Simultaneous Identification of 29 Prevalent Invasive Pneumococcal Serotypes or Pairs of Serotypes by Hybridization-Ligation PCR

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A hybridization-ligation PCR assay was developed for the simultaneous detection and identification of 21 pneumococcal serotypes and 8 pairs of serotypes in the same serogroup: 1, 2, 3, 4, 5, 6A, 6B, 6C-6D, 7F-7A, 8, 9A-9V, 9N-9L, 11A, 14, 15B-15C, 16F, 17F, 18B-18C, 19A, 19F, 20, 21, 22A-22F, 23A, 23B, 23F, 28A-28F, 35B and 38. This novel assay was validated with 185 serotyped pneumococcal invasive clinical isolates and 57 culture-negative pleural fluids previously typed by real-time PCR.

Conjugate vaccines have caused a decline in invasive pneumococcal disease, but with a significant emergence of nonvaccine serotypes that has dramatically changed the distribution of invasive pneumococci (9, 14). Mainly, the prevalence of serotypes 1, 3, 5, 6A, 7F, and 19A has increased in recent years (4, 10, 12). The aim of this study was to develop a hybridization-ligation PCR method for the simultaneous and highly sensitive identification of the most prevalent 37 pneumococcal serotypes or serotype pairs. These are of particular significance because of their involvement in invasive pneumococcal disease (IPD) worldwide (1, 2, 3). This assay is based on the previously described multiplex ligation-dependent probe amplification (MLPA) technology (11).

A total of 185 pneumococcal invasive clinical isolates, five strains of each of the 37 serotypes, were selected from the collection of the National Centre of Microbiology (Majadahonda, Spain) during the period from 2007 to 2011. Pneumococci were serotyped by the Quellung reaction (5). To assess the specificity of the method, seven isolates of *Streptococcus* spp., including *S. pyogenes*, *S. oralis*, *S. gordonii*, *S. sanguinis*, *S. salivarius*, *S. agalactiae*, and *S. mitis*, and another seven pathogens, namely, *Staphylococcus aureus*, *Staphylococcus warneri*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Mycobacterium tuberculosis*, *Escherichia coli*, and *Mycoplasma pneumoniae*, were selected. We evaluated the assay with 57 pleural fluid samples which were available from 88 culture-negative pleural fluids (PFs) from children with pneumococcal empyema (PE) during 2003 to 2006 in three Spanish hospitals and which had been analyzed by real-time PCR in a previous study (8, 13).

Synthetic specific probes were designed with the program Raw-Oligo (Raw-Probe, version 0.15B; Raw-Soft) based on all available capsular genes from the GenBank database (<http://www.ncbi.nlm.nih.gov>), aligned by using ClustalW (6), and synthesized by Sigma-Genosys (Cambridge, United Kingdom).

One probe comprised two adjacent half probes that should adjacently hybridize to the specific DNA region. If half probes hybridize with a template, a complete probe is produced by a ligation reaction. This results in one template for subsequent PCR. Each probe yields an amplification product of unique size from 78 to 260 bp. One half probe contained a common primer sequence (forward or reverse) and a hybridization sequence (left or right). In order to obtain different lengths, we included an unrelated stuffer sequence (with sizes from 7 to 83 bp) in some half probes, obtained from the sequence of the *Prunus* necrotic ringspot virus. Construction and characteristics of probes are detailed in Fig. 1, panel I, and the supplemental material.

We designed common primers from sequences of the *Prunus* necrotic ringspot virus *cp* gene, isolate AprIt.caf1 (GenBank no. AJ133199.1), as follows: forward primer (5'-3'), GCTGGTGGATGCCGTTCTTG (nucleotides 71 to 90); reverse primer (5'-3'), CTCCATTCGGATGGCACTTCTTG (nucleotides 91 to 113).

