other technique that required an automated sequencer for pneumococcal capsular typing was the PCRSeqTyping [13]. The effectiveness of this technique was 100%. Compared with the 31 serotypes detected individually with the FAF-mPCR, the PCRSeqTyping is able to detect 59 serotypes individually in a single step. For the remaining 32 serotypes, a second PCR of 10 possible is needed.

The Quellung reaction is the most used technique and the gold standard for pneumococcal serotyping [17]. A study performed in 2005 in 11 European cities to evaluate the quality of serotyping concluded that the most frequent mistakes included a wrong serotype within the correct serogroup as a consequence of not using the entire factor sera needed to correctly identify each strain of pneumococcus [18].

Although there are many reports in the literature on *S. pneumoniae* serotyping methods, few of them compare their efficacy. In this work, we have compared three molecular techniques with the Quellung reaction, still the gold standard today. The three molecular tests showed 100% specificity but all of them needed a second step for a full serotype identification: a Quellung reaction in the S. PneumoStrip™ and the FAF-mPCR tests, and a second PCR in the PCRSeqTyping.

When a capsular type is identified by a molecular technique, it must be confirmed with the Quellung reaction, still the gold-standard technique for serotype identification. Nevertheless, molecular test showed 100% specificity in their targeted serotypes, greatly reduced the number of Quellung reactions to be performed, and allowed the possibility of working in batches of isolates greatly reducing the serotyping turnaround time. The three molecular typing methods studied showed similar performance results, and the selection of one of them to improve pneumococcal typing should be done according to the technical capabilities of each laboratory.

Declaration of Interest:
The authors have declared no competing interests.

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