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A reverse-hybridization test for the identification of 76 pneumococcal serotypes, 42 individually and 34 in pairs

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ABSTRACT

The *S. PneumoStrip* test is a recently developed reverse hybridization strip-based commercial assay that allows for the identification of 76 pneumococcal serotypes, 42 individually and 34 in pairs, according to their specific gene sequences. The test was validated with reference strains of 92 different pneumococcal serotypes and with a selection of 75 clinical isolates representing 55 serotypes, showing 100% sensitivity and specificity. The test was also applied to 64 pneumococcal invasive isolates (23 different serotypes) consecutively collected between June 2016 and March 2017, with 60 (93.8%) being serotyped. Four isolates belonging to serotypes 13, 29, and 35B (2 isolates), which are not included in the test, did not produce a hybridization signal with serotype specific probes. The identification of most serotypes causing invasive pneumococcal disease together with the simplicity of performance and results interpretation, and the use of routine laboratory equipment make this test very suitable for most clinical and research laboratories.

1. Introduction

Streptococcus pneumoniae (pneumococcus) is a human pathogen that frequently causes respiratory diseases such as community acquired pneumonia or otitis media, and less frequently invasive disease as meningitis or bacteremia (Centers for Disease Control and Prevention, n.d.). The external polysaccharide capsule constitutes the pneumococcal major virulence factor, being a highly immunogenic component (Griffith, 1928). Pneumococcal capsule is composed of complex polysaccharides (Kamerling, 2000) and more than 90 different capsular polysaccharide types (serotypes) have been described to date according to their reaction with specific antisera in the Quellung reaction.

Pneumococcal conjugate vaccines (PCV) commercialized since 2000 contain the polysaccharides of the serotypes most frequently causing invasive pneumococcal disease (IPD) in children, as the polysaccharide of all serotypes cannot be included in a unique vaccine. Although PCV have been very successful decreasing the incidence of IPD due to vaccine serotypes in vaccinated and unvaccinated populations due to herd protection, a replacement of serotypes causing disease has been observed (Flasche et al., 2011; Weinberger et al., 2011; Weil-Olivier et al., 2012). This serotype replacement necessitates continuous surveillance of serotypes responsible for IPD, not only to monitor the replacement events but to determine current vaccines´ efficacy, to design future vaccines and to control the possible emergence of particularly virulent serotypes. To achieve this goal, robust capsular typing tools capable of determining a wide range of serotypes in an easy way are needed. Ideally, these tools should be quick and simple and with a high sensitivity and specificity in order to be implemented in the daily work of clinical laboratories.

Genes for pneumococcal capsular synthesis are located in the capsular polysaccharide synthesis (*cps*) locus, which shows enough genetic divergence between serotypes to enable the determination of serotypes on the basis of molecular techniques (Kong et al., 2005; Tarragó et al., 2008; Tomita et al., 2011; Leung et al., 2012; Marimón et al., 2016). In general, a very good agreement between results obtained by molecular methods and the Quellung reaction has been demonstrated. Of the molecular techniques described, multiplex-PCR is one of the most simple and frequently used, identifying different number of serotypes on the basis of the different length of the amplicons obtained (Marimón et al., 2016; Brito et al., 2003; Pai et al., 2006; Coskun-Ari et al., 2012; Richter et al., 2013). However, the different multiplex-PCR designed showed the same problem that the similarity of capsular genes between related serotypes gave amplification products of the same length, making impossible to identify individually some serotypes. Amplicon

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Table 1
Distribution of controls and serotypes or serogroups in the three strips of the S. PneumoStrip test.

Line	Strip A	Strip B	Strip C
control	Color development	Color development	Color development
control	*lytA*[a]	*ply*[b]	*lytA*[a]
control	*cpsA*[c]		
1	1	2	7B
2	3	8	7C/40
3	4	9L/9N	15A
4	5	10A	15F
5	6A	10B	16F
6	6B	10F/10C	19B/19C
7	6C	11A/11D	21
8	6D	11B	25F/25A
9	7F/7A	11C	38
10	9A/9V	11F	24A
11	14	12A/46	24B/24F
12	18A	12B/44	31
13	18B/18C	12F	32F/32A
14	18F	15B/15C	33B/33D
15	19A	17F	33C
16	19F	20	35A
17	23A	22F/22A	35C
18	23B	33F/33A	35F
19	23F	37	47F
20			41A
21			41F

[a] *lytA*: *Streptococcus pneumoniae* autolysin gene.
[b] *ply*: *Streptococcus pneumoniae* pneumolysin gene.
[c] *cpsA:* capsular gene.

hybridization with serotype-specific probes could distinguish between amplicons of the same size but with different sequences without the need of using more sophisticated techniques as sequencing. The objective of this work was to develop and assess the performance of the "S. PneumoStrip test", a commercial strip reverse-hybridization assay for easy and robust pneumococcal typing from culture, based on multiplex-PCR followed by semi-automated strip hybridization.