DNA was extracted with a QIAamp DNA minikit (Qiagen, Hilden, Germany). The Nanodrop method (Nanodrop Technologies, Wilmington, DE) was used to determine DNA concentrations and detection limits (Fig. 1, panel I). The assay was performed in a DNA Engine Dyad thermal cycler (Bio-Rad Laboratories, Hercules, CA) and involved three stages, as follows. (i) Hybridization was performed at 95°C for 1 min followed by 60°C for 16 h with 5 µl of DNA template (10 to 75 ng), which had been denatured at 98°C for 5 min and cooled to 25°C, and hybridization master mix, which contained 1.5 µl of hybridization buffer (PerfectHyb Plus hybridization buffer; 1×; Sigma-Genosys, Madrid, Spain) and 1.5 µl of all half probes (0.01 µM concentration of each probe). (ii) Ligation was performed for 15 min at 54°C using 0.5 U of thermostable ligase (*Taq* DNA ligase; New England Biolabs), followed by 5 min at 98°C, and then the sample was held at 4°C. (iii) PCR was performed with 5 µl of ligation product, 0.2 µM concentrations of forward and reverse primers, and Illustra Puretaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, United Kingdom). The thermocycling profile was as follows: 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by 72°C for 20 min and cooling at 4°C. PCR products were analyzed using a QIAxcel multicapillary gel electrophoresis system (Qiagen, Hilden, Germany). All samples were unambiguously assigned according to their predicted molecular weight (Fig. 1, panel II). The system was able to analyze 96 samples per run.

From the 29 probes designed to target specific serotypes, 21 were completely specific for the targeted serotype: 1, 2, 3, 4, 5, 6A, 6B, 8, 10A, 14, 16F, 17F, 19A, 19F, 20, 21, 23A, 23B, 23F, 35B, and 38. Eight probes identified pairs of serotypes of the same sero-

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I.

Vaccines	ST/2 STs	bp	Det. lim.	Target gene	LHS - RHS	GenBank Accession
P	28A/28F	78	22	<i>wzy</i>	11321-11355/11013-11047	CR931692.1/CR931693.2
	17F	84	50	<i>wcrT</i>	12494-12534	CR931670.1
	21	93	35	<i>wzy</i>	12416-12465	CR931680.1
PCV10	7A/7F	100	32	<i>wcwA</i>	14106-14162/14102-14158	CR931643.1/CR931640.1
P, PCV13	19A	106	21	<i>wzy</i>	9893-9955	CR931675.1
P	22A/22F	112	45	<i>wzy</i>	13717-13785	CR931682.1/CR931681.1
P, PCV7, PCV10, PCV13	14	118	25	<i>lrp</i>	15370-15444	CR931662.1
P	9N/9L	123	25	<i>wzx</i>	12722-12793	CR931647.1/CR931646.1
P, PCV13	3	129	17	<i>galU</i>	9360-9430	CR931634.2
P, PCV7, PCV10, PCV13	19F	135	20	<i>wzy</i>	11591-11667	CR931678.1
P, PCV10, PCV13	1	141	13	<i>capK</i>	13724-13777	CR931632.1
P, PCV10, PCV13	5	147	6	<i>capK</i>	13060-13115	CR931637.1
P, PCV7, PCV10, PCV13	9A/9V	153	37	<i>tnp</i>	15297-15356/14980-15039	CR931648.1/CR931645.1
P	20	159	7	<i>wciL</i>	10247-10306	CR931679.1
P, PCV7, PCV10, PCV13	4	165	30	<i>cps4G</i>	9284-9345	CR931635.1
P	8	171	30	<i>capK</i>	12705-12758	CR931644.1
P	2	177	8	<i>wchG</i>	8473-8522	CR931633.1
P	6C/6D	183	10	<i>wchA</i>	7215-7268/1035-1088	EF538714.1/FJ899600.1
P	35B	189	20	<i>wcrH</i>	11199-11259	CR931705.1
	38	195	8	<i>wcyV</i>	18575-18648	CR931710.1
	15B/15C	201	9	<i>wzy</i>	7948-8007	CR931665.1/CR931664.1
P, PCV7, PCV10, PCV13	23F	207	7	<i>wzy</i>	9197-9267	CR931685.1
PCV13	6A	213	30	<i>wciP</i>	8872-8931	CR931638.1
P, PCV7, PCV10, PCV13	6B	219	20	<i>wciP</i>	8769-8838	CR931639.1
P, PCV7, PCV10, PCV13	18B/C	225	15	<i>gct</i>	15826-15895	CR931672.1/CR931673.1
P	23A	231	15	<i>wzy</i>	8003-8063	CR931683.1
	16F	243	14	<i>wzy</i>	11612-11678	CR931668.1
	10A	249	9	<i>wcrG</i>	12234-12290	CR931650.1
	23B	260	12	<i>wzy</i>	9956-10005	CR931684.1
detection c.		119	10	<i>ply</i>	1868847-1868894	CP002176.1

