Universitat de Barcelona

Características clínicas y microbiológicas de la enfermedad neumocócica invasiva pediátrica en Barcelona en la era de la vacuna heptavalente conjugada

Mariona Fernández de Sevilla Estrach

Características clínicas y microbiológicas de la enfermedad neumocócica invasiva pediátrica en Barcelona en la era de la vacuna heptavalente conjugada

Memoria presentada por

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para optar al título de Doctora en Medicina

Trabajo realizado bajo la dirección del **Dr Juan José García García** (Servicio de Pediatría) y la **Dra Carmen Muñoz Almagro** (Departamento de Microbiología Molecular) del Hospital Sant Joan de Déu.

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CERTIFICAN

Que **MARIONA FERNÁNDEZ DE SEVILLA ESTRACH**, licenciada en Medicina por la Universitat de Barcelona, ha realizado bajo su dirección la presente Tesis Doctoral, titulada **"Características clínicas y microbiológicas de la enfermedad neumocócica invasiva pediátrica en Barcelona en la era de la vacuna heptavalente conjugada"**

Esta tesis está concluida y se considera apta para su lectura y defensa pública.

Dr Juan José García García   Dra Carmen Muñoz-Almagro

Barcelona, 25 de Junio de 2012

Als meus pares que m'ho han donat tot,

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Abreviaturas

ENI: Enfermedad neumocócica invasiva

ADN: Acido desoxirribonucleico

VIH: Virus de la inmunodeficiencia humana

IgG: Inmunoglobulina G

IgM: Inmunoglobulina M

IgA: Inmunoglobulina A

LCR: Líquido cefalorraquídeo

PCR: Reacción en cadena de la polimerasa

CLSI: Clinical and Laboratory Standards Institute

MLST: Multi Locus Sequence Type

ST: Secuenciotipo

SLV: Single locus variant

DLV: Doble locus variant

CC: Complejo clonal

PCV7: Vacuna antineumocócica heptavalente conjugada

EEUU: Estados Unidos de América

cols: Colaboradores

SV: Serotipos incluidos en la PCV7

SNV: Serotipos no incluidos en la PCV7

UCI: Unidad de Cuidados Intensivos

DE: Desviación estándar

CMI: Concentración Mínima Inhibitoria

VSFT: Vacunados según ficha técnica

IC: Intervalo de confianza

1. INTRODUCCIÓN

La enfermedad neumocócica está producida por la bacteria *Streptococcus pneumoniae*. Evolutivamente este patógeno ha acumulado diferencias genéticas por lo que actualmente distinguimos 93 serotipos de *S.pneumoniae* con agresividad y adaptación al ser humano diferente. La identificación del serotipo se basa en la cápsula polisacárida que envuelve la pared celular. Aunque se han identificado 93 serotipos, la mayor parte de enfermedad neumocócica está producida por relativamente pocos de ellos. La predominancia de serotipos cambia con el tiempo y la edad y puede ser diferente según la región geográfica[1],[2].

1.1-Epidemiología y etiopatogenia

El nicho ecológico natural de *S.pneumoniae* es la nasofarínge humana, en especial la del niño pequeño y por tanto principal fuente de diseminación humano a humano. El índice de colonización es estacional y aumenta a mediados de invierno. El estado de portador nasofaríngeo varía de un 11% a 93% dependiendo de la edad, región geográfica y población [3],[4],[5]. Como factores que aumentan el estado de portador se han descrito la edad menor a 2 años, la asistencia a guardería o colegio, el hacinamiento, el invierno y tener padres fumadores[3]. La duración del estado de portador depende del serotipo y es inversamente proporcional a la edad, suele ser de 2 a 4 meses pero puede durar hasta 17 meses [6],[7],[8].

Actualmente *S. pneumoniae* es uno de los patógenos más frecuentes en las infecciones adquiridas en la comunidad, tanto en la población pediátrica como en la adulta. En nuestro entorno constituye el agente causal más frecuente de neumonía simple y complicada, meningitis, otitis y sinusitis aguda[9]. Otras infecciones

neumocócicas menos comunes son las infecciones de tejidos blandos (celulitis orbitarias y otras), erisipelas, infecciones gingivales y glositis, artritis, osteomielitis, peritonitis primaria, salpingitis y endocarditis. Se define enfermedad neumocócica invasiva (ENI) cuando es posible aislar o detectar ADN de *S. pneumoniae* en cualquier líquido corporal habitualmente estéril (sangre, líquido cefalorraquídeo, líquido pleural, líquido peritoneal, líquido articular u otros). (EC No.22/253/EC del Parlamento Europeo 19 Marzo 2002).

La mayor tasa de ataque de ENI ha tenido lugar tradicionalmente en los primeros años de vida, con un pico de incidencia entre los 6 y 11 meses [1]. La incidencia de ENI resulta especialmente elevada en niños menores de 2 años, alcanzado cifras superiores a los 80-100 casos / 100.000 habitantes-año. En la mayor parte de estudios de ENI los niños se afectan más que las niñas.

La infección por neumococo posee una morbimortalidad considerable, en el 2005 se estimó que fue responsable de 700.000 a 1.000.000 de muertes en niños menores de 5 años, la mayor parte de ellos vivían en países desarrollados [10]. Unicamente el 2,3% de los pacientes que sobreviven a un primer episodio de ENI tendrán episodios recurrentes de ENI. El mayor riego de recurrencia se ha descrito en infectados por el VIH y en niños menores de 5 años con infección crónica[11].

La mayor parte de la infección neumocócica es episódica pero se han descrito epidemias en poblaciones hacinadas como campamentos militares y cárceles[12] , y se han reportado agrupaciones de casos en niños que asisten a guarderias[13].

Los factores más importantes en el desarrollo de la enfermedad neumocócica en niños son la virulencia del serotipo (dependiente de la composición y cantidad del polisacárido capsular), la presencia de infección viral del tracto respiratorio y la ausencia de inmunidad humoral específica. La incidencia de la enfermedad neumocócica cursa de forma paralela a las infecciones virales del tracto respiratorio, por lo que son más comunes en invierno y primavera [14]. El principal mecanismo de defensa del huésped frente al neumococo es la fagocitosis activando la opsonización iniciada por anticuerpos de tipo IgG, IgM e IgA así como activando la vía clásica del complemento, neutrófilos y células fagocíticas a nivel de pulmón, hígado y bazo. Los niños menores de 2 años son un grupo de riesgo fundamental porque no han desarrollado del todo su inmunidad y suelen tener una alta tasa de colonización nasofaríngea por *S. pneumoniae*.

Las diferencias inmunológicas del polisacárido capsular han permitido clasificar el neumococo en 93 serotipos diferentes. La pertenencia a uno u otro de estos serotipos hace que presenten variaciones en la resistencia a la fagocitosis, en la activación de la vía alternativa del complemento y en la penetración tisular. De este modo hay serotipos que colonizan mejor la nasofaringe pero son poco invasivos y otros que colonizan poco pero tienen una mayor predisposición a producir enfermedad invasiva[15]. En cambio, el impacto que tiene el tipo clonal en la invasividad del neumococo es controvertida[15,16] . Por clon se entiende el conjunto de bacterias que son idénticas desde el punto de vista genético; el estudio clonal permite caracterizar de forma precisa los aislamientos de neumococo en base a sus características genéticas.

Hay varias condiciones médicas que favorecen al desarrollo de ENI como son: infección por VIH, drepanocitosis, asplenia o disfunción esplénica, síndrome nefrótico, fístula de LCR, inmunodepresión o terapia inmunosupresora, neoplasias (leucemia, linfoma), trastornos cardiacos, pulmonares e implante coclear.

1.2-Métodos diagnósticos

Las herramientas que disponemos para caracterizar el neumococo son:

1. Métodos microbiológicos tradicionales:

   a. Siembra en medio de agar sangre ya sea en condiciones anaerobias o en dióxido de carbono al 5%

   b. Tinción de Gram

   c. Aglutinación en látex

   d. Sensibilidad a la optoquina

   e. Estudio de sensibilidad antibiótica. El estudio de sensibilidad a penicilina y otros antibióticos se realiza según los puntos de corte meníngeos del Clinical and Laboratory Standards Institute (CLSI) del año 2008[17]. Las muestras con resistencia intermedia o alta resistencia se consideran no sensibles. Existían unos puntos de corte que se cambiaron para adaptarlos mejor a la realidad clínica. Dichos puntos de corte "tradicionales" o "meníngeos" siguen utilizándose con criterios epidemiológicos y para infecciones del SNC. Los puntos de corte establecidos en 2008 ("no meníngeos") se utilizan para el resto de infecciones. En la tabla siguiente se detallan los puntos de corte de la sensibilidad a la penicilina y cefotaxima diferenciando los criterios tradicionales (puntos de corte "meníngeos") de los nuevos puntos de corte introducidos en el 2008 válidos para infecciones neumocócicas excepto meningitis (puntos de corte "no meníngeos").

CMI	SENSIBLE	RESISTENCIA INTERMEDIA	ALTA RESISTENCIA
Puntos de corte meníngeos			
Penicilina	≤ 0,06µg/ml	0,12µg/ml	≥ 2µg/ml
Cefotaxima	≤ 0,5µg/ml	1µg/ml	≥ 2µg/ml
Puntos de corte **no** meníngeos			
Penicilina	≤ 2µg/ml	4µg/ml	≥ 8µg/ml
Cefotaxima	≤ 1µg/ml	2µg/ml	≥ 4µg/ml

Tabla 1: *Puntos de corte de la sensibilidad a la penicilina y cefotaxima.*

    f. Serotipado de los aislados por cultivo a partir de la reacción de Quellung.

2. Métodos moleculares (*disponibles y utilizados en nuestro laboratorio*):

    g. PCR en tiempo real. Se realiza utilizando el Robot AbiPrism7300 de laboratorios Applied Biosystem según protocolo del departamento de Microbiología Molecular de nuestro hospital[18]. El gen seleccionado para el diagnóstico de *S. pneumoniae* es el de la neumolisina. A todas las muestras que presentan PCR positiva para neumolisina se les realiza la confirmación mediante la PCR para el gen capsular wzg, si éste resulta negativo se excluyen ya que podría tratarse de microorganismos como *S.mitis* parecidos genéticamente al neumococo y ser por tanto falsos positivos.

    h. Estudio del serotipado través de la PCR multiplex secuencial[19] para muestras detectadas mediante PCR con cultivo negativo. Mediante esta técnica pueden distinguirse 24 serotipos (1, 3, 4, 5, 6A/C, 6B/D, 7F/A, 8, 9V/A/N/L, 14, 15B/C, 18C/B, 19A, 19F/B/C, 23A y 23F).

    i. Genotipado por "Multi Locus Sequence Type" (MLST) (www.mlst.net) El estudio de composición clonal de las cepas se

realiza mediante el uso de múltiples locus de tipificación de secuencia (MLST). La técnica de MLST consiste en el análisis mediante secuenciación del ADN de un número determinado de genes. Estos genes codifican distintas enzimas metabólicas bacterianas (*housekeeping genes*). La comparación de estas secuencias entre distintos aislados permite establecer identidades o diferencias clonales de utilidad en el análisis epidemiológico bacteriano. El hecho de utilizar enzimas metabólicas, no sometidas a presión selectiva, permite detectar variaciones neutras que definen líneas clonales relativamente estables. Por lo tanto, MLST es un marcador molecular de aplicación en epidemiología global o a largo plazo. Para cada especie bacteriana, es preciso establecer un grupo de genes metabólicos a estudiar. El esquema de MLST desarrollado para cepas de neumococo utiliza fragmentos internos de los siguientes genes: **aroE** (shikimate deshidrogenasa), **gdh** (glucosa-6-fosfato deshidrogenasa), **gki** (glucosa kinasa), **recP** (trasketolase), **spi** (signal peptidase I), **xpt** (xantina fpsfpribosiltransferasa), **ddl** (D-alanina D-alanina ligasa). La asignación de los alelos y tipos de secuencias o secuenciotipos (ST) se lleva a cabo utilizando el software en la página web: www.mlst.net. Los secuenciotipos que comparten seis de los siete alelos se consideran Single Locus Variant (SLV) y si comparten cinco de siete alelos se consideran Doble Locus Variant (DLV). Los SLV o DLV son asignados como complejos clonales. El análisis de los ST y la asignación de los complejos clonales (CC) se realiza con el programa eBURST (Feil EJ, 2004). En el presente

estudio se realiza la asignación más restrictiva, considerándose complejo clonal los SLV.

1.3-Vacuna antineumocócica conjugada heptavalente

Una de las principales armas contra *S.pneumoniae* han sido las vacunas, pero lamentablemente la clásica vacuna (23 valente), que previene contra 23 de los 93 serotipos, sólo es eficaz en población adulta ya que por la inmadurez del sistema inmunológico infantil (se trata de una vacuna polisacárida incapaz de inducir inmunidad en los niños pequeños) no hay adecuada respuesta tras la vacuna en los niños.

La importante magnitud del problema de la infección neumocócica en niños menores de 2 años y la resistencia creciente del neumococo a los antibióticos[20] estimularon la obtención de vacunas conjugadas frente al neumococo. La unión covalente del polisacárido con una proteína hace que sean inmunogénicas en los menores de 2 años, inducen células de memoria y reducen el estado de portador de serotipos vacunales. En el año 2000 se publicaron los datos de un estudio sobre eficacia de una nueva vacuna, la vacuna heptavalente conjugada (PCV7) (Prevenar®;Wyeth Lederle Vaccines), que por su diseño inducía una respuesta efectiva en el niño pequeño. Los resultados del estudio que incluyó a 38000 niños, demostraban en el análisis por intención de tratar el significativo descenso de la ENI, 39 casos en el grupo control versus 3 en el grupo de PCV7 [21]. A partir de estos datos diversas sociedades científicas recomendaron la vacunación universal en niños a los 2,4,6 y 18 meses de edad y numerosos países incluyeron esta vacuna en el calendario vacunal sistemático. Esta vacuna cubre los serotipos 4, 6B, 9V, 14, 18C, 19F y 23F, que son los que con mayor frecuencia se detectaban como colonizadores de nasofaringe e históricamente se relacionaban con enfermedad invasiva en países desarrollados, especialmente en EEUU[22,23].

En el año 2010, debido a la emergencia de serotipos no vacunales que estaban siendo los responsables de gran parte de la ENI en los últimos años, se comercializaron dos nuevas vacunas antineumocócicas conjugadas, la vacuna decavalente, la cual cubre los serotipos de la heptavalente, 4, 6B, 9V, 14, 18C, 19F y 23F, más los serotipos 1,5 y 7F y la vacuna trecevalente que, aparte de cubrir los 10 serotipos anteriores, ofrece cobertura frente a los serotipos 3, 6A y 19A.

Los artículos discutidos en la presente tesis han sido realizados previo a la introducción de la vacuna decavalente y trecevalente por lo que la tesis se centra en el impacto que la vacuna conjugada heptavalente ha tenido en la enfermedad neumocócica invasiva. Este conocimiento es de suma importancia para preveer la repercusión que las nuevas vacunas tendrán en la epidemiología de la ENI.

Los resultados de diferentes estudios efectuados antes de la introducción de la vacuna PCV7 demostraron que la mayoría de los serotipos causantes de ENI en niños en Estados Unidos y Canadá eran los incluidos en la vacuna PCV7 (SV) (88.7% de SV). En Europa la cifra era sensiblemente inferior (74.4% de SV)[24] por lo que el impacto teórico de la vacuna podría ser inferior[25]. En nuestro hospital los SV fueron responsables del 65% de la ENI durante el período 1997-2001.

Cabe destacar, también, que España, antes de la introducción de la vacuna en 2001, era una de las áreas del mundo con mayor grado de resistencia a los antibióticos[26]. Dado que en nuestro país el 83% de las resistencias del neumococo a la penicilina se concentraba en los serotipos 6,9,14 y 23 y todos ellos están incluidos en la vacuna PCV7, los expertos atribuyeron a la vacunación con vacuna neumocócica conjugada heptavalente de la población infantil un importante potencial de reducción de la

proporción de cepas de neumococo resistentes a los antibióticos causantes de ENI en

nuestro país[27].