2. Materials and methods

The commercially available "S. PneumoStrip test" (OPERON S.A., Zaragoza, Spain) is a reverse-hybridization based technique that allows for the identification of pneumococcal serotypes according to their specific *cps* gene cluster sequences. The design of the primers and probes of the different serotypes included in the test was based on the sequences of the genes encoding the pneumococcal capsule (the *cps* gene cluster) by the Sanger Institute (Bentley et al., 2006) and hosted at the NCBI webpage accession numbers from CR931632 (serotype 1) to CR931722 (serotype 48). The identification of serotypes 6C and 6D was based on the sequence of the *wciN* gene (Park et al., 2007).The design of the primers and probes was performed by Operon S.A. (Zaragoza, Spain) and are protected for commercial purposes. All Quellung reactions were performed at the Microbiology Department of University Hospital Donostia (UHD) using polyclonal rabbit antisera form the Statens Serum Institute (SSI, Copenhagen, Denmark).

2.1. S. PneumoStrip procedure

The procedure for the S. PneumoStrip test comprises three steps: DNA extraction, amplification by multiplex-PCR and strip reverse-hybridization. Briefly, DNA was extracted by boiling for 15 min a suspension of approximately 5 separated colonies in 100 µl of distilled water. After centrifugation at 12,000 rpm for 2 min, 5 µl of the supernatant were used in the PCR. Target amplification was performed by a multiplex-PCR containing the 43 pair of primers for the 76 serotypes and 3 identification genes in a unique reaction tube using a conventional thermocycler (Applied Biosystems) and the following conditions: denaturation step of 5 min at 96 °C, 40 cycles of 15 s at 96 °C, 1 min at 58 °C and 30 s at 72 °C and a final extension step of 10 min at 72 °C. Denatured amplicons were subjected to reverse hybridization using nylon strips with the serotype-specific probes immobilized in specific positions and an automated procedure on an Auto-LIPA 48 instrument (Innogenetics N.V., Gent, Belgium).

The S. PneumoStrip test contains three strips for each sample named A, B and C, each one containing a development control line and probes for controls, serogroups and/or serotypes. Strip A was designed to contain the serotypes included in the PCV13; strip B to contain serotypes included in the 23-valent pneumococcal polysaccharide vaccine (PPV23) and strip C to contain the serotypes more commonly causing invasive disease in our region (Gipuzkoa, northern Spain) during 2015 and 2016. Besides the specific lines for serotype identifications (Table 1), strips A and C included a *lytA* (autolysin gene) probe line to identify pneumococci. Strip A also included a *cpsA* probe line, indicating whether the isolate is a capsulated or unencapsulated pneumococcus, except for serotypes 25F, 25A and 38 that have an altered *cpsA* gene (Leung et al., 2012). Strip B also included a *ply* (pneumolysin gene) probe line to confirm the identification of the isolate as *S. pneumoniae*.

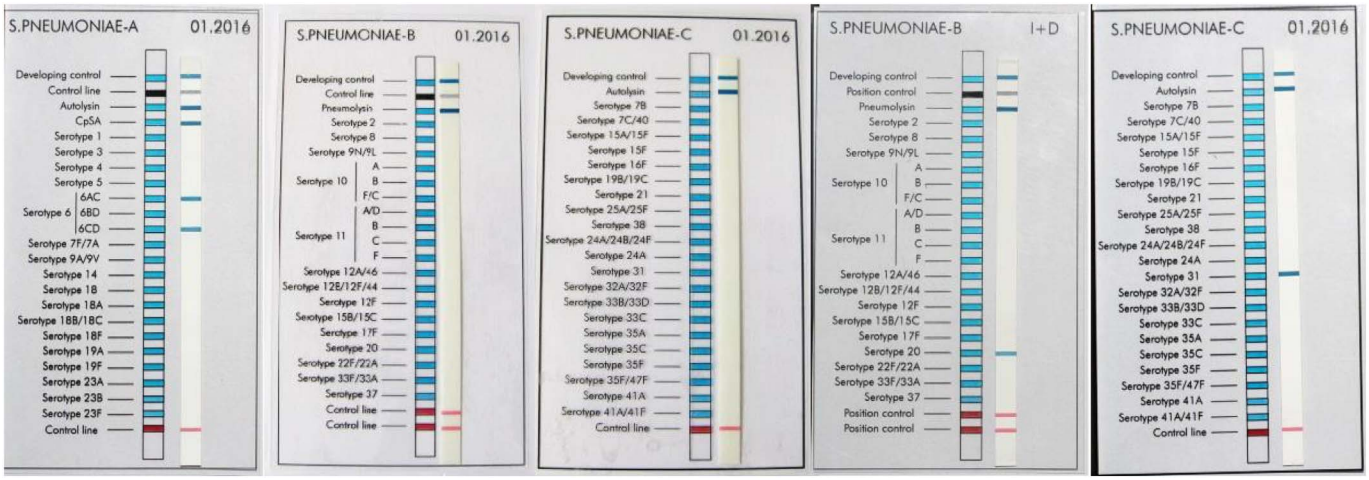

Fig. 1. Results and interpretation of the serotyping of three *Streptococcus pneumoniae* isolates serotypes 6C, 20, and 31 using the S. PneumoStrip test. For serotype 6C, the results of strips A, B, and C are shown. For serotypes 20 and 31, only the results of the strips showing hybridization with the corresponding serotype band, strips B and C respectively, is shown.