II.

serotype / serotypes pairs

FIG 2 Results of a hybridization-ligation PCR assay with 57 culture-negative pleural fluids, previously analyzed by real-time PCR. (I) Diagram showing the results of the PCR assay. (II) Visualization with the QIAxcel system. The probes were mixed with three homemade markers (lanes 2, 3, and 4). The first lane corresponds to a 10-bp commercial marker (Invitrogen, Life Technologies).

group: 6C-6D, 7A-7F, 9A-9V, 9N-9L, 15B-15C, 18B-18C, 22A-22F and 28A-28F. The high specificity of probes was demonstrated by the fact that all clinical isolates were correctly typed and no nonpneumococcal isolates hybridized with any pneumococcal probe. A control for *Streptococcus pneumoniae* detection was included in the study (Fig. 1, panel I).

PCR products were sequenced in a 3730XL sequencer using a BigDye Terminator cycle sequencing kit, v3.1 (Applied Biosystems, Foster City, CA). The resulting sequences exactly matched the corresponding published sequences. The lower detection limit of this method was 5 ng, corresponding to 25 bacteria (Fig. 1, panel I). Homemade molecular weight markers were created with a mixture of all serotype-specific amplification products (Fig. 2, panel II).

In order to assess the usefulness of the assay in culture-negative

clinical samples, 57 previously studied PFs from children diagnosed with PE were tested (8, 13). The results are shown in Fig. 2, panel I, and the supplemental material. Compared with the study by Obando et al. (8), the most relevant finding was the identification of a serotype for seven samples of 21 previously unidentified serotypes. Furthermore, all serotypes of serogroup 9 were detected in two independent reactions: 9A-9V and 9N-9L. Serotypes responsible for PE were fully covered by the assay (7). The sensitivity of the assay was sufficient to allow it to be used directly on clinical samples but was lower than that noted with real-time PCR (13). This could be because the PE samples were repeatedly frozen and thawed as a consequence of having been used in a previous study. However, this is compensated for by its overall capacity as a 29-multiplexed assay in a single tube. To our knowledge, the method described here includes the largest number of pneumococcal se-

FIG 1 Construction and characteristics of probes, with their visualization by the QIAxcel capillary gel electrophoresis system, for each serotype or serotype pair. (I) Characteristics of probes. Vaccines are listed as P (polysaccharide vaccine) or PCV7, PCV10, or PCV13 (7-valent, 10-valent, and 13-valent pneumococcal conjugate vaccines). Serotypes (ST) are listed; pairs are listed where two serotypes could not be distinguished. The detection limit (Det. lim.) is the sensitivity of the assay, expressed as the number of bacteria. Left and right hybridization sequences (LHS and RHS) are the nucleotide positions of sequences from the corresponding GenBank accession number. They refer to the 5' starting nucleotide for the LHS and the 3' ending nucleotide for the RHS. The last row gives data for a *Streptococcus pneumoniae* detection control. (II) Visualization with the QIAxcel capillary gel electrophoresis system. The first lane corresponds to a 10-bp commercial marker (Invitrogen).

rototypes detected in a single assay, comprising about 95% and 80% of the IPD serotypes in Spain and worldwide, respectively. Therefore, the assay could be useful in colonization studies in which cocolonization with some pneumococcal serotypes is common. Furthermore, this novel assay is highly reproducible. It is also reasonably cost-effective, because only a thermocycler and electrophoresis equipment are needed. This means that it could easily be introduced into most clinical laboratories.

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