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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*.

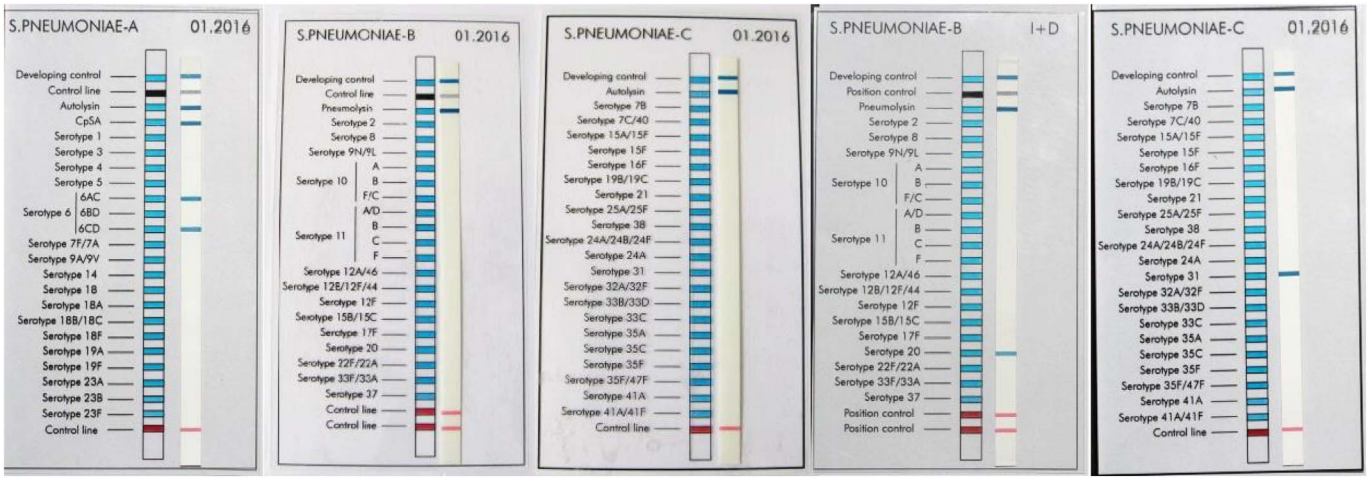

Fig. 1. Results and interpretation of the serotyping of three *Streptococcus pneumoniae* isolates serotypes 6C, 20, and 31 using the S. PneumoStrip test. For serotype 6C, the results of strips A, B, and C are shown. For serotypes 20 and 31, only the results of the strips showing hybridization with the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

Table 2
Comparison of clinical isolates serotyped by the Quellung reaction and the S. PneumoStrip test.

Serotype	No. of selected clinical isolates (University Hospital Donostia collection)	No. of unselected invasive clinical isolates prospectively collected (June 2016–March 2017)	Serotype by S. PneumoStrip
1	2		1
2	1		2
3	5	9	3
4	1	1	4
5	1		5
6A	1		6A
6B	1		6B
6C	2	2	6C
7F	1		7F/A
7A	1		7F/A
8	1	7	8
9A	1		9A/V
9L	1		9N/L
9N	1		9N/L
9V	1	1	9A/V
10F	1		10F/C
10A	1	4	10A
10B	1		10B
11A	1	7	11A/D
11D	1		11A/D
12F	1	4	12F
12A	1		12A/46
13[a]		1	None
14	1	2	14
15A	3		15A/F
15B	1	3	15B/C
15C	1		15B/C
16F	3		16F
17F	4		17F
17A[a]	1		None
18A	1		18A
18B	1		18B/C
18C	1	1	18B/C
19F	1	1	19F
19A	2	2	19A
20	1		20
21	2		21
22F	1	8	22F/A
22A	1		22F/A
23F	1		23F
23A	2		23A
23B	1	1	23B
24F	1	4	24F/24B
24B	1		24F/24B
25F	1		25A/F
28A[a]	1		None
29[a]	1	1	None
31	2	1	31
33A		1	33F/A
33F	1		33F/A
34[a]	1		None
35F	1	1	35F
35A	1		35A
35B[a]	1	2	None
37	1		37
38	1		38
48[a]	1		None
Unencapsulated	3		None[b]
Total isolates	75	64	

[a] Serotypes not included in the S. PneumoStrip test.
[b] Hybridization observed in *lytA* and *ply* control bands, absent in *cpsA* control band and any specific serotype band.

2.2. Validation of S. PneumoStrip test

The initial validation study was performed using reference isolates of the 76 *S. pneumoniae* serotypes included in the S. PneumoStrip and of another 16 serotypes not included, to confirm the specificity of the assay with the 92 serotypes for which currently there are specific antisera available at the SSI. Reference isolates included isolates from the SSI (n = 42), or from the Centers for Disease Control and Prevention, CDC, Atlanta, GA, USA (n = 13), from the American Type Culture Collection, Manassas, VA, USA (n = 8), or invasive clinical isolates from the collection at UHD (Marimón et al., 2016) previously characterized and serotyped by the Quellung reaction (n = 29). To check the specificity of the *lytA*, *ply* and *cpsA* genes included in the strips as amplification controls, the S. PneumoStrip test was also tested with a set of 12 viridans group streptococci clinical isolates collected at UHD including two isolates each of *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus sanguinis*, *Streptococcus pseudopneumoniae*, *Streptococcus salivarius* and *Streptococcus anginosus*.