1.4-Enfermedad neumocócica tras la introducción de la vacuna heptavalente

1.4.1-Situación en Estados Unidos

Aplicada de forma masiva a la población de Estados Unidos, la PCV7 demostró ser efectiva en la reducción de la incidencia de ENI [28,29]. En la población vacunada norteamericana se comprobó un descenso del 80% en la tasa de incidencia de ENI en niños menores de 2 años (2007 vs 1998/99)[30]. La reducción obtenida fue mayor que la esperada según las coberturas vacunales alcanzadas probablemente por la reducción de la prevalencia de portadores en los colectivos de población vacunados. La vacuna proporciona, pues, un importante grado de protección indirecta a través de la inmunidad de grupo[31,32]. No obstante, cabe recalcar que aunque disminuye el estado de portador de serotipos vacunales, con el tiempo aumenta el estado de portador de serotipos no vacunales [1].

Aunque tras la introducción de PCV7 en Estados Unidos la incidencia de ENI disminuyó de forma radical, en los últimos años se ha descrito un incremento significativo en la incidencia de ENI por serotipo 19A[33,34]. El aumento de la ENI producida por éste y otros serotipos no vacunales no ha llegado a los niveles de la época prevacunal. Posiblemente otros factores aparte de la vacuna han jugado un papel en el incremento dado que este aumento del 19A se ha descrito en otros países sin vacunación sistemática como Corea e Israel[35,36].

1.4.2-Situación en España

En España la PCV7 se comercializó a partir de junio de 2001, pero, a diferencia de Estados Unidos, en España la vacuna neumocócica conjugada heptavalente no fue incorporada a los calendarios de vacunaciones sistemáticas, excepto en la Comunidad de Madrid que lo incluyó en noviembre de 2006. En la mayoría de Comunidades Autónomas, como Catalunya, la vacunación antineumocócica se realiza de manera individualizada y sin financiación por parte del sistema público, por indicación de los pediatras, variando las coberturas vacunales en función de las recomendaciones de los mismos y de la aceptación de la vacunación por parte de los padres. Se estima que en la mayoría de las Comunidades Autónomas las coberturas vacunales frente al neumococo en los niños menores de 2 años son de alrededor el 50%[37] .

La información epidemiológica disponible en la actualidad, 11 años después de la introducción de PCV7, parece señalar que la estrategia de vacunación indicada, seguida hasta el presente en España, no ha tenido ni mucho menos, el impacto obtenido con la vacunción universal adoptada en Estados Unidos. No obstante, cabe destacar, que en España la ENI no es una enfermedad de declaración obligatoria lo que constituye una limitación para los estudios epidemiológicos.

Desde la comercialización de la vacuna PCV7 se ha observado una emergencia de la enfermedad invasiva neumocócica por serotipos no incluidos en la vacuna heptavalente conjugada en nuestra área geográfica[38] . En un estudio reciente realizado en nuestro centro y utilizando tan solo datos procedentes de cultivos, se pudo constatar este hecho, observándose como en el rango de edad <2 años la incidencia de ENI pasaba de 32.4 episodios/100.000 habitantes-año en el período prevacunal

(1997-2001) a 51.3 episodios/100.000 habitantes-año en el período postvacunal (2002-2006) (incremento del 58%; IC 95%, 2%-145%,)[38]. Por lo que respecta a la franja de edad entre 2 y 4 años la incidencia aumentó de 11.3 episodios/100.000 habitantes-año a 26.5 episodios por 100.000 habitantes-año (incremento de 135%; IC 95%, 31%-320%)[38]. Este fenómeno ha sido constatado por otros centros de nuestro entorno[39] y en nativos de Alaska[40] pero es discrepante con estudios norteamericanos que no encuentran este incremento. En un estudio colaborativo reciente realizado en Madrid[41] tras la implantación obligatoria de la PCV7 se ha podido constatar que el 60% de la ENI que se produjo en Madrid entre mayo de 2007 y abril 2008 estuvo causada por tres serotipos (1,5 y 19A), todos ellos no incluidos en la PCV7.

En España, la emergencia de la enfermedad invasiva posiblemente está siendo producida por la expansión de clones virulentos, preexistentes y nuevos, que expresan SNV y que además producen un cambio en las manifestaciones clínicas[38]. Existe la duda de si se trata de reemplazo por ocupación del nicho o son variaciones cíclicas, dado que también ha ocurrido en poblaciones donde no se utilizó la vacuna y ya se percibía esa tendencia cíclica antes de iniciarse la vacunación en el año 2000. Esta expansión podría estar favorecida de forma indirecta por la menor circulación de los 7 serotipos vacunales a nivel global. Esta posibilidad está siendo publicada con datos retrospectivos por centros americanos que iniciaron una vacunación masiva a principios de la década actual[42,43,]. Byington y cols han revisado retrospectivamente todos los casos de ENI con cultivo positivo diferenciando el período prevacunal del postvacunal (con un 54% de cobertura en el grupo menor de 3 años). Al comparar los dos períodos encuentran un descenso (27%) significativo del total de ENI y de la proporción de SV cusantes de ENI. Sin embargo, observan un incremento

significativo de la enfermedad grave 57% vs 71% (p<0.015) y de neumonías con empiema 16% vs 30% (p=0.015) [30].

Este aumento significativo de incidencia de enfermedad neumocócica invasiva está siendo monitorizado "en tiempo real" en nuestro centro. Con tal de conocer de forma más precisa el verdadero impacto de la ENI en nuestro medio se incluyen no sólo los casos diagnosticados mediante cultivo, sino también los casos diagnósticados mediante la técnica de Real-Time PCR, específica del gen neumolisina y el gen capsular wzg de *S.pneumoniae,* lo que nos permite con elevada sensibilidad y especificidad incrementar la confirmación microbiológica de los casos.

En resumen, de las conclusiones de los diferentes trabajos realizados en los últimos años en España parece obtenerse algunos datos evidentes:
-Ha habido una reducción de ENI por SV en la población vacunada.
-Ha habido reducción de ENI por los SV en población general, debida a la inmunidad de grupo.
-Ha habido un aumento de los SNV.

En resumen, la ENI constituye un problema grave de Salud Pública en la edad infantil, con una morbimortalidad considerable[10]. En los últimos años se han introducido vacunas adecuadas para la edad infantil por lo que es de gran importancia realizar una buena monitorización de la ENI tras la introducción de las mismas.

La presente tesis pretende a partir del análisis de la ENI que ha tenido lugar en los últimos años en nuestro medio, en un área sin vacunación sistemática, identificar los factores clínicos y microbiológicos asociados a la emergencia de ENI por SNV.

2. HIPÓTESIS DE TRABAJO

-La incidencia de ENI continúa aumentando en nuestro medio debido a la emergencia de los SNV.

-La emergencia de estos SNV podría explicarse por dos mecanismos:

 *El crecimiento excesivo de algunos SNV como el 1 y el 5 que tienen una composición clonal homogénea y se aislan raramente en la nasofaringe de portadores. No obstante, tienen un gran poder en causar ENI y podrían producir brotes, sobre todo en un área geográfica localizada.

 *El incremento de algunos SNV como el 19A, el cual tiene una composición clonal heterogénea y a menudo se aisla en portadores nasofaríngeos. Su diseminación en la comunidad podría ser un fenómeno generalizado.

-Nos encontramos ante un cambio de epidemiología molecular de la ENI en nuestro medio que se correlaciona con un cambio en las manifestaciones clínicas.

 -La utilización de las técnicas de microbiología molecular como la reacción en cadena de la polimerasa (PCR) permite identificar más casos de ENI.

-El aumento en nuestro medio del serotipo 19A se debe a la introducción de clones multirresistentes por ser un buen colonizador.

3. OBJETIVOS

1-Determinar la incidencia de ENI en menores de 5 años en nuestro medio.

2-Describir las principales características clínicas de la ENI en nuestro medio.

3-Determinar los factores microbiológicos relacionados con la ENI en menores de 5 años en nuestro medio:

      3.1-Serotipos causantes

      3.2-Composición clonal

      3.3-Perfil de resistencias de los aislados

4-Describir las características clínicas y moleculares de la ENI producida por el serotipo 19A en nuestro medio.

4. ARTÍCULOS

Clinical Presentation of Invasive Pneumococcal Disease in Spain in the Era of Heptavalent Conjugate Vaccine

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Background: The aim of this study was to analyze the rate of incidence, clinical presentation, serotype, and clonal distribution of invasive pneumococcal disease (IPD) in the era of heptavalent pneumococcal conjugate vaccine (PCV7) in Barcelona, Spain.

Methods: This was a prospective study comprising all children <5 years with IPD who were managed in 2 tertiary-care, pediatric hospitals between January 2007 and December 2009. IPD was defined as the presence of clinical findings of infection together with isolation or detection of DNA of *Streptococcus pneumoniae* in a sterile fluid sample.

Results: In this study, 319 patients (53.3% male), mean age 29.6 months, were included. Comparing rates in 2007 and 2009 (76.2 and 109.9 episodes/100,000 population, respectively), an increase of 44% (95% confidence interval, 10%–89%) was observed. The main clinical presentation was pneumonia (254 episodes, 79.6%), followed by meningitis (29, 9.1%), and bacteremia (25, 7.8%). The diagnosis was made by positive culture in 123 (38.6%) patients and in 196 (61.4%) by real-time polymerase chain reaction. Serotype study was performed in 300 episodes, and 273 (91%) were non-PCV7 serotypes. The most frequent serotypes were 1 (20.7%), 19A (15.7%), and 3 (12.3%). A minimal inhibitory concentration ≥0.12 μg/mL to penicillin was detected in 34.4% of isolates. Sequence type 306 expressing serotype 1 was the most frequent clonal type detected (20.3% of studied strains).

Conclusions: IPD continues to increase in Barcelona, and the rate is higher than previously reported as a result of low sensitivity of bacterial culture. Non-PCV7 serotypes were responsible for 91% of episodes and pneumonia was the main clinical presentation.

Key Words: *Streptococcus pneumoniae*, pneumococcal conjugate vaccine, pneumonia, serotype, MLST

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*S*treptococcus pneumoniae is a major cause of morbidity and mortality worldwide, especially among young children, despite the availability of antibiotic treatment and vaccines. The World Health Organization estimates that every year more than one million children younger than 5 years die of invasive pneumococcal disease (IPD), mainly in developing countries.[1]

The imbalance between host factors and virulence of the pathogen is partly responsible for the production of IPD. The main virulence factor of pneumococcus is the polysaccharide capsule, with 93 serotypes with differing pathogenicity.[2]

Following introduction of pneumococcal conjugate vaccine (PCV7) in the United States, there was a dramatic decline in IPD rates and drug-resistant pneumococci.[3,4] However, in Spain and other countries, we observed a significant increase in the rate of IPD caused by non-PCV7 serotypes and a slight reduction in the rate of IPD caused by PCV7 serotypes.[5] There was a change in the main serotypes associated with IPD, and this change was associated with changes in clinical types of IPD,[6] a reduction in the rate of antibiotic-resistant strains causing IPD, and the emergence of previously established virulent clones of non-PCV7 serotypes.[5]

The introduction of real-time polymerase chain reaction (PCR)-based methods that specifically identify capsular DNA in direct sample offer a sensitive, rapid, and simple approach for the surveillance of IPD.[7] Different authors have reported that molecular methods can be used directly on sterile biologic samples, improving the ability to diagnose IPD.[8–12] At present, little is known about the epidemiologic characteristics, clinical presentation, and outcome of IPD including episodes with negative bacterial culture. The purpose of this study was to determine the epidemiologic variables, clinical presentation, current trends, and serotypes and clones of *S. pneumoniae* among children in Barcelona, Spain, after the implementation of PCV7, in 2001, including patients with negative culture who were diagnosed by real-time PCR.

MATERIALS AND METHODS

Patients and Definitions

We performed a prospective study comprising all children <5 years with IPD managed in 2 tertiary-care pediatric hospitals in Barcelona (Spain) during a 3-year period (January 2007-December 2009). These 2 centers serve a pediatric referral population of 134,662 children <5 years (around 27% of the Catalan pediatric population <5 years).[13]

An episode of IPD was defined as the presence of clinical findings of infection together with isolation and/or DNA detection of pneumolysin (*ply*) gene and an additional capsular gene of *S. pneumoniae* by real-time PCR in any sterile body fluid such as blood, cerebrospinal fluid, pleural fluid, or articular fluid.

Data Collected and Analyzed

Epidemiologic characteristics included age, gender, immunization status against *S. pneumoniae* (when written records were available), underlying medical condition, group child care attendance, antibiotic treatment and/or respiratory infection before the diagnosis of IPD, history of breast-feeding, household size, and exposure to tobacco smoke.

Clinical characteristics including clinical presentation, intensive care unit (ICU) admission, complications, antibiotic treatment and duration, days of hospitalization, and clinical outcome were also recorded.

Microbiologic Bacterial Culture and Antimicrobial Susceptibility Studies

All pneumococcal isolates were identified by standard microbiologic methods that remained constant during the study period. Agar dilution technique was used to determine the minimal inhibitory concentration (MIC) of several antibiotics, including penicillin and cefotaxime. American Type Culture Collection 49619 (serotype 19F) was used as a control. Susceptibility to penicillin and other antibiotics was defined according to the 2008 meningeal break points by the Clinical Laboratory Standards Institute.[14] Isolates with intermediate- or high-level resistance were defined as nonsusceptible.

DNA Detection of *S. pneumoniae* by Real-time PCR

Detection of *ply* gene of *S. pneumoniae* was performed by real-time PCR according to a published assay.[7]

Serotype Identification

Serotyping of strains isolated by culture was carried out by the Quellung reaction, using antisera provided by the Statens Serum Institut (Copenhagen, Denmark), or by dot-blot serotyping.[15] MICs and serotyping of the strains were performed at the National Center for Microbiology (Majadahonda, Spain). Detection of pneumococcal serotypes in negative culture clinical samples but *ply* pneumococcal gene positive was performed according to a published multiplex real-time PCR methodology.[16] This procedure includes the DNA detection of conserved *wzg* capsular gene of *S. pneumoniae* and other different genes selected to distinguish 24 serotypes (1, 3, 4, 5, 6A/C, 6B/D, 7F/A, 8, 9V/A/N/L, 14, 15B /C, 18C/B, 19A, 19F/B/C, 23A, and 23F).

Serotypes were classified into the following groups: PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F), PCV10 serotypes (PCV7 serotypes plus 1, 5, 7F), and PCV13 serotypes (PCV10 serotypes plus 19A, 6A, 3).

Clonal Study

Clonal composition of strains was analyzed using multilocus sequence typing (MLST) as reported elsewhere.[17] The assignment of alleles and sequence types (ST) was carried out using the software at the pneumococcal web page www.mlst.net. Analysis of ST and assignment to clonal complex (CC) were performed with the eBURST program.[18] STs that shared 6 of 7 alleles (single locus variants) were considered a CC.

Statistical Analysis

Rates of IPD, defined as the number of episodes per 100,000 population, were calculated using the annual estimates of pediatric population obtained from the Department of Statistics in Catalonia[13] and the percentage of capture of both hospitals among total hospitalization in children <5 years. In Catalonia county, these hospitals captured, during the study period, 25.4% of all pediatric hospitalizations <2 years and 32.2% of pediatric hospitalizations between 2 to 5 years.

We used the χ^2 test or Fisher exact test to compare proportions and Student *t* test to compare means. Statistical analyses were performed using SPSS for Windows, version 17.0 (SPSS), and Epi Info, version 6.0 (Centers for Disease Control and Prevention). We calculated 95% confidence intervals (CIs), and 2-sided *P* values ≤0.05 were considered to be statistically significant.

RESULTS

During the study period, 319 episodes of IPD were identified in 319 patients, including 170 male patients (53.3%) and 149 female patients (46.7%), with a mean age of 29.6 months (standard deviation [SD]: 15.7). One hundred ninety-two episodes (60.2%) were in children aged 24 to 59 months, 99 (31.0%) in children aged 7 to 23 months, and 28 (8.8%) in children <6 months of age.

There was clearly seasonal variation. 73% of episodes were detected during cool months (October to March) versus 27.2% during warm months (April-September), $P < 0.001$.

Two hundred forty-four (76.5%) patients reported group child care attendance, 144 (45.1%) patients had a viral respiratory infection by history during the month before IPD, and 44 (13.8%) had received antibiotic treatment the month before IPD.

Two hundred twenty-five (70.5%) patients reported a history of breast-feeding, and 122 (38.2%) patients had been exposed to tobacco smoke. The mean household size was 4 cohabitants (SD: 1.2, range: 2–10).

According to the criteria of the American Academy of Pediatrics,[19] only 5 of 319 (1.5%) children were at high risk of IPD, including 2 children with malignant disease who were receiving immunosuppressive therapy, 1 with diabetes mellitus, 1 with congenital cyanotic cardiopathy, and 1 with pulmonary emphysema.

Concerning immunization status for *S. pneumoniae*, 168 (52.8%) cases had received at least 1 dose of PCV7, although only 141 (44.3%) were considered fully vaccinated by age.

Incidence

Rates of IPD increased between 2007 and 2009; the incidence of IPD in <5 years in 2007 was 76.2 cases/100,000 population; in 2008, it was 82.2 cases/100,000 population; and in 2009, it was 109.9 cases/100,000 population. Comparing rates in 2007 and 2009, there was an increase of 44% (95% CI: 10%–89%; $P = 0.008$). There was a significant increase in the rate of pneumonia during the study period: an increase of 81% (95% CI: 33%–148%; $P = 0.001$) comparing 2007 versus 2009. There were no significant changes in the rates of meningitis and bacteremia during the study (Table, Supplemental Digital Content 1, http://links.lww.com/INF/B14 shows rate of IPD in children according to age group during 2007–2009).

Clinical Presentation

Overall, the clinical diagnosis of patients included in this study was pneumonia in 254 (79.6%) patients, meningitis in 29 (9.1%), bacteremia in 25 (7.8%), arthritis or osteomyelitis in 6 (1.9%), sepsis in 3 (0.9%), and cellulitis in 2 (0.6%). Among pneumonia cases, 51 (20.1%) were noncomplicated pneumonia, 171 (67.3%) were empyema, and 32 (12.6%) parapneumonic pleural effusion.

Table 1 shows the distribution of positive samples detected by culture and by real-time PCR according to main clinical presentations.

Children were admitted to the hospital for 310 (97.2%) of the 319 episodes. The mean length of stay was 10.8 days (SD: 7.5). The longest mean stay by clinical presentation was 18.25 days (SD: 13.19) for meningitis. Of note, patients with noncomplicated pneumonia have no statically differences in the median age,

TABLE 1.   Distribution of Positive Samples Detected by Culture and by Real-time PCR According to Main Clinical Presentations

	Positive Blood Culture	Positive Plasma Real–time PCR	Positive Pleural Effusion Culture	Positive Pleural Effusion Real–time PCR
Noncomplicated Pneumonia (n = 51)	18	39		
Parapneumonic pleural effusion (n = 32)	5	18	0	12
Empyema (n = 171)	18	75	32	151
			CSF Culture	CSF Real–Time PCR
Meningitis (n = 29)	15	18	20	19
Bacteremia (n = 25)	25	2		

gender, days of hospitalization, and total days of antibiotic in the groups "positive blood culture" and "only plasma real-time PCR positive."

The mean of days of antibiotic therapy (including extrahospital treatment) was 17.8 days (SD: 6.8). Arthritis and osteomyclitis were the diagnosis with the longest duration of antibiotic therapy (28.17 days, SD: 11.78).

Forty-four children (13.8%) were admitted to the pediatric intensive care unit. Overall, 27 of 29 episodes of meningitis (93.1%), 14 of 254 (5.5%) episodes of pneumonia, and 3 of 3 (100%) episodes of sepsis were admitted to ICU.

Among children admitted to ICU, 22 (51.2%) had received at least 1 dose of PCV7, but only 19 (44.2%) were fully vaccinated for age.

Of the 319 patients, there were 4 (1.3%) deaths, 3 patients with meningitis and 1 with sepsis. Thirty-four patients (10.7%) had sequelae associated with *Streptococcus pneumoniae*: neurologic sequelae in 15 of 29 (51.7%) meningitis episodes and pulmonary sequelae in 17 of 254 (6.7%) children with pneumonia.

Serotypes, Molecular Study, and Antibiotic Susceptibility

Diagnosis was established in 123 (38.6%) episodes by culture and in 196 (61.4%) by real-time PCR.

The serotyping study was done in 300 (94%) of the total IPD episodes. In 120 (40%), the serotyping study was carried out with strain isolates from culture, whereas 180 (60%), were done with direct samples by multiplex, real-time PCR. Overall, 23 different serotypes were identified. Nevertheless, there was a large number (76, 25.3%) of samples with *ply* and *wzg* gene positive but no specific gene of 24 serotypes tested, so we considered these as "other serotypes." The most frequent among identified serotypes were serotype 1 (62; 20.7%), 19A (47; 15.7%), and 3 (37; 12.3%). Of the 300 episodes, 27 (9%) were caused by PCV7 serotypes and 273 (91%) were caused by non-PCV7 serotypes. One-hundred nineteen (39.7%) were caused by PCV10 serotypes and 209 (69.7%) by PCV13 serotypes. Of 27 patients who had IPD attributed to PCV7 serotypes, 5 were well vaccinated. The characteristics of vaccinated children with IPD caused by PCV7 serotypes are shown in Table 2.

There were significant differences in the clinical presentation among the most prevalent serotypes detected in the study: serotype 1 and serotype 3 were significantly associated with pneumonia, whereas the clinical presentation of episodes caused by serotype 19A was more diverse (Table 3). Among episodes resulting in death, 3 were caused by non-PCV7 serotypes (serotypes 7F, 27, and 6A) and 1 by vaccine serotype 23F in an unvaccinated child.

PCV7 serotypes were significantly present in younger children (mean age, 21.2 vs. 30.4 months in IPD caused by non-PCV7 serotypes; P = 0.004). In addition, IPD by PCV7 serotypes was

TABLE 2.   Characteristics of Vaccinated Children With Invasive Pneumococcal Disease Caused by PCV7 Serotypes

Sex	Age (mo)	Clinical Presentation	Previous Disease	Serotype
Female	5	Bacteremia	Methylmalonic acidosis	19F
Female	13	Bacteremia	Retinoblastoma (neutropenia)	19F
Female	45	Pneumonia	No	19F
Male	29	Pneumonia	No	14
Female	50	Pneumonia	No	14

associated with a higher rate of sequelae than non-PCV7 serotypes (25.9% vs. 9.9%; P = 0.02). In contrast, non-PCV7 serotypes were associated mainly with pneumonia: 81.3% of total episodes caused by non-PCV7 versus 48.1% of episodes caused by PCV7 serotypes; P < 0.001 (Table 3).

Molecular analysis by MLST was performed for 108 of 123 (87.8%) strains isolated by culture. Overall, when comparing our data with isolates listed in the MLST database, there were 46 different STs, including 8 new ST profiles (ST3437, serotype 23F; ST3436, serotype 38; ST4827, ST2948, and ST4826, serotype 19F; ST4676, serotype 27; ST5195, serotype 19A; ST4834 serotype 7F). Of these, 50% new ST expressed PCV7 serotypes. eBURST analysis using the stringent 6/7 identical loci definition grouped the 46 ST into 6 CCs and 34 singletons (Fig. Supplemental Digital Content 2, http://links.lww.com/INF/B15, shows clonal distribution of 108 invasive isolates from pediatric patients obtained by use of the output of eBURST, version 3. Each circle represents single MLST, with the area proportional to the number of isolates of that ST. Black lines represent single-locus variants).

Six CCs or ST accounted for 55.9% of total collection: ST306 (n = 22 isolates serotype 1), ST320 (n = 9 isolates serotype 19A), CC289 (n = 8 isolates serotype 5), ST191 (n = 8 isolates serotype 7F), ST1201 (n = 7 isolates serotype 19A), and CC276 (n = 5 isolates serotype 19A and 1 serotype 24B).

Comparative analysis of our serotype and ST results with those published in the MLST database showed that 5 of our STs expressed serotypes different than those previously reported (capsular switching): ST101 (serotype 15C), ST109 (serotype 23F), ST230 (serotype 24B), ST433 (serotype 28), and ST2372 (serotype 23F). Antibiotic susceptibility was available for 120 of 123 (97.5%) strains.

None of the 120 strains was fully resistant (MIC >8 μg/mL) and 3 (2.5%) were intermediately penicillin-resistant according to nonmeningeal breakpoints. Two of these strains be-

TABLE 3. Epidemiologic Data and Clinical Characteristic of 300 Episodes of IPD Caused by PCV7 Serotypes, Non-PCV7 Serotypes, and the 5 Main Serotypes Detected in the Study

Serotype	No. Episodes	Age (Mean SD)	Sex (Males) N (%)	Clinical Presentation*				PICU Admission N (%)	Outcome	
				Pneumonia N (%)	Bacteremia N (%)	Meningitis N (%)	Others N (%)		Sequelae	Death
PCV7	27	21.2 (13.68)	16 (59.3%)	13 (48.1%)	7 (25.9%)	6 (22.2%)	1 (3.7%)	6 (22.2%)	7 (25.9%)	1 (3.7%)
Non–PCV7	273	30.4 (15.84)	145 (53.1%)	222 (81.3%)	18 (6.6%)	23 (8.4%)	10 (3.6%)	38 (13.9%)	27 (9.9%)	3 (1.1%)
Serotype 1	62	41 (10.48)	34 (54.8%)	62 (100%)	0	0	0	2 (3.2%)	3 (4.8%)	0
Serotype 19A	47	19.21 (10.54)	28 (59.6%)	32 (68.1%)	6 (12.8%)	6 (12.8%)	3 (6.3%)	9 (19.1%)	4 (8.5%)	3 (2.4%)
Serotype 3	37	29.03 (14.11)	16 (43.2%)	36 (97.3%)	0 (0%)	1 (2.7%)	0	6 (16.2%)	5 (13.5%)	0
Serotype 7FA	21	24.71 (16.2)	16 (76.2%)	12 (57.1%)	4 (19%)	4 (19%)	1 (4.9%)	5 (23.8%)	4 (19%)	1 (4.8%)
Serotype 14	12	24.71 (13.3)	9 (75%)	11 (91.7%)	1 (8.3%)	0	0	0	3 (25%)	0

*Other clinical presentations were arthritis or osteomyelitis, sepsis, and cellulitis.
Statistically significant differences (χ^2 test for categorical variables and Student *t* test for continuous variables) were found for the following.
Mean age: PCV7 versus non-PCV7 serotypes ($P = 0.004$); serotype 1 versus other serotypes ($P < 0.001$); serotype 19A versus other serotypes ($P < 0.001$).
Gender: serotype 7F/A versus other serotypes ($P = 0.03$).
Clinical presentation: serotype 1 versus other serotypes ($P = 0.001$).
PICU admission: serotype 1 versus other serotypes ($P = 0.002$).
Outcomes: PCV7 versus non-PCV7 serotypes ($P = 0.02$).

longed to ST320 expressing serotype 19A and the other belonged to ST2948 expressing serotype 19F. Forty-one isolates (34, 4%) had an MIC ≥ 0.12 μg/mL, and 18 of these isolates (43.9%) were serotype 19A. Regarding cefotaxime, only 2 isolates (1.7%) showed an MIC ≥ 4, and both belonged to ST320-expressing serotype 19A. Regarding meningeal breakpoints, 21 isolates (17.5%) showed a diminished susceptibility to cefotaxime, and serotype 19A account 66.7% of these episodes.

DISCUSSION

This is a prospective study that updates the information about IPD in children in a geographical area without systematic vaccination. The inclusion of episodes with negative culture and only detected by real-time PCR has allowed us to gain greater insight into the burden of the disease and the main serotypes causing IPD in Barcelona. We think that molecular methods can be used directly not only on samples as cerebrospinal fluid, pleural effusion, or arthritis fluid but also in plasma improving the ability to diagnose IPD. The usefulness of real-time PCR in blood has been discussed because some authors found a high rate of detection of pneumococcal DNA in healthy controls associated with nasopharyngeal carriage.[20] However, we consider plasma PCR–positive patients with noncomplicated pneumonia and negative culture as patients with pneumococcus pneumonia and not false positive from pneumococcus colonization. All these patients are clinically compatible with pneumococcus pneumonia (all of them have high fever, cough, crackling, or hypophonesis in the auscultation and radiologic image of alveolar condensation). Moreover, our patients with noncomplicated pneumonia have no statically differences in clinical variables in the groups "positive blood culture" and "only plasma real-time PCR positive." In the same way, other authors have described previously the validity of plasma PCR in diagnosing IPD.[8,11] A low bacterial load could explain the negativity of the culture in these patients. However, more studies in this area will be required to confirm the validity of plasma PCR in determining deep-seated pneumococcal infection.

The incidence of IPD continues to increase in our geographic area. The incidence is higher than previously reported, presumably as a result of low sensitivity of the bacterial culture, which was the only microbiologic criterion for definition of IPD in previous studies.[5] The hospitals included in the study are the most important ones in pediatrics in Catalonia. Non-PCV7 serotypes cause most IPD episodes, whereas PCV7 serotypes cause only a minority of cases. The change in pneumococcal serotypes causing IPD is associated with a change in clinical presentation and in some epidemiologic characteristics.

Concerning clinical manifestations, the proportion of pneumococcal bacteremia and meningitis is relatively stable, but a significant increase in pneumonia was observed. These changes were observed by others in the United States.[21] The increase in pneumonia has also been observed in other regions of Spain[22] and in other countries such as Denmark and United States.[23,24] A significant proportion of these pneumonias is complicated by empyema, and some of the children (5.5%) developed pulmonary sequelae and required intensive care management. This high proportion of empyema and parapneumonic pleural effusion could also be explained because the study was performed in 2 tertiary-care pediatric hospitals. Of concern is the increase in complicated pneumonias caused by non-PCV7 serotypes. It is important that vaccines against *S. pneumoniae* include serotypes associated with pneumonia, such as serotypes 1 and 3.

The mean age of children with IPD is higher than previously reported in the prevaccine era,[25] and the majority of children with IPD is healthy without any recognized risk factors. This high proportion of healthy children is different from what was recently reported by Kaplan et al.[21] These differences are caused in part by the introduction of a virulent clone of serotype 1, with proven capacity to produce outbreaks, just before the implementation of PCV7 in our country.[5] Serotype 1, which is associated with pneumonia in older healthy children,[22] was the main serotype detected in our series, whereas in Kaplan et al's study, serotype 1 was infrequent.

It is remarkable how many serotypes are involved, which demonstrates the great diversity of pneumococcus. The detection of only 24 serotypes by multiplex real-time PCR methodology is a limitation of the study and raises concern about the high diversity of pneumococcus and the need for accurate surveillance of this disease in the coming years, including new molecular methods to detect a wider range of serotypes.

Despite the low vaccination coverage (approximately 50%), a low rate of infections due to vaccine serotypes were found. These data confirm, as have many other studies, that PCV7 is highly effective against IPD caused by vaccine serotypes, because this vaccine also prevents IPD in adult contacts and nonvaccinated siblings through indirect effect (herd immunity) on pneumococcal transmission.[26]

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Regarding the 5 cases of IPD in vaccinated children, it is important to note that 2 of them had a previous disease and had not completed the vaccination schedule, which might explain this failure.

As to the clonal study, it also showed great genetic diversity in the strains that produce IPD in our pediatric population, including the appearance of new ST and capsular switches.

As reported before,[5] ST306 is the most important ST in our population. This ST relates to an increase of empyema.[22] Recently, our group showed that this increase of empyema associated with ST306 may be because of the presence of PsrP,[27] a pneumococcal virulence factor not present in all STs of *S. pneumoniae*. PsrP is an adhesine related to the invasion of pulmonary cells by pneumococcus.

As for the study of antibiotic susceptibility, we previously reported a decrease in the global rate of penicillin resistance if we compare the present rate with that of the prevaccine period.[5] Nevertheless, the presence of strains of serotype 19A, especially those with ST320 having multiple antibiotic resistances, is grounds for concern. PCV13 includes serotype 19A, which we hope will be controlled once the new vaccine is implemented.

One of the limitations of the study is that not all episodes of IPD are detected, as blood cultures and/or PCR *S. pneumoniae* are not performed in all children with fever or suspected of pneumonia. Therefore, some bacteremias and pneumonias have presumably not been diagnosed. However, our guidelines for evaluating children with fever did not substantially change during the study period.

In conclusion, IPD continues to increase in Barcelona, and the rate is much higher than previously reported due to low sensitivity of bacterial culture. Non-PCV7 serotypes were responsible for 91% of episodes, and pneumonia was the main clinical presentation.

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4.2- Artículo_2

Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype

19A among children in Barcelona

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Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A among children in Barcelona[☆]

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Summary *Objective:* To describe the epidemiology of invasive pneumococcal disease (**IPD**) caused by *Streptococcus pneumoniae* serotype 19A.

Methods: We studied all children and adolescents with **IPD** caused by serotype 19A who were admitted to a Children's Hospital in Barcelona (1997—2007). Serotyping, antibiotic susceptibility and clonal analysis were performed.

Results: Comparing the pre-vaccine period (1997—2001) with the early vaccine period (2002—2004) and the late vaccine period (2005—2007) there was an increase of **IPD** caused by serotype 19A: 1 of 58 episodes (1.7%) vs. 8 of 54 episodes (14.8%) vs. 27 of 123 episodes (21.9%), respectively ($P = 0.002$). All *S. pneumoniae* serotype 19A isolated in the pre-vaccine and early vaccine periods ($n = 9$) were penicillin susceptible, while in the late vaccine period, 12 of 27 (44%) were penicillin nonsusceptible ($P = 0.01$).

A clonal analysis revealed 15 different sequence types (STs) expressing serotype 19A. 10 of them were preexisting STs associated with serotype 19A including the multidrug-resistant ST320 and ST276.

Conclusion: There was an increase of **IPD** caused by *S. pneumoniae* serotype 19A which was mainly related with the emergence of preexisting clones several of them closely related with

international multidrug-resistant clones. These results should be considered when selecting the new conjugate pneumococcal vaccines.

Introduction

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide, especially among young children. There are 91 different pneumococcal serotypes that can be distinguished by their polysaccharide capsule, but only around one third produces invasive pneumococcal disease (**IPD**).[1]

In the year 2000 the 7-valent pneumococcal conjugate vaccine (**PCV7**) against serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F was licensed in the United States for young children. In Spain **PCV7** was introduced in June 2001 and the current estimated vaccination uptake is around 50%.[2] In pre-vaccine years the **PCV7** serotypes were responsible for 50—80% of all **IPD** varying widely depending on geographic location.[1] In addition, **PCV7** serotypes were often penicillin and multidrug-resistant isolates. Following introduction of **PCV7** in the USA there was a dramatic decline in **IPD** rates and drug-resistant pneumococci.[3,4] However, recent reports from the USA and other countries have shown an emergence of **IPD** caused by non-**PCV7** serotypes[5,6] and the increase of drug-resistant pneumococcal clones expressing non-**PCV7** serotypes in nasopharyngeal carriage and invasive isolates.[7—10]

It has been shown that **PCV7** protects against pneumococcal nasopharyngeal colonization by vaccine serotypes.[11] Therefore, the risk of colonization and consequently the risk of **IPD** by **PCV7** serotypes decrease both in the vaccine target age group (young children) and in older children and adults because of herd immunity.[12]

Reducing carriage of **PCV7** serotypes may produce a vacant ecological niche which may be filled by non-**PCV7** serotypes, and this phenomenon is called "serotype replacement". Replacement colonization has been reported,[13] but little is know about the relevance of serotype replacement in the epidemiology and clinical findings of **IPD**.

Recently, some reports have shown that serotype 19A is becoming one important cause of pneumococcal disease in the USA population with high vaccine uptake.[5,14—16] However, it has also been reported an increase of pneumococcal disease caused by serotype 19A in Korea and Israel[17,18] where PCV7 is not used which suggests that other non-vaccine factors such as antibiotic consumption may play a role in this increase. It is worrisome that some of these serotype 19A pneumocci are multidrug-resistant isolates.[19]

The aim of this study was to describe the clinical and molecular epidemiology of *S. pneumoniae* serotype 19A causing **IPD** among children in Barcelona.

Patients and methods

Patients and setting

We studied all children and adolescents with invasive pneumococcal disease (**IPD**) who were admitted to Sant Joan de Deu Hospital in Barcelona (January 1997 to December 2007), and we selected for the present study those infected with serotype 19A. A detailed description of our institution and the geographic area was reported elsewhere.[2] The Clinical Microbiology Laboratory monitored all culture-proven pneumococcal infections and several variables are routinely registered including demographic data, identification hospital number, type of infection, and antimicrobial susceptibility. Serotyping, antimicrobial susceptibility testing and clonal analysis by multilocus sequence typing (MLST) were performed as previously described,[2] and summarized below.

PCV7 is not currently subsidized by the Spanish Health Service. **PCV7** uptake has increased since its introduction in June 2001 with an estimated **PCV7** coverage in the year 2007 about 45—50%.[2]

Definitions

Invasive pneumococcal disease (**IPD**) was defined as the presence of clinical findings of infection together with isolation of *S. pneumoniae* in blood, cerebrospinal fluid or any other sterile fluid by culture. No other microbiological techniques, such as polymerase chain reaction (PCR), were used for the diagnosis of **IPD**.

IPD was classified according to the International Classification of Disease, Ninth Revision (ICD-9) specific for diseases caused by *S. pneumoniae* including: meningitis, pneumonia, parapneumonic empyema, occult bacteremia, sepsis, arthritis, peritonitis, and endophthalmitis. We reviewed the electronic medical records and registered demographic and clinical variables including: age, sex, date of admission, clinical manifestations, outcomes and vaccination status. Data were recorded following the guidelines of the Hospital's Ethical Committee.

Serotyping and antimicrobial susceptibility

All isolates were serotyped by Quellung reaction. In addition, isolates identified as serogroup 19 during the pre-vaccine period were also tested by specific Real Time PCR of serotype 19A according to a published assay.[20]

Agar dilution technique was used to determine the minimal inhibitory concentrations (MICs) of penicillin and other antibiotics. Antibiotic susceptibility was defined according to the 2008 breakpoints by the Clinical Laboratory Standards Institute.[21] Isolates with intermediate or high level resistance were defined as nonsusceptible. Multidrug resistance was defined as nonsusceptible to three or more antimicrobial agents.

Clonal analysis

MLST was performed as reported elsewhere.[22] The assignment of alleles and sequence types (ST) were carried out using the software at the pneumococcal web page:

Table 1 Invasive Pneumococcal Disease (IPD) caused by serotype 19A in three periods (pre-vaccine period, early vaccine period, and late vaccine period).

	Serotype 19A episodes/Total episodes (%)			
	Pre-vaccine (1997—2001)	Early vaccine (2002—2004)	Late vaccine (2005—2007)	P value[a]
Overall episodes	1/58 (1.7)	8/54 (14.8)	27/123 (21.9)	0.002
Clinical manifestations				
- Meningitis	0/18 (0)	1/8 (12.5)	4/24 (16.6)	0.198
- Pneumonia (overall)	1/15 (6.6)	1/22 (4.5)	11/58 (18.9)	0.169
without empyema	1/8	0/9	5/24	
with empyema	0/7	1/13	6/34	
- Bacteremia/sepsis	0/17 (0)	6/16 (37.5)	9/32 (28.1)	0.024
- Others[b]	0/8 (0)	0/8	3/9 (33.3)	0.048

[a] Chi-square test (two by three tables).
[b] Others (arthritis, appendicitis, and endophthalmitis).

www.mlst.net. Analysis of ST and assignment to clonal complex was performed with the eBURST program.[23] STs that shared five of seven allelic identities (double locus variants [DLV]) or shared six of seven allelic (single locus variants [SLV]) were considered a clonal complex.

Statistical analysis

The study period was divided into three periods: the pre-vaccine period (1997—2001), the early vaccine period (2002—2004) and the late vaccine period (2005—2007). We used the Chi-square test or Fisher's exact test, when appropriated, to compare proportions, and Student t-test to compare means. Statistical analyses were performed using SPSS for windows, version 14.0. Rates of IPD (episodes/100,000 population)

were calculated using children population in the southern Barcelona area as reported elsewhere.[2] Two-sided P values $\leq$0.05 were considered statistically significant.

Results

During the 11-year study period, there were 235 episodes of invasive pneumococcal disease (IPD) occurring in 230 children; the mean age was 3.1 years (range 1 month—17 years) and 60% were males. Overall, there were 35 different serotypes, and serotype 19A was recovered from 15.3% of the episodes (36 of 235 isolates).

Comparing the pre-vaccine period (1997—2001) with the early vaccine period (2002—2004) and the late vaccine period (2005—2007) there was a significant

Table 2 Characteristics of patients with invasive pneumococcal disease (IPD) caused by serotype 19A vs. other serotypes.

	Episodes (%) Serotype 19A ($n = 36$)	Episodes (%) Other serotypes ($n = 199$)	P value
Age (yrs) ($\pm$SD)	1.8 ($\pm$2.2)	3.4($\pm$3.4)	0.01
Sex (males)	23 (63.9)	119 (56.7)	0.64
Underlying conditions	1 (2.8)[a]	19 (9.5)[b]	0.18
PCV7 vaccination[c]	3 (9.1)	20 (11)	0.73
Clinical Manifestations of IPD			
- Meningitis	5 (13.9)	45 (22.6)	0.23
- Pneumonia (overall)[d]	13 (36)	82 (41.2)	0.56
with empyema	7	47	
without empyema	6	35	
- Bacteremia/sepsis	15 (41.7)	50 (25.1)	0.04
- Others	3 (8.3)	22 (11)	0.62
PICU admission	4 (11.1)	49 (24.6)	0.07
Days of hospital stay (mean$\pm$SD)	9.2 ($\pm$6.8)	12.1($\pm$9.1)	0.07
Mortality	1 (2.8)	8 (4.1)	0.7

PICU: pediatric intensive care unit.
Statistical methods: Chi-square test (categorical variables) and Student t-test (continuous variables).
[a] A child with chronic pulmonary disease.
[b] Includes 8 IPD episodes in 7 children with malignant disease and innmunosupressive therapy, 5 IPD episodes in 2 children with CSF leakage, 2 with HIV infection, 1 with chronic pulmonary disease, 2 with chronic cardiac disease, and 1 with chronic renal failure.
[c] The PCV7 vaccinations status was available in 214 (33 with serotype 19A and 181 with others serotypes).
[d] Pneumonia with positive blood and/or pleural fluid cultures.

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increase of **IPD** caused by serotype 19A: 1 of 58 episodes (1.7%) vs 8 of 54 episodes (14.8%) vs 27 of 123 episodes (21.9%), respectively ($P = 0.002$). In terms of rates of **IPD** per 1000 blood cultures performed, serotype 19A also increased (from 0.04 episodes per 1000 blood cultures during pre-vaccine period to 0.48 during early vaccine period and 1.39 during late vaccine period; $P < 0.001$). Among children <5 years, rates of **IPD** caused by serotype 19A, comparing early vaccine period and late vaccine period, increased 147%; 95% CI, 11—448% (from 4.8 to 11.9 episodes per 100,000 population, $P = 0.02$). Table 1 shows the clinical manifestations of **IPD** in the three periods. Although the numbers were small, a statically significant increase was observed for bacteremia/sepsis and other infection group.

IPD caused by serotype 19A compared with other serotypes

As shown in Table 2, children with **IPD** caused by serotype 19A were younger than those infected with other serotypes: mean age of 1.8 years (range 1 month—11 years) vs 3.4 years (range 1 month—17 years), respectively, $P = 0.01$. Regarding the clinical manifestations, bacteremia/sepsis was more frequently found in the group of patients infected by serotypes 19A ($P = 0.04$). There were no statistically significant differences in underlying conditions (defined according to the criteria of the American Academy of Pediatrics),[24] vaccination uptake with PCV7 and mortality. Although it did not reach statistical significance the PICU admission rate and length of hospital stay tended to be greater in the other serotypes group.

Emergence of drug-resistant serotype 19A

Table 3 shows antibiotic resistance including CLSI (Clinical laboratory Standard Institute) meningeal and nonmeningeal breakpoints. According to meningeal breakpoints, in the pre-vaccine and the early vaccine periods, all serotype 19A isolates ($n = 9$) were penicillin susceptible, whereas in the late vaccine period 12 of 27 isolates (44%) were penicillin nonsusceptible ($P = 0.01$) (Table 2); 8 of these 12 isolates (66.6%) showed multidrug resistance (defined as nonsusceptible to three or more antimicrobial agents). Of note, 3 of the 8 multidrug-resistant isolates had a cefotaxime MIC of 2 μg/mL and were fully resistant to penicillin, erythromycin and tetracycline; these 3 isolates belonged to ST320 or ST276 clones (see below).

Molecular analysis of serotype 19A isolates

Of the 36 isolates, 35 (97%) were available for molecular analysis (Fig. 1). Overall, there were 15 different sequence types (STs) expressing serotype 19A: 10 preexisting STs associated with serotype 19A including the multidrug-resistant ST320 and ST276; 2 preexisting STs (ST30 and ST1793) not previously associated with serotype 19A; and 3 new STs (ST2589, ST2618 and ST3438).

Among the 24 penicillin susceptible 19A isolates, ST1201 was detected in 10 isolates. A clonal group identified by eBURST (that include ST199 as the primary founder of the group) was detected in another 8 isolates, including ST416, ST450, ST274, ST199, and the new ST2618. Clonal compositions of the remaining 6 isolates were: ST202, ST2589, ST1793, and ST30 (Fig. 1).

The analysis of the 12 penicillin nonsusceptible 19A isolates, revealed that of all them have identical allelic profiles or are single locus variants or double locus variants of several resistant international clones included in the PMEN (pneumococcal molecular epidemiology network) (Table 4). Thus, we detected the well-known Spain[23F]-1 (ST81) in 2 isolates and the multiresistant clone ST320 which is a DLV of Taiwan[19F]-14 in 2 isolates. In addition, there were 3 unusual clones: a single locus variant of Denmark[14]-32 (ST276) in 4 isolates, a double locus variant of the same clone Denmark[14]-32 (ST2013) in 3 isolates and one additional strain with a new MLST profile (ST3438) that was submitted to the curator of MLST for designation which was detected as a DLV of Columbia[23F]-26. Table 4 shows detailed information of the clinical manifestations, clonal composition, and antimicrobial susceptibility patterns.

Table 3 Antimicrobial susceptibility of serotype 19 A pneumococal strains ($n = 36$).

| MIC (mg/L)	Pre-vaccine and early vaccine period	Late vaccine period	P Value
	No. of strains (%) $n = 9$	No. of strains (%) $n = 27$	
Penicillin G (meningeal breakpoints)			
≤0.06	9 (100)	15 (56)	0.01
≥0.12	0	12 (44)	
Penicillin G (nonmeningeal breakpoints)			
≤2	9 (100)	26 (96.3)	0.5
4	0	1 (3.7)	
≥8	0	0	
Cefotaxime (meningeal breakpoints)			
≤0.5	9 (100)	22 (82)	0.4
1	0	3 (11)	
≥2	0	2 (7)	
Cefotaxime (nonmeningeal breakpoints)			
≤1	9 (100)	25 (92.6)	0.4
2	0	2 (7.4)	
≥4	0		
Erythromycin			
≤0.25	3 (33)	18 (66.6)	0.07
0.5	1 (11)	0	
≥1	5 (56)	9 (33.3)	
Tetracycline			
≤2	3 (33.3)	14 (57.8)	0.4
4	0	1 (3.7)	
≥8	6 (66.6)	12 (40.5)	
Chloramphenicol			
≤4	7 (78)	25 (93)	0.2
≥8	2 (22)	2 (7)	

Statistical Methods: Chi-square test (2 by k tables).
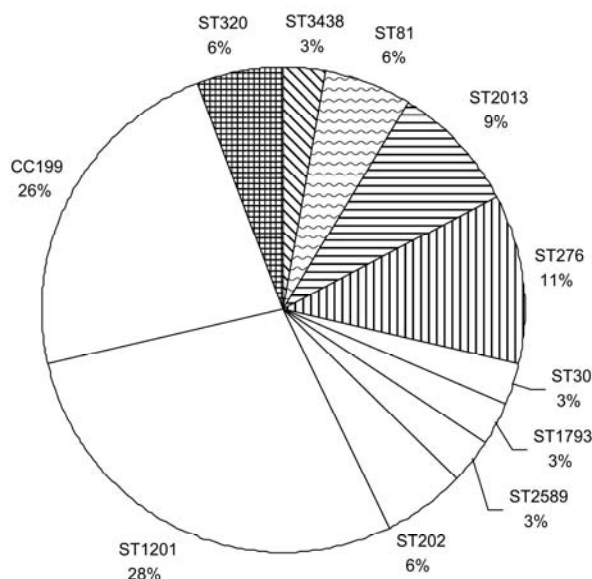
Figure 1 Clonal composition of 35 *Streptococcus pneumoniae* strains of serotype 19A isolated from children with invasive pneumococcal disease (**IPD**). Penicillin nonsusceptible strains are marked with grid. Clonal complex with ST199 as founder (**CC199**) includes ST199, ST416, ST274, ST2618 and ST450.