A second validation was performed with 75 selected clinical isolates, 46 from invasive and 29 from non-invasive pneumococcal diseases (including 3 unencapsulated isolates from patients with conjunctivitis) representing 55 different serotypes previously confirmed by the Quellung reaction, the maximum number of different serotypes available in the *S. pneumoniae* clinical isolates collection from the Microbiology Department at UHD.

A third study was performed prospectively testing 64 non-selected invasive isolates consecutively collected from blood cultures at the Microbiology Department at UHD between June 2016 and March 2017. S. PneumoStrip test results of this prospective study were also confirmed by the Quellung reaction.

3. Results and discussion

The S. PneumoStrip test is based on the reverse-hybridization technique that allows for the identification of 76 pneumococcal serotypes according to their *cps* locus sequences. The test recognizes 42 serotypes individually and another 34 serotypes in pairs, 28 of the same serogroup, as serotypes 7F/7A in strip A, and 6 of different serogroups, as serotypes 7C/40 in strip C.

In this study a total of 231 pneumococcal isolates were analyzed: 92 reference strains, each of different serotype, and 139 clinical isolates. The reference strains of the 92 different serotypes gave the expected results (Fig. 1) while the 12 viridans group streptococci did not hybridize to the *ply*, *lytA* and *cps* control genes, except for one *S. mitis* isolate, which hybridized to the *lytA* probe. The presence of the *lytA* gene in isolates from nonpneumococcal species of the *Streptococcus mitis* group is not uncommon (Obregón et al., 2002).

The 139 selected and non-selected clinical isolates represented 57 serotypes (136 isolates) and 3 unencapsulated isolates (Table 2) and all of them gave the expected results on the strips except one isolate of serotype 48 that showed a light hybridization in the band of serotype11F.

Of note, the three unencapsulated isolates hybridized to the *lytA* and *ply* probes, but neither to the *cpsA* nor any of the serotype specific probes. Similarly, serotypes 25A, 25F and 38 did not hybridize to the *cpsA* probe as it is known that these serotypes have mutations and deletions in the *cpsA* gene (Leung et al., 2012) but in contrast to unencapsulated isolates clearly hybridized to their corresponding serotype band in lines 8 or 9 in strip C.

The 64 invasive isolates prospectively collected between June 2016 and March 2017 belonged to 22 different serotypes and only 4 (6.2%) isolates belonging to serotypes 13, 29, 35B (2 isolates) could not be serotyped with the strips as these serotypes are not yet included in the test (Table 2). Nonetheless, this percentage of isolates serotyped might vary between different regions according to different local serotype frequencies.

Among the advantages of the S. PneumoStrip test are the use of common laboratory devices (conventional PCR thermocycler and hybridization device) and the easy, objective and recordable results as compared with the Quellung reaction. The need of an automated

hybridization device could be regarded as a problem but the reverse hybridization can also be performed in manually operated incubators.

Nonetheless, the S. PneumoStrip test has some limitations. From a technical point of view, some serotypes whose capsular genes are similar, as with serotypes 15B and 15C, could not be differentiated by means of this technique. However, due to the hybridization step, it is possible to differentiate between serotypes not differentiable by only PCR, as serotypes 12A, 12F, 44 and 46 generally identified together by PCR (Marimón et al., 2016; Selva et al., 2012; Ziane et al., 2015). In addition, there are at least another 16 serotypes that will have to be included in a new strip to identify all distinct serotypes. Finally, we have not tested the S. PneumoStrip directly on clinical samples to study, in addition, the performance when more than one serotype is present as it is frequently found in pneumococcal nasopharyngeal carriage studies (Ercibengoa et al., 2012).

In conclusion, the validation result of a new commercial technique for the identification of 76 pneumococcal serotypes is described. As compared with the Quellung reaction, the sensitivity and specificity were 100%, both with reference and clinical isolates and was able to identify the serotypes of 93% of 64 recently and consecutively obtained invasive clinical isolates. The reverse-hybridization S. PneumoStrip test showed therefore to be a good and useful technique for the identification of most *S. pneumoniae* serotypes. The minimum technical requirements for its performance and the simplicity of the interpretation of the results make the S. PneumoStrip reverse-hybridization test a good technique for clinical laboratories not familiar with pneumococcal serotyping techniques.

Conflict of interest

The industrial and intellectual property rights of the S. PneumoStrip test are shared by OPERON S.A., Zaragoza, Spain (Susan Gamen and Ana Manrique) and the Biodonostia Health Research Institute (Jose Maria Marimón, Maria Morales, Maria Ercibengoa and Gustavo Cilla). The S. PneumoStrip test is manufactured and commercialized by OPERON S.A.

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