Discussion

The implementation of **PCV7** for children has been associated with a decline in invasive pneumococcal disease (**IPD**)[3,4] and nasopharyngeal colonization rates[11] caused by vaccine serotypes. An important question is whether or not the non-vaccine serotypes (or which of these serotypes) reach an advantage to colonize the nasopharynx and/or to produce **IPD** (the called replacement phenomenon). It seams reasonable to think that if this replacement occurs it could be especially significant for non-vaccine serotypes that were common inhabitants of the nasopharynx and/or had a high potential to produce outbreaks and were widely disseminated in the community before PCV7.

Recently an increase of **IPD** caused by non-**PCV7** serotypes in USA and Spain has been described.[2,5,6,16,25] At least two different models could explain the emergence of these non-vaccine serotypes.

First, the overgrowth of some non-vaccine serotypes, such as serotypes 1 and 5, which have a homogeneous clonal composition and are rarely isolated in healthy nasopharyngeal carriers. However, these serotypes (serotype 1 and 5) have a high potential to cause **IPD** and may produce outbreaks, particularly in a localized geographical area.[26]

Second, the enhancement of some non-vaccine serotypes, such as serotype 19A, which have a diverse clonal composition and are often isolated from healthy nasopharyngeal carriers. These serotypes (like serotype 19A) can produce **IPD** and their dissemination in the community could be expected to become a generalized phenomenon.

The present study shows an increase of **IPD** caused by serotype 19A, and a special concern about the emergence of serotype 19A variants of internationally multiresistant clones of

PMEN. Recent reports have documented an increase of antibiotic resistance among non-vaccine pneumococcal serotypes such as serotype 19A.[14,27] This may occur by different mechanisms: 1) capsular switching from an antibiotic resistant clone expressing a vaccine serotype in the pre-vaccine era that express a non-vaccine serotype in the vaccine era. It has been observed in serotype 19A variants circulating in USA[28]; 2) by emergence of minor antibiotic resistant clones existing prior to vaccination; and 3) the appearance of new clones.

Although we observed the three mechanisms in our study, the most important was the emergence of unusual antibiotic resistant clones expressing serotype 19A that had been considered as a minor cause of **IPD** in the pre-vaccine era.

In brief, the international multiresistant clone Spain[23F]-1 (ST81) was widely encountered in Spain and other countries in the pre-vaccine era,[29,30] but it was expressing vaccine serotypes, mainly serotype 23F, while in the present study we detected this clone expressing serotype 19A. Also we observed an emergence of minor clones existing in the pre-vaccine era such as ST276 reported in Portugal,[31] France and Turkey (www.mlst.net) or ST2013 reported in Egypt according also the web page of MLST. Of note, the multiresistant clone ST320 had been previously reported in Korea,[17] Australia and Norway,[32] and recently identified in USA[15,33] which aroused special concern.

The capsule of pneumococci is a major virulence factor and this may explain why certain serotypes have greater potential to cause **IPD**.[34] Up to date, there is a controversy about the impact of clonal type on the invasive disease potential of pneumococci.[34,35] We do not know if the minor clones found in the present study may continue with an international spread or they could be sporadic cases. Interestingly, the first highly penicillin resistant clones isolated in 1977 were

Table 4 Characteristics of patients, clonal compostition and antimicrobial susceptibility in 36 IPD episodes caused by serotype 19A.

Pt	Months	Sex	Year of isolation	Prior PCV7	Prior antibiotic therapy	Clinical manifestation	ST	PMEN clone	PEN MIC	CTX MIC	CHL MIC	ERY MIC	TET MIC
1	96	M	2001	Non	AZY	Pneumonia	202	DLV-Taiwan19F-14	0.015	0.03	4	0.5	64
2	12	M	2002	Non	AMX/CLV	Bacteremia/sepsis	30[a]	non-related	0.015	0.015	16	>128	16
3	6	M	2002	N.A	Non	Bacteremia/sepsis	1201	non-related	0.015	0.015	16	>128	64
4	24	M	2002	N.A	CLA	Empyema	2589[b]	non-related	0.015	0.03	4	>128	32
5	7	F	2002	Non	Non	Bacteremia/sepsis	N.A	non available data	0.015	0.03	4	64	32
6	18	M	2003	N.A	Non	Bacteremia/sepsis	416	DLV Netherlands15B-37	0.030	0.015	4	0.12	0.25
7	12	F	2003	Yes	Non	Meningitis	1793[a]	TLV-Netherlands14-35	0.015	0.015	4	0.12	0.25
8	12	M	2004	Non	Non	Bacteremia/sepsis	202	DLV-Taiwan19F-14	0.03	0.03	4	128	32
9	1	F	2004	Non	Non	Bacteremia/sepsis	1201	non-related	0.03	0.015	4	0.12	0.50
11	12	F	2005	Non	Non	Empyema	81	Spain23F-1	1	0.5	4	0.25	8
10	6	M	2005	Non	Non	Meningitis	81	Spain23F-1	0.50	0.25	8	0.12	8
12	1	M	2005	Non	Non	Bacteremia/sepsis	199	Netherlands15B-37	0.015	0.015	4	0.12	0.25
14	72	M	2005	Non	Non	Arthrits	276	Denmark14-32	2	2	4	128	16
15	6	M	2005	Non	AMX/CLV	Bacteremia/sepsis	276	Denmark14-32	0.5	0.5	4	128	8
13	24	M	2005	Non	Non	Empyema	276	Denmark14-32	1	1	4	128	16
18	24	M	2005	Yes	Non	Bacteremia/sepsis	1201	non-related	0.015	0.015	16	128	64
16	8	M	2005	Non	Non	Pneumonia	1201	non-related	0.015	0.015	4	0.12	0.25
17	48	M	2005	Non	Non	Pneumonia	1201	non-related	0.015	0.015	4	0.12	0.25
19	11	M	2006	Non	Non	Bacteremia/sepsis	199	Netherlands15B-37	0.015	0.015	4	0.12	0.25
20	10	M	2006	Non	Non	Bacteremia/sepsis	274	SLV Netherlands15B-37	0.015	0.015	4	128	32
21	10	F	2006	Non	Non	Bacteremia/sepsis	276	Denmark14-32	1	0.5	4	128	32
22	12	F	2006	Non	Non	Empyema	416	DLV Netherlands15B-37	0.015	0.015	4	0.12	0.25
23	24	F	2006	Non	Non	Pneumonia	1201	non-related	0.015	0.015	4	0.12	0.25
24	24	F	2006	Non	Non	Empyema	2013	DLV-Denmark14-32	0.25	0.12	4	0.12	64
25	36	M	2006	Non	Non	Empyema	2013	DLV-Denmark14-32	0.5	0.12	4	0.12	32
26	6	M	2006	Non	Non	Meningitis	2618[b]	TLV Netherlands15B-37	0.03	0.015	4	0.12	0.25
27	24	F	2006	Non	Non	Arthritis	3438[b]	DLV columbia23F-26	0.5	0.12	4	0.12	0.25
28	25	M	2007	Non	CEF	Empyema	320	DLV-Taiwan19F-14	4	2	4	128	32
29	132	F	2007	Non	Non	Meningitis	416	DLV Netherlands15B-37	0.015	0.015	4	0.12	0.25
30	6	F	2007	Yes	Non	Pneumonia	450	DLV Netherlands15B-37	0.015	0.015	4	0.12	0.25
31	16	F	2007	Non	Non	Bacteremia/sepsis	1201	non-related	0.015	0.015	4	0.12	0.25
32	12	F	2007	Non	Non	Bacteremia/sepsis	1201	non-related	0.015	0.015	4	0.12	0.25
33	3	M	2007	N.A	Non	Meningitis	1201	non-related	0.015	0.015	4	0.12	0.25
34	11	M	2007	Non	CLA	Pneumonia	2013	DLV-Denmark14-32	1	1	4	128	4
35	17	M	2007	Non	Non	Bacteremia/sepsis	320	DLV-Taiwan19F-14	2	1	4	128	32
36	15	M	2007	Non	Non	Arthritis	1201	non-related	0.015	0.015	4	0.12	0.25

Abbreviations: M, Male; F, Female; PEN, penicillin; AMX/CLV, amoxicillin/clavulanic; CTX, cefotaxime; CHL, choramphenicol; ERY, erythromycin; AZY, azithromycin; CLA,claritromicin; TET, tetracycline; MIC, minimun inhibitory concentration (µg/ml).

A summary of clonal analysis of 14 of these 36 serotype 19A strains had been included in a previous report.[2]

[a] These sequence types have only been associated with serotypes other than 19A.

[b] New sequence types that were identified in this study and deposited in the international multilocus sequence type database.

associated with serotypes 14 and 19A but international spread was more common for serotype 14.[36]

In conclusion, our study and others[14,15,19,27,33] show that *S. pneumoniae* serotype 19A is spreading rapidly and is becoming one important cause of **IPD** in the **PCV7** era. The spread of serotype 19A was related with the emergence of multiple penicillin susceptible and nonsusceptible clones, several of them closely related with well-known international multiresistant clones.

The possibility that the emergence of serotype 19A causing IPD is co-incidental and not related to vaccination should be considered. However, based on the reported experience and in our own data one might think that the vaccine is at least an additional factor that may contribute in selecting the most successfully serotypes for global spreading.

Continued surveillance of pneumococcal serotypes causing **IPD** is mandatory for the new conjugate vaccine strategies.

Acknowledgments

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5. DISCUSIÓN DE LOS RESULTADOS

Se trata de dos estudios prospectivos realizados en una región sin vacunación sistemática, a diferencia de otros estudios americanos publicados con anterioridad, y permite conocer la evolución que ha experimentado la ENI tras la introducción, irregular, de PVC7 en nuestra área geográfica.

En el primer artículo, a diferencia también de otros trabajos, no se ha utilizado tan sólo el cultivo para el diagnóstico de ENI de forma que se han utilizado también técnicas de biología molecular (PCR) para incrementar los diagnósticos. Este hecho ha permitido tener una idea más aproximada del impacto real de la enfermedad así como obtener un incremento en el serotipado de *S. pneumoniae*, con lo que se ha logrado caracterizar mejor la ENI.

El segundo artículo se centra en la caracterización del segundo serotipo más frecuente en nuestro medio, el serotipo 19A, de extraordinario interés por su emergencia tras la introducción de PCV7 debido a un fenómeno de reemplazo capsular. Este serotipo se asocia a multirresistencia a antibióticos por lo que su estudio es relevante.

A continuación se discuten los resultados obtenidos en ambos estudios.

5.1- Objetivo 1

Determinar la incidencia de ENI en menores de 5 años en nuestro medio

Se incluyeron en el primer estudio 319 pacientes (53.3% varones), la media de edad fue de 30 meses (DE 15.6 meses). La edad media es más alta que la descrita en estudios previos a la introducción de la vacuna[44] , lo que confirma la sospecha de un cambio en la epidemiología de la ENI tras la introducción de PCV7.

192 casos (60.2%) tuvieron lugar en niños entre 24 y 59 meses, 99 (31%) en niños entre 7 y 23 meses y 28 (8.8%) en niños menores de 6 meses.

Se aprecia una clara tendencia estacional. 73% de los episodios tuvieron lugar durante los meses fríos (octubre a marzo) mientras que 27.2% en los meses cálidos (abril-septiembre), p>0.001.

Según los criterios de la Academia Americana de Pediatría[45], sólo 5 de 319 (1.5%) pacientes presentaban factores de riesgo para ENI, incluyendo 2 niños con neoplasia que recibían tratamiento quimioterápico, 1 paciente con diabetes mellitus, 1 paciente con cardiopatía congénita cianosante y 1 con enfisema pulmonar. La alta proporción de pacientes sin enfermedad predisponente es diferente a la referida por Kaplan et al[46] y avala la necesidad de implantación de campañas de vacunación sistemáticas dirigidas a toda la población infantil y no sólo a grupos de riesgo.

En cuanto al antecedente de vacunación con PCV7, 168 (52.8%) pacientes habían recibido al menos una dosis de PCV7 aunque sólo 141 (44.3%) estaban correctamente vacunados según ficha técnica (VSFT).

Según ficha técnica, la serie primaria en niños consiste en tres dosis, de 0,5 ml cada una, administrándose habitualmente la primera dosis a los 2 meses de edad y con un intervalo de, al menos, 1 mes entre dosis. Se recomienda una cuarta dosis en el segundo año de vida.

En niños previamente no vacunados: Niños de 7 a 11 meses de edad: dos dosis, de 0,5 ml cada una, con un intervalo de al menos 1 mes entre dosis. Se recomienda una tercera dosis en el segundo año de vida. Niños de 12 a 23 meses de edad: dos dosis, de 0,5 ml cada una, con un intervalo de al menos 2 meses entre dosis. Niños de 24 meses a 5 años de edad: una dosis única.

El estado vacunal frente a *S.pneumoniae* por grupo de edad puede verse en la siguiente tabla:

Grupo de edad	VSFT	No VSFT	**TOTAL**
3 a 6 m	9 (32.1%)	**19 (67.9%)**	**28 (100%)**
7 a 23m	41 (41.8%)	**58 (58.2%)**	**99 (100%)**
24 a 59 m	91 (47.4%)	**101 (52.6%)**	**192 (100%)**
TOTAL	141 (44.3%)	**178 (55.7%)**	**319 (100%)**

Tabla 2. *Estado vacunal frente a S.pneumoniae por grupo de edad*

Comparando las incidencias del 2007 y 2009 (76.2 y 109.9 casos/100.000 habitantes, respectivamente) se observa un incremento del 44% (IC 95%, 10%-89%, p: 0.008).

Se aprecia un incremento significativo en la incidencia de neumonía durante el período de estudio: un incremento del 81% (IC 95%, 33%-148%; p: 0.001) comparando el 2007 con el 2009. Por el contrario, no hay cambios significativos en la incidencia de bacteriemia y meningitis durante el período de estudio. En la siguiente tabla se detallan las incidencias globales y por manifestaciones clínicas en los diferentes años del estudio.

Con estos resultados es evidente que la incidencia de ENI ha continuado aumentando en nuestra área geográfica, a expensas, sobre todo, de las neumonías. La incidencia es mayor que la descrita previamente, en parte debido a la baja sensibilidad del cultivo, único método diagnóstico utilizado en estudios previos[38].

Tabla 3. *Incidencia de la enfermedad neumocócica invasiva (ENI) en niños según el grupo de edad en los años 2007,2008 y 2009*

	2007		2008		2009		(2007 vs 2009)	
	Episodios	Incidencia[a]	Episodios	Incidencia[a]	Episodios	Incidencia[a]	% Cambio (95% IC)	p
NIÑOS < 24 meses								
Toda la ENI	41	100.5	35	84.1	52	120	21% (-20 a 82%)	0.3
Neumonía	14	34.3	21	50.5	35	80.8	138% (28 a 343%)	0.004
Neumonía no complicada	5	12.3	5	12	15	34.6	186% (4 a 687%)	0.003
Neumonía complicada	9	22.1	16	38.4	20	46.1	112% (-4 a 365%)	0.05
Meningitis	11	27.0	7	16.8	7	16.2	-39% (-76a 57%)	0.2
Bacteriemia oculta/sepsis	12	29.4	5	12	8	18.5	-36% (-74 a 56%)	0.3
Otros	4	9.8	2	4.8	2	4.6	-52% (-91a 160%)	0.3
							(2007 vs 2009)	
NIÑOS 24-59meses	2007		2008		2009		% Cambio (95% IC)	p
Toda la ENI	47	62.9	63	81.1	81	104.2	64% (15 a 135%)	0.006
Neumonía	46	61.6	59	76	79	101.6	64% (14 a 136%)	0.007
Neumonía no complicada	5	6.7	5	6.4	16	20.6	205% (12 a 733%)	0.02
Neumonía complicada	41	54.9	54	69.5	63	81	47% (-1 a 117%)	0.05
Meningitis	1	1.3	3	3.9	0	-	-	
Bacteriemia oculta/sepsis	0	-	1	1.3	2	2.6	-	
Otros	0	-	0	0	0	-	-	
NIÑOS <60 meses								
Toda la ENI	88	76.2	98	82.2	133	109.9	44% (10 a 89%)	0.007
Neumonía	60	52.0	98	67.1	114	94.2	81% (33 a 148%)	0.0001

[a] Episodios por 100.000 niños que viven en el area geográfica del Hospital Sant Joan de Déu según los datos del Institut Català d'Estadística (www.idescat.net).

Métodos Estadísticos: calculamos el IC 95% y los valores p con Epi Info version 6.0 (CDC).

Describir las principales características clínicas de la ENI en nuestro medio

Los diagnósticos clínicos de los pacientes incluidos en el estudio pueden verse en la siguiente figura:
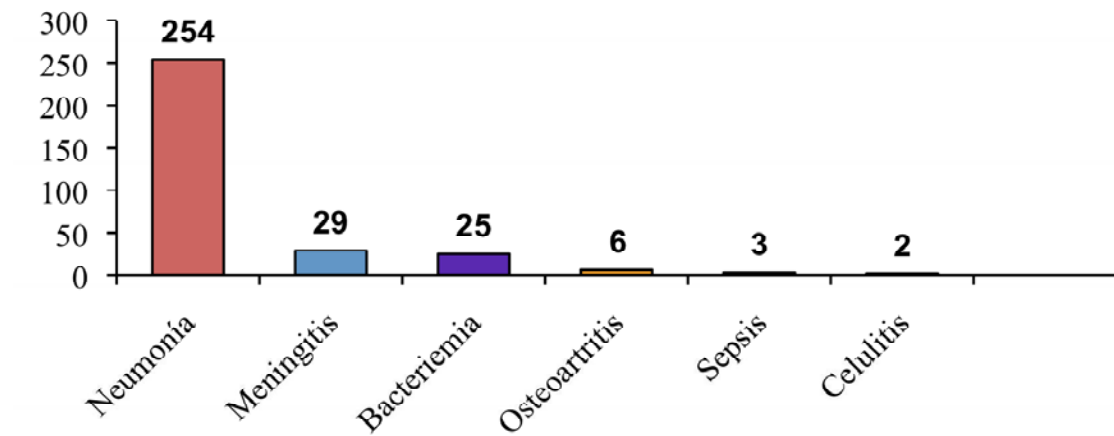

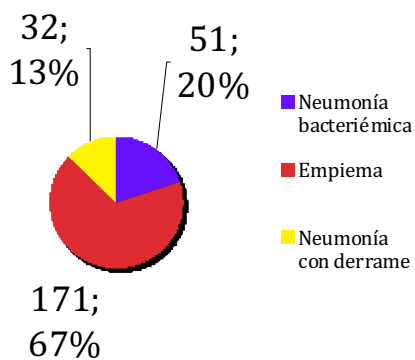**Figura 1:** *Diagnósticos clínicos*

Figura 2: *Distribución de los diferentes tipos de neumonía*

En los últimos años se aprecia un incremento significativo en el número de neumonías, mientras que el número de bacteriemias y meningitis permanece relativamente estable. Estos cambios se han observado también en otras zonas de España[47] y en otros países como Dinamarca y Estados Unidos[48,49].

Cabe destacar que una gran proporción de las neumonías se complicaron con la aparición de empiema y algunos de ellos requirieron ingreso en UCI. La elevada proporción de empiemas y derrames pleurales en nuestra serie puede ser debida, en parte, a que el estudio se ha realizado en 2 hospitales de tercer nivel, que son considerados los más importantes en Pediatría en Catalunya.

Los principales diagnósticos por grupo de edad se muestran en la siguiente gráfica. En los niños menores de 2 años de edad (n: 127) podemos observar un polimorfismo clínico con 3 formas clínicas: neumonía ya sea simple o complicada con derrame pleural o empiema, en un 53.5% de los casos totales; meningitis, en un 19.7%; y bacteriemia (17.3%). En el 9.4% restante cabe destacar 6 formas osteoarticulares, 4 sepsis y 2 celulitis Por el contario, en las formas clínicas de los niños con edades comprendidas entre los 24 y 59 meses (n: 192) se observó un monoformismo clínico: el 96.4% de las formas clínicas fueron neumonías.
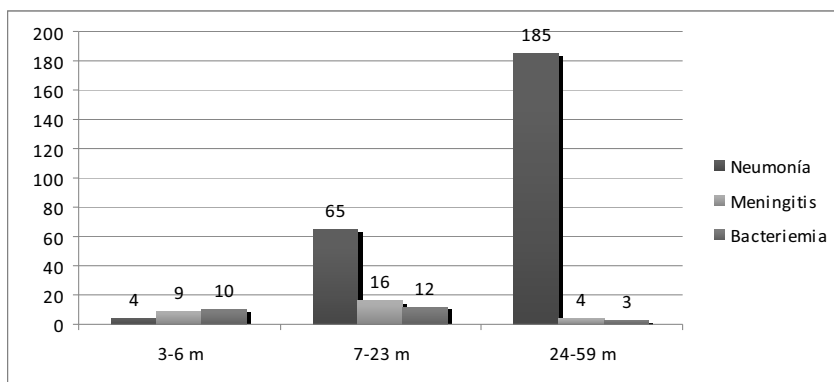

Figura 3: *Distribución de los principales diagnósticos clínicos por grupo de edad*

A continuación se muestra la tabla que describe cómo se ha realizado el diagnóstico de las diferentes manifestaciones clínicas. Podemos observar como la PCR permite incrementar considerablemente el diagnóstico de la neumonía en todas sus formas (neumonía simple, neumonía con derrame pleural y empiema).

	Hemocultivo positivo	PCR en plasma positiva		
Neumonía no complicada (n=51)	18	39	**Cultivo de líquido pleural positivo**	**PCR en líquido pleural positiva**
Neumonía con derrame (n=32)	5	18	0	12
Empiema (n=171)	18	75	32	151
			Cultivo de LCR positivo	**PCR en LCR positiva**
Meningitis (n=29)	15	18	20	19
Bacteriemia (n=25)	25	2		

Tabla 4. *Distribución de las muestras positivas detectadas por cultivo y por PCR en tiempo real según las principales presentaciones clínicas.*

Ingresaron en el hospital 310 (97.2%) pacientes. Los 9 pacientes que no ingresaron tenían neumonías simples o bacteriemias. La estancia media hospitalaria fue de 10.8 días (DE 7.5). La mayor estancia hospitalaria correspondió a las meningitis (18.2 días, DE 13.9). La media de días totales de antibiótico fue de 17.8 días (DE 6.8). Esta cifra incluye tanto los días de tratamiento hospitalario como extrahospitalario. Las artritis/osteomielitis fueron los diagnósticos con mayor duración de tratamiento antibiótico (28.2 días, DE 11.8).

Requirieron ingreso en UCI pediátrica 44 pacientes (13.8%). Ingresaron con mayor frecuencia en la UCI los pacientes entre 3-6 meses (32.1%, 9/28) y el grupo entre 7-

23 meses (22.2%, 22/99), mientras que sólo ingresaron en UCI el 6.8% (13/192) de los pacientes entre 24-59 meses. Esto es debido, en parte, a los diferentes tipos de ENI que tienen lugar según el grupo etario, los niños pequeños tienen mayor proporción de infecciones graves como meningitis y sepsis mientras que estos diagnósticos son menos frecuentes en los niños mayores. Ingresaron en UCI 27/29 (93.1%) casos de meningitis, 14/254 (5.5%) casos de neumonía y 3/3 (100%) de los episodios de sepsis.

En la siguiente tabla se observa el pronóstico al alta según las diferentes formas clínicas:

Forma Clínica	Curación	Curación con secuelas	Defunción	TOTAL
Neumonía	**237 (93.3%)**	17(6.7%)	-	**254 (100%)**
Meningitis	11 (37.9%)	**15 (51.7%)**	3 (10.3%)	**29 (100%)**
Bacteriemia	**25 (100%)**	-	-	**25 (100%)**
Infección osteoarticular	**6 (100%)**	-	-	**6 (100%)**
Sepsis	-	2 (66.7%)	1 (33.3%)	**3 (100%)**
Celulitis	**2 (100%)**	-	-	**2 (100%)**
Total	**281(88.1%)**	**34 (10.7%)**	**4(1.3%)**	**319 (100%)**

Tabla 5: *Pronóstico al alta según las diferentes formas clínicas*

Podemos observar la importante morbimortalidad de las meningitis y sepsis neumocócicas con presencia de secuelas o éxitus en más de la mitad de los casos.

El análisis de la evolución de la ENI según el grupo de edad constató que la enfermedad resulta significativamente más grave en los niños menores de 2 años, por lo que debe incidirse sobre la importancia de la vacunación temprana. No en vano, las 4 defunciones, 3 meningitis y 1 cuadro de sepsis, que se produjeron se localizaron en este grupo de edad. Es más, el mayor número de secuelas, fundamentalmente respiratorias, paquipleuritis y neumotórax, todas normalizadas tras 6 meses de

evolución, y neurológicas de la meningitis, hidrocefalia e hipoacusia, también tuvieron lugar en el grupo de pacientes menores de 2 años. En consecuencia, la probabilidad de muerte y de curación con secuelas es más elevada en los niños menores de 24 meses.

5.3-Objetivo 3

Determinar los factores microbiológicos relacionados con la ENI en menores de 5 años en nuestro medio: serotipos relacionados, composición clonal y perfil de resistencias de los aislados

El diagnóstico se realizó mediante cultivo en 123 (38.6%) casos y en 196 (61.4%) exclusivamente por PCR en tiempo real. La inclusión de casos con cultivo negativo y diagnosticados gracias a la PCR en tiempo real ha permitido conocer la carga real de la enfermedad y ha contribuido a su mejor caracterización.

5.3.1-Serotipos

Pudo realizarse el serotipado en 300 (94%) de los 319 casos de ENI. En 120 (40%) el serotipado se realizó a partir de cepas aisladas por cultivo mediante la reacción de Quellung, mientras que en 180 (60%) se realizó a partir de muestra directa mediante la PCR multiplex. Se identificaron un total de 23 serotipos diferentes. No obstante, hubo un gran número (76, 25.3%) de muestras identificadas únicamente por PCR con los genes *ply* y *wzg* positivos pero que no correspondían a ninguno de los 24 serotipos identificados mediante la PCR multiplex, por lo que fueron considerados como "otros serotipos".

En la siguiente figura podemos ver la distribución por serotipos, en la que podemos observar como los serotipos 1,19A y 3 fueron los más frecuentes.
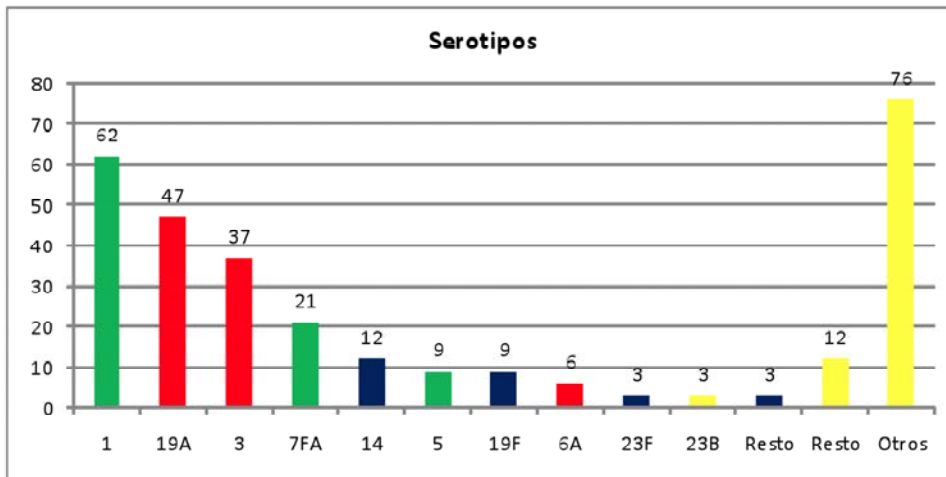
Figura 4: *Distribución de serotipos*

Figura 5: *Serotipos incluidos en las 3 vacunas antineumocócicas conjugadas*

El serotipo 1, asociado a neumonías en niños sanos mayores de 2 años, es el principal serotipo detectado en nuestra serie, mientras que es infrecuente en series americanas como en el estudio de Kaplan[46]. El serotipo 1, capaz de provocar epidemias, empezó a provocar ENI de forma destacable en nuestro medio justo antes de la implementación de PCV7 en nuestro país[38].

Cabe destacar el alto número de serotipos implicados en la ENI en nuestro medio, lo que confirma la alta diversidad del neumococo. La detección de sólo 24 serotipos mediante la PCR multiplex es una limitación del estudio y avisa sobre la necesidad de continuar con la vigilancia epidemiológica de la enfermedad en los próximos años, incluyendo la introducción de nuevas técnicas moleculares que permitan detectar más serotipos. Es necesario continuar estudiando esta enfermedad para determinar si, con la comercialización de vacunas que protegen frente a un mayor número de serotipos, se produce un desplazamiento de la ENI hacia serotipos que, por el momento, son minoritarios.

En las siguientes figuras observamos la distribución de serotipos teniendo en cuenta su inclusión en las 3 vacunas antineumocócicas conjugadas:
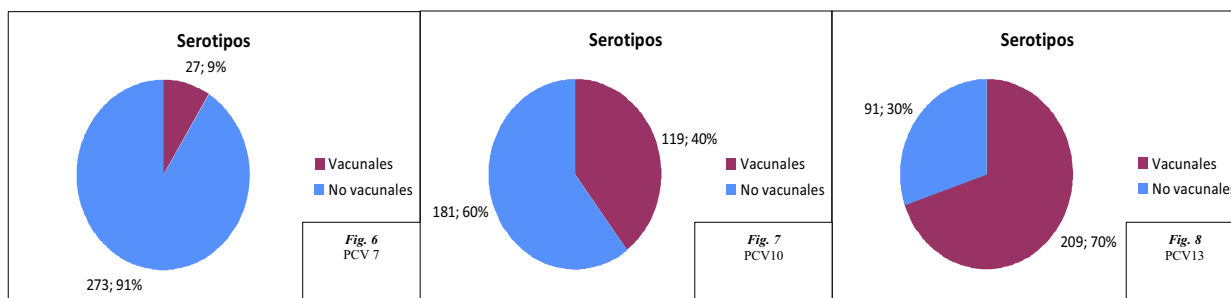
Podemos observar (*figura 6*) como los serotipos no incluidos en la PCV7 son los responsables de la mayoría de episodios de ENI en nuestra serie. Este cambio en los serotipos responsables conlleva un cambio en las manifestaciones clínicas y en la epidemiología como se analiza a continuación.

A pesar de la baja cobertura vacunal (aproximadamente 50%) la tasa de ENI debida a SV es baja. Este dato confirma, como muchos otros estudios, que la PCV7 es muy

efectiva frente a la ENI producida por SV porque esta vacuna previene también la ENI en contactos adultos y niños a través de la inmunidad de grupo[29].

De las *figuras 7 y 8* en las que se aprecia la cobertura de serotipos con las actuales vacunas decavalente y trecevalente se desprende la necesidad de recomendar estas vacunas en la población pediátrica.

Cabe destacar que de los 27 pacientes que tuvieron ENI debida a serotipos incluidos en la PCV7, 5 estaban vacunados. Las características de estos 5 pacientes se resumen en la tabla siguiente, es importante destacar que 2 de ellos tenían una enfermedad de base y no habían completado el esquema vacunal lo que podría explicar el fallo vacunal.

Sexo	Edad (meses)	Presentación clínica	Enfermedad previa	Serotipo	Pauta recibida
Niña	5	Bacteriemia	Acidosis metilmalónica	19F	3 meses (m)
Niña	13	Bacteriemia	Retinoblastoma (neutropenia)	19F	2, 4, 6 m
Niña	45	Neumonía	No	19F	8, 10, 15 m
Niño	29	Neumonía	No	14	7, 10, 21 m
Niña	50	Neumonía	No	14	26 m

Tabla 6: *Características de los 5 pacientes vacunados con PCV7 y que tuvieron ENI por SV*

Existen diferencias significativas en cuanto a la presentación clínica relacionada con los diferentes serotipos. En la siguiente tabla observamos la presentación clínica de los serotipos más frecuentes de nuestra serie.

	1	19A	3	7FA	14	5	19F	6A	23F	Otros	TOTAL
Neumonía	62	32	36	12	11	6	1	2	0	92	254
Meningitis	0	6	1	4	0	1	2	1	2	12	29
Bacteriemia oculta	0	6	0	4	1	2	5	2	1	4	25

***Tabla 7:** Presentación clínica de los serotipos más frecuentes de nuestra serie*

Así, los serotipos 1 y 3 se relacionan fuertemente con neumonías, mientras que la presentación clínica de los episodios causados por serotipo 19A es más variable.

El serotipo 1 es universalmente sensible a la penicilina y el 3 suele serlo también, por lo que el comportamiento agresivo que demuestran estos serotipos, con producción de empiemas y neumonías necrotizantes, no se debe a una disminución en la susceptibilidad a los antibióticos prescritos sino a factores propios de virulencia de la cepa[50]. Por tanto en estos casos más que una escalada en los antibióticos a utilizar deben utilizarse otro tipo de medidas como el drenaje precoz del derrame pleural y la utilización de fibrinolíticos o de videotoracoscopia en caso necesario.

Tras la neumonía la meningitis es la forma clínica más frecuente. Los serotipos que más se asocian a meningitis son el 19A y el 7F. Ninguna meningitis estuvo producida por el serotipo 1 y tan sólo una por el 3. En España, Casado-Flores y colaboradores, en un estudio efectuado en cinco comunidades autónomas (Catalunya, Galicia, Madrid, Euskadi y Navarra), detectan una disminución del 54% en la incidencia de

meningitis neumocócica entre 2001 y 2006[51]. Una tendencia similar se ha observado en Francia, con descensos de la incidencia de meningitis en menores de dos años del 85%[52]. En el momento actual el serotipo 19A es el principal productor de meningitis neumocócica en nuestro país, donde ocupa el segundo lugar como causante de ENI tras el serotipo 1, y es la principal causa de ENI en otros países como Estados Unidos[53].

Actualmente la bacteriemia oculta ocupa el tercer lugar dentro de la ENI. Se ha descrito un claro descenso en la incidencia de bacteriemia neumocócica desde la introducción de PCV7. Benito y colaboradores, en un trabajo retrospectivo en que se compara la incidencia de bacteriemia oculta en niños febriles de 3 a 36 meses, entre la época prevacunal y postvacunal, observan una reducción del 57.5% en la bacteriemia neumocócica y del 79% en la bacteriemia producida por serotipos incluidos en PCV7, sin observar un aumento significativo en la bacteriemia producida por serotipos no vacunales[54]. Es posible que esta disminución de la incidencia se haya producido también en parte por un cambio en los protocolos diagnósticos encaminados a su detección, de forma que al descender mucho la incidencia de bacteriemia oculta se tiende a adoptar enfoques diagnósticos menos agresivos que llevan a la práctica de menos hemocultivos y analíticas[55]. Los serotipos 19A, 7F y 19F son los predominantes en la bacteriemia oculta.

Entre los casos que fueron éxitus, 3 fueron causados por serotipos no incluidos en PCV7 (serotipos 7F,27 y 6A) y 1 por el serotipo vacunal 23F en un niño que no había sido vacunado.

A continuación se muestra una tabla comparativa de los SV y SNV, así como de los serotipos más frecuentes de nuestra serie (1, 19A, 3, 7, 14). Si comparamos los SV con los SNV, observamos como los SV se dieron en pacientes más pequeños (edad media 21.2 versus 30.4 meses en ENI causada por SNV; p: 0.004). Los SNV se asocian principalmente con neumonías: 81.3% del total de episodios causados por SNV versus 48.1% de los episodios causados por SV, p: 0.001.

La ENI por SV se asocia a un mayor porcentaje de secuelas que la ENI producida por SNV (25.9% vs 9.9%; p: 0.02).

Tabla 8. Características clínicas y microbiológicas de los 300 episodios de ENI causados por SV, SNV y los 5 serotipos principales detectados en el estudio.

Serotipo	Nº de episodios	Edad media (DE)	Sexo (niños) N (%)	Presentación clínica*				UCI Ingresos N (%)	Pronóstico	
				Neumonía N (%)	Bacteriemia N (%)	Meningitis N (%)	Otros N (%)		Secuelas	Defunción
SV	27	21.2 (13.68)	16 (59.3%)	13 (48.1%)	7 (25.9%)	6 (22.2%)	1(3.7%)	6 (22.2%)	7 (25.9%)	1 (3.7%)
SNV	273	30.4 (15.84)	145 (53.1%)	222 (81.3%)	18 (6.6%)	23 (8.4%)	10 (3.6%)	38 (13.9%)	27 (9.9%)	3 (1.1%)
Serotipo 1	62	41 (10.48)	34 (54.8%)	62 (100%)	0	0	0	2 (3.2%)	3 (4.8%)	0
Serotipo 19A	47	19.21 (10.54)	28 (59.6%)	32 (68.1%)	6 (12.8%)	6 (12.8%)	3 (6.3%)	9 (19.1%)	4 (8.5%)	3 (2.4%)
Serotipo 3	37	29.03 (14.11)	16 (43.2%)	36 (97.3%)	0 (0%)	1 (2.7%)	0	6 (16.2%)	5 (13.5%)	0
Serotipo 7FA	21	24.71 (16.2)	16 (76.2%)	12 (57.1%)	4 (19%)	4 (19%)	1 (4.9%)	5 (23.8%)	4 (19%)	1 (4.8%)
Serotipo 14	12	24.71 (13.3)	9 (75%)	11 (91.7%)	1 (8.3%)	0	0	0	3 (25%)	0

* Otras presentaciones clínicas: artritis o osteomielitis, sepsis y celulitis.

Se encontraron diferencias estadísticamente significativas (prueba chi-cuadrado para variables categóricas y t-Student para variables continuas):

Edad media: SV versus SNV (P: 0.004); serotipo 1 versus otros serotipos (p<0.001); serotipo 19A versus otros serotipos (p<0.001)

Sexo: serotipo 7F/A versus otros serotipos (p: 0.03)

Presentación clínica: serotipo 1 versus otros serotipos (p: 0.001)

Ingreso en UCI: serotipo 1 versus otros serotipos (p: 0.002)

Pronóstico: SV versus SNV (p: 0.02)

5.3.2-Composición clonal

El análisis molecular mediante MLST se realizó en 108 de 123 (87.8%) muestras identificadas por cultivo. Al comparar nuestros datos con los registrados en la base de datos internacional www.mlst.net se identifican 46 secuenciotipos (ST) diferentes. De los 46 ST, 8 fueron nuevos ST (ST3437, serotipo 23F; ST3436, serotipo 38; ST4827, ST2948 y ST4826, serotipo 19F; ST4676, serotipo 27; ST5195, serotipo 19A; ST4834 serotipo 7F). Destaca por tanto, que aunque los serotipos PCV7 fueron minoritarios en nuestra colección (9%), el 50% de los nuevos clones estaban expresando serotipos vacunales, reflejo de la alta dinámica y adaptabilidad genética de los serotipos sometidos a la presión de la vacuna.

El análisis eBURST agrupó a estos 46 ST en 6 complejos clonales y 34 singletons.

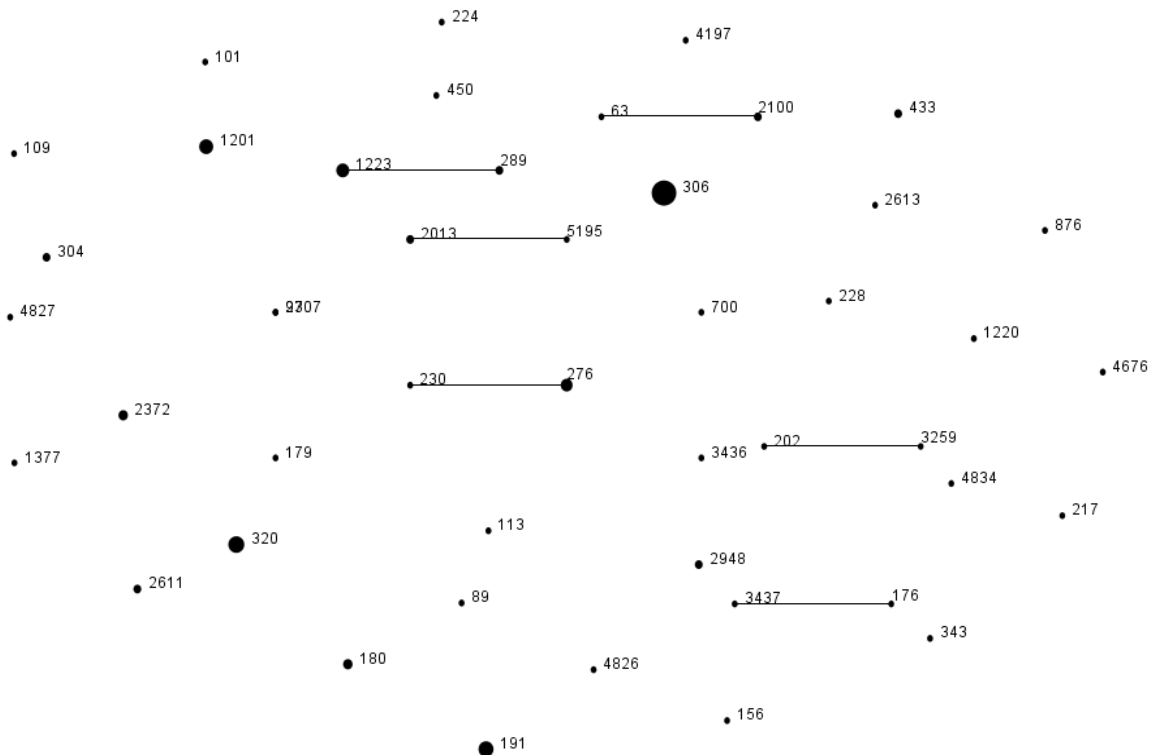Composición Clonal de 108 aislados invasivos de S.pneumoniae

Figura 9: Distribución clonal de los 108 aislados obtenidos con la aplicación eBurst, version 3. Cada círculo representa un único MLST, con el área proporcional al número de aislados de cada ST. Las líneas negras representan SLV.

Seis ST o complejos clonales representaron el 55.9% de la colección: ST306 (n: 22 aislamientos del serotipo 1), ST320 (n: 9 aislamientos serotipo 19A), CC289 (n: 8 aislamientos serotipo 5), ST191 (n: 8 aislamientos serotipo 7F), ST1201 (n: 7 aislamientos serotipo 19A) y CC276 (n: 5 aislamientos serotipo 19A y 1 serotipo 24B).

Como vemos el ST306 es el más importante en nuestra población. Este ST se relaciona con un aumento de neumonía complicada con empiema[47] . Recientemente nuestro grupo demostró que este aumento del empiema asociado a ST306 podría ser causado por la presencia de PsrP[50], un factor de virulencia del neumococo no presente en todos los ST de *S.pneumoniae.* PsrP es una adhesina relacionada con la invasión del neumococo a las células pulmonares.

El análisis comparativo de los serotipos de nuestros ST con aquéllos publicados en la base de datos internacional mostró que 5 de los ST de nuestra colección expresaban diferentes serotipos a los registrados en la base de datos internacional, considerándose un posible fenómeno de capsular switching: ST101 (serotipo 15C), ST109 (serotipo 23F), ST230 (serotipo 24B), ST433 (serotipo 28) y ST2372 (serotipo 23F).

De los datos anteriores se extrae la alta diversidad genética de las cepas que producen ENI en nuestra población, incluyendo la aparición de nuevos ST y fenómeno de capsular switching o recambio capsular.

5.3.3-Perfil de resistencias de los aislados

El estudio de sensibilidad antibiótica pudo realizarse en 120 de las 123 (97.5%) cepas. Ninguna de las 120 cepas era resistente a la penicilina (CMI ≥8 µg/ml) y 3 (2.5%) presentaban resistencia intermedia a la penicilina según los puntos de corte no meníngeos. 2 de estas cepas pertenecían al ST320 que expresaba el serotipo 19A y la otra pertenecía al ST2948 que expresaba el serotipo 19F. 41 cepas (34.4%) tenían una CMI ≥ 0.12 µg/ml (puntos de corte meníngeos) y 18 de estas cepas (43.9%) correspondían al serotipo 19A. En cuanto a la sensibilidad a la cefotaxima, sólo 2 cepas (1.7%) mostraban una CMI ≥4µg/ml y las 2 pertenecían al ST320 que expresa el serotipo 19A. En cuanto a los puntos de corte meníngeos, 21 cepas (17.5%) mostraban una sensibilidad disminuida a la cefotaxima, representando el serotipo 19A el 66.7% de los episodios.

Al analizar específicamente los casos de meningitis, pudo hacerse estudio de sensibilidad antibiótica en 21/29 casos de meningitis (72.4%). 9/21 (31%) presentaban una CMI a la penicilina ≥0,12 µg/ml (4/9 correspondían al serotipo 19A). No obstante, todas las cepas eran sensibles a la cefotaxima excepto 2 (ambas correspondían al serotipo 19A) que presentaban una resistencia intermedia. En nuestra serie todos los casos de meningitis fueron tratados inicialmente con cefotaxima+vancomicina.

Nuestro grupo ya había publicado un descenso en la tasa global de resistencia a la penicilina si comparamos la tasa actual con la del período prevacunal[38]. Además, con los nuevos puntos de corte sugeridos para las neumonías y el resto de formas clínicas

de ENI (salvo para las meningitis) a la práctica no existiría ningún problema de resistencia antibiótica, ya que ninguna cepa en nuestro estudio mostró una CMI para penicilina igual o superior a 8 µg/ml. De estos datos se desprende que los betalactámicos deben continuar siendo el tratamiento de elección de la ENI. No obstante, es de especial preocupación que el segundo clon predominante en nuestra muestra sea el clon multirresistente ST320, clon que expresa el serotipo 19A. Las clonas multirresistentes tienen la capacidad de diseminarse con rapidez a nivel mundial. Este serotipo no está incluido en la PCV7 pero sí en la vacuna 13-valente, por lo que es de esperar que pueda ser controlado una vez se instaure la nueva vacuna.

Describir las características clínicas y moleculares de la ENI producida por serotipo 19A en nuestro medio

La incidencia de ENI producida por serotipo 19A ha aumentado de forma significativa tras la introducción de la vacuna PCV7. No obstante, otros factores aparte de la PCV7 son responsables de este incremento dado que este aumento del 19A se ha descrito en países sin vacunación sistemática como Corea e Israel[35,36]. En EEUU, antes de la introducción de PCV7, el serotipo 19A era el noveno serotipo en frecuencia responsable de ENI en niños menores de 5 años, sólo precedido de los SV y el serotipo relacionado 6A[44]. El serotipo 19A era, pues, un colonizador habitual de la nasofaringe incluso antes de la introducción de PCV7[56]; la reducción de la colonización por SV podría haber facilitado su expansión por ocupación del nicho. Otro factor que puede haber contribuido a su expansión es la variabilidad clínica que tiene el serotipo 19A, el cual es capaz de producir tanto colonización nasofaríngea, como otitis media aguda como enfermedad invasiva[57,58].

En definitiva, la emergencia de 19A en diferentes poblaciones es debida a múltiples y combinados factores, incluyendo la prevalencia basal de 19A y de sus clones virulentos, diferentes modelos de uso de antibiótico y tendencias temporales. La introducción de PCV7 en una población puede ser seguida de la emergencia de SNV como el 19A, pero no hay datos suficientes para implicar a PCV7 como el único o incluso el factor más importante[35,36].

En el segundo artículo se incluyen todos los niños <18 años con ENI por serotipo 19A diagnosticados mediante cultivo en el Hospital Sant Joan de Déu de 1997 a 2007. El estudio, por tanto, abarca 3 períodos diferentes: la época prevacunal (1997-2001), la época vacunal inicial (2002-2004) y la época vacunal tardía (2005-2007). La proporción global de casos de ENI causados por serotipo 19A frente el total de casos de ENI fue de 36/235 (15.3%).

5.4.1- Epidemiología y formas clínicas

En la tabla siguiente podemos ver la distribución de casos en los diferentes períodos de estudio:

	Casos serotipo 19A/total casos (%)			
	Prevacunal (1997-2001)	Vacunal inicial (2002-2004)	Vacunal tardía (2005-2007)	Valor p^a
Casos totales	1/58 (1.7%)	8/54 (14.8%)	27/123 (21.9%)	0.002
Presentación clínica				
- Meningitis	0/18 (0%)	1/8 (12.5%)	4/24 (16.6%)	0.198
- Neumonía (total)	1/15 (6.6%)	1/22 (4.5%)	11/58 (18.9%)	0.169
- sin empiema	1/8	0/9	4/25	
- con empiema	0/7	1/13	6/34	
- Bacteriemia/sepsis	0/17 (0%)	6/16 (37.5%)	9/32 (28.1%)	0.024
- Otros[b]	0/8 (0%)	0/8 (0%)	3/9 (33.3%)	0.048

[a] Test Chi-quadrado
[b] Otros (artritis, apendicitis y endoftalmitis)

Tabla 9: *Distribución de casos por serotipo 19A en los diferentes períodos de estudio*

Se aprecia como la ENI por el serotipo 19A ha ido incrementándose en los últimos años de forma significativa. Resultados similares se han obtenido en países como Corea del Sur, donde la PCV7 se introdujo a finales de 2003, y donde la proporción de ENI producida por 19A era de 0% en el período 1991-1994, del 8-10% en 1995-2000, 26% en la época prevacunal inmediata 2001-2003 y 20% en 2004-2006[35]. En Corea del Sur la prevalencia de ENI producida por serotipo 19A empezó a aumentar antes de la introducción de PCV7. En EEUU la incidencia de ENI producida por 19A aumenta de 0.8 casos/100.000 habitantes en 1998 a 2.5 casos/100.000 habitantes en 2005 ($p<0.05$)[53]. En Noruega la PCV7 fue introducida en 2006 y la incidencia de ENI por 19A aumentó de 0.3 casos/100.000 habitantes en 2004 a 1.67 casos/100.000 habitantes en 2010 (IRR 5.59; IC 95% 3.04-10.26)[59].

Al analizar las diferentes formas clínicas se aprecia como la bacteriemia por 19A aumenta de forma significativa a lo largo de los 3 períodos mientras que no se encuentra aumento significativo en las meningitis y neumonías. Es importante recalcar la capacidad que tiene el serotipo 19A de producir diferentes manifestaciones clínicas.

Si comparamos la ENI producida por el serotipo 19A con la ENI producida por otros serotipos, observamos como:

-El serotipo 19A es propio de niños más pequeños (1.8 años versus 3.4 años, p: 0.01). Este resultado concuerda con lo descrito en estudios previos[39,60].

-La bacteriemia/sepsis es más frecuente en el grupo de niños con ENI por serotipo 19A (p: 0.04).

-No se consiguen diferencias estadísticamente significativas pero hay tendencia a la significación estadística en ingresos en UCI (menor en niños con 19A) y estancia media (menor en niños con 19A) debido al tipo de ENI que produce el 19A.

5.4.2-Sensibilidad antibiótica y análisis molecular

En cuanto al estudio de sensibilidad antibiótica utilizando los puntos de corte meníngeos:

-En la época prevacunal y vacunal inicial todos los serotipos 19A aislados eran sensibles a la penicilina mientras que en la época vacunal tardía 12/27 (44%) no eran sensibles a la penicilina (p: 0.01).

-De estos 12 serotipos 19A no sensibles a la penicilina, 8/12 (66.6%) eran multirresistentes (resistencia a ≥3 antibióticos). De estos 3/8 presentaban una CMI para la cefotaxima ≥2µg/ml y alta resistencia a eritromicina y tetraciclinas. Estos 3 aislados tenían el clon ST320 o ST276.

El análisis molecular pudo hacerse en 35 de las 36 cepas con serotipo 19A. Se obtuvieron 15 clones diferentes expresando el serotipo 19A:

-10 preexistentes que expresaban el 19A en la época prevacunal (incluye los clones multirresistentes ST320 y ST276).

-2 clones preexistentes (ST30 y ST1793) no asociados previamente al serotipo 19A.

-3 clones nuevos (ST2589, ST2618 y ST3438).

Al analizar los clones según la sensibilidad antibiótica:

1-De los 24/36 serotipos 19A sensibles a la penicilina

    *10/24: ST1201

    *8/24: grupo clonal con ST199 como primer clon que se encuentra y que incluye ST416, ST450, ST274, ST199 y el clon nuevo ST2618. El aumento de ENI por 19A en Noruega, país donde hay una política restrictiva en cuanto a la

prescripción antibiótica, se ha producido sobre todo por el CC199, sensible a la penicilina[59]. El CC199 también se ha encontrado como principal responsable del incremento post PCV7 del 19A en EEUU[33,53] aunque la contribución relativa de este complejo clonal ha disminuido desde 2005[61].

*6/24: ST202, ST2589, ST1793 y ST30

2-De los 12/36 serotipos 19A no sensibles a la penicilina

*2/12: Spain[23F]-1 (ST81). Este clon multirresistente se había visto ampliamente en España y otros países en la época prevacunal[62,63] pero expresando un serotipo vacunal (23F), mientras que en este estudio expresaba el 19A lo que constituye un fenómeno de capsular switching.

*2/12: ST320. Es una variante del clon Taiwan[19F]-14-ST236 y la mayor parte de cepas del CC320 son resistentes a la penicilina. El CC320 ha sido identificado también en Corea[35] y Noruega[64] (antes de la introducción de PCV7), en Francia[65], en Australia, y recientemente en EEUU[33,34], lo cual ha sido motivo de gran preocupación.

*4/12: ST276. Se trata de un clon minoritario reportado en Portugal[66], Francia o Turquía en la época prevacunal.

*3/12: ST2013. Clon minoritario reportado en Egipto en la época prevacunal según la página web del MLST.

*1/12: ST438

Es de especial preocupación la emergencia en nuestro medio de estos clones multirresistentes del serotipo 19A. En un estudio multicéntrico sobre la ENI producida por serotipo 19A realizado tras la introducción de PCV7 en la Comunidad

de Madrid, los 2 clones multirresistentes ST320 y ST276 eran ya los más frecuentes[67].

Estos clones asociados a multirresistencia varían dependiendo del área geográfica, así mientras que en Corea la expansión el 19A también ha sido debida al ST320[35], en EEUU, aparte del 320, uno de los clones relacionados con el serotipo 19A y asociados a multirresistencia es el ST2722[68], no aislado en nuestro medio.

Este aumento de clones resistentes en los últimos años podría explicarse por 3 mecanismos distintos:

a) Capsular switching de un clon resistente que expresaba un SV en la época pre-vacunal y ahora expresa un SNV. Esto se ha observado fundamentalmente en EEUU[69].

b) Emergencia de clones resistentes minoritarios que existían antes de la vacunación.

c) Aparición de nuevos clones.

Aunque nosotros observamos los 3 mecanismos en nuestro estudio el más importante fue la emergencia de clones resistentes minoritarios que existían antes de la vacunación.

6. CONCLUSIONES

1- Se ha observado un aumento de la ENI en Barcelona a lo largo del período de estudio. Es una enfermedad con una incidencia en nuestro medio de 109.9 casos/100.000 niños menores de 5 años en el año 2009.

2- La ENI tiene una alta morbimortalidad, sobre todo en la forma clínica de meningitis que produce:

   -Estancia media hospitalaria superior a 1 semana

   -93.1% de ingresos en UCI

   -10.3% de mortalidad

3- La neumonía es el diagnóstico clínico más importante (79.6%).

4- La PCR en tiempo real incrementa considerablemente el diagnóstico de la ENI (de 123 casos diagnosticados por cultivo a 319 casos añadiendo la PCR).

5- El 48.6% de los serotipos identificados corresponden a los serotipos 1,19A y 3.

6- Los SNV constituyen el 91% de los casos de ENI.

7- En los últimos años se ha producido un cambio en la distribución de serotipos causantes de ENI lo cual representa no sólo un hallazgo microbiológico sino un cambio en las manifestaciones clínicas y en los niveles de resistencia a los antibióticos. Por tanto a serotipos diferentes le corresponden manifestaciones clínicas diferentes.

8- Debido a estos cambios se ha modificado la edad de máxima incidencia. De esta forma, dada la elevada prevalencia de neumonías y empiemas, la ENI es ahora más frecuente en el intervalo de edad de 2 a 5 años que en los menores de 2 años.

9- Existe una alta diversidad genética de las cepas que están causando ENI en nuestra población pediátrica incluyendo la aparición de nuevos clones y recambio capsular de los mismos.

10- En la práctica clínica, en el momento actual no hay problema de resistencia antibiótica, ya que ninguna cepa en nuestro estudio tiene una CMI para penicilina igual o superior a 8 µg/ml.

11- El serotipo 19A se está extendiendo rápidamente y se está convirtiendo en una causa importante de ENI en la era vacunal. Puede dar lugar a cualquier forma clínica pero respecto el resto de serotipos produce con mayor frecuencia bacteriemias o sepsis.

12- El aumento del 19A se relaciona con la emergencia de clones sensibles y resistentes, varios de ellos relacionados con clones multirresistentes conocidos.

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8. ANEXOS

8.1- Cuadernillo de recogida de datos (Artículo 1)

**ESTUDI EFECTIVITAT VACUNA
ANTINEUMOCÒCCICA 7- VALENT**

FITXA EPIDEMIOLÒGICA DELS CASOS

Centre hospitalari ___ __ __ __ __ __ __ __ __ __ __ __ __ __

Hospital de la Vall d' Hebrón	**1.** ☐
Hospital de Sant Joan de Déu	**2.** ☐

Núm. Cas __ __ __

Dades personals

CIP : _ _ _ _ _ _ _ _ _ _   **Número d'història Clínica** _____

Nom _____ 1r Cognom _____ 2n Cognom _____

Data de naixement: (_ _ / _ _ / _ _)   Sexe: **1.** ☐ Home   **2.** ☐ Dona

Domicili: _____ Codi Postal: _____

Municipi: _____ Telèfon:_____

País de naixement: _____

País de naixement del pare: _____ de la mare:_____

És usuari de

Centre de Salut públic 1. ☐ Sí. Nom del pediatra _____ Nom Centre _____

                                                     Telèfon_____

           2. ☐ No

Centre de salut privat o metge Privat? 1. ☐ Sí. Nom del pediatra _____ telèfon_____

                     2. ☐ No

Dades clíniques

Data d' inici de símptomes : (_ _ / _ _ / _ _)

Data d' hospitalització: (_ _ / _ _ / _ _)

Forma clínica: Meningitis	**1.** ☐ Sí	**2.** ☐ No
Sèpsia	**1.** ☐ Sí	**2.** ☐ No
Pneumònia no complicada	**1.** ☐ Sí	**2.** ☐ No
Pneumònia complicada:		

	Amb Empiema	1. ☐ Sí	2. ☐ No
	Sense Empiema	1. ☐ Vessament pleural	
		2. ☐ Pneumotòrax	
		3. ☐ Pneumatocele	
		4. ☐ Atelectasia lobar	

Bacterièmia oculta   1. ☐ Sí   2. ☐ No

Infecció osteo articular   1. ☐ Sí   2. ☐ No

Altres   1. ☐ Sí   2. ☐ No   Especificar_____

Data d' alta :   (_ _ / _ _ / _ _)

Ingrés a UCI   1. ☐ Sí
                2. ☐ No

    Data d'ingrés a UCI: (_ _ / _ _ / _ _)

    Data d'alta a UCI: (_ _ / _ _ / _ _)

Complicacions: 1. ☐ Sèpsia

        2. ☐ Coagulació Intravascular Disseminada

        3. ☐ Hidrocefàlia

        4. ☐ Insuficiència respiratòria aguda que requereix ventilació mecànica

        5. ☐ Altres. Especificar_____

Tractament:

 • Tractament previ a l' ingrés: 1. ☐ Sí   2. ☐ No

        Antibiòtic 1*: _ _ En cas que l'antibiòtic sigui codi 16, especificar_____
            Dosi: _____   Dies: _____
        Antibiòtic 2*: _ _ En cas que l'antibiòtic sigui codi 16, especificar_____
            Dosi: _____   Dies: _____

 • Tractament hospitalari

        Antibiòtic 1*: _ _ En cas que l'antibiòtic sigui codi 16, especificar_____
            Dosi: _____   Dies: _____
        Antibiòtic 2*: _ _ En cas que l'antibiòtic sigui codi 16, especificar_____
            Dosi: _____   Dies: _____

  * *veure codi de l'antibiòtic en manual de procediment CASOS pàg 5*

Canvi d'antibiòtic a l'hospital: 1. ☐ Sí   2. ☐ No

Antibiòtic 1*: _ _ En cas que l'antibiòtic sigui codi 16, especificar_____

       Dosi: _____   Dies: _____

Antibiòtic 2*: _ _ En cas que l'antibiòtic sigui codi 16, especificar_____

       Dosi: _____   Dies: _____

Dies totals de tractament antibiòtic (intra i extrahospitalari) _ _

Diagnòstic Principal: _____

Variables evolutives:

Evolució a l'alta: **1.** ☐ Curació

                  2. ☐ Curació amb seqüeles

                  3. ☐ Defunció

      Seqüeles: **1.** ☐ Neurològiques: ☐ Dèficit motor   ☐ Convulsions   ☐ Retràs psicomotor

                                ☐ Altres   Especificar_____

                2. ☐ Pulmonars: ☐ Paquipleuritis   ☐ Altres. Especificar_____

                3. ☐ Osteoarticular: ☐ Coixesa   ☐ Altres. Especificar_____

                4. ☐ Pèrdua d'audició

                5. ☐ Altres. Especificar_____

Estudi microbiològic

1. Sang.   Cultiu: 1. ☐ positiu   2. ☐ positiu no viable   3. ☐ negatiu

                      3. ☐ No realitzat   4. ☐ no valorable

              PCR: 1. ☐ positiu   2. ☐ negatiu

                        3. ☐ No realitzat   4. ☐ no valorable

2. Líquid Cefaloraquidi. Cultiu: 1. ☐ positiu   2. ☐ negatiu

                         3. ☐ no realitzat   4. ☐ no valorable

Detecció d'antigen: 1. ☐ positiu   2. ☐ negatiu

                          3. ☐ no realitzat   4. ☐ no valorable

PCR: 1. ☐ positiu   2. ☐ negatiu

        3. ☐ No realitzat   4. ☐ no valorable

3. Líquid pleural.   Cultiu: 1. ☐ positiu   2. ☐ negatiu

                               3. ☐ no realitzat   4. ☐ no valorable

        PCR: 1. ☐ positiu   2. ☐ negatiu

                3. ☐ No realitzat   4. ☐ no valorable

4. Líquid peritoneal. Cutliu: 1. ☐ positiu   2. ☐ negatiu

                              3. ☐ no realitzat   4. ☐ no valorable

        PCR: 1. ☐ positiu   2. ☐ negatiu

                3. ☐ No realitzat   4. ☐ no valorable

5. Líquid articular. Cultiu: 1. ☐ positiu   2. ☐ negatiu

                              3. ☐ no realitzat   4. ☐ no valorable

        PCR: 1. ☐ positiu   2. ☐ negatiu

                3. ☐ No realitzat   4. ☐ no valorable

6. Altres mostres. Especificar _____

        Cultiu: 1. ☐ positiu   2. ☐ negatiu

                3. ☐ no realitzat   4. ☐ no valorable

        PCR: 1. ☐ positiu   2. ☐ negatiu

                3. ☐ No realitzat   4. ☐ no valorable

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Malaltia de base   1. ☐ Sí   2. ☐ No

GRUP D'ALT RISC O GRUP 1

1. ☐ ANÈMIA DE CÈL·LULES FALCIFORMES. Mesos del diagnòstic (_ _)

2. ☐ ASPLÈNIA ANATÒMICA O FUNCIONAL. Mesos del diagnòstic (_ _)

3. ☐ SIDA I ALTRES IMMUNODEFICIÈNCIES. Mesos del diagnòstic (_ _)

4. ☐ IMPLANTS COCLEARS. Mesos del diagnòstic (_ _)

GRUP DE PRESUMIBLE ALT RISC O GRUP 2

5. ☐ MALALTIA AUTOINMUNE CONGÈNITA (DÈFICIT DE LINFÒCITS B, DÈFICIT DE LINFÒCITS T, DÈFICIT DE COMPLEMENT O DESORDRE FAGOCITARI)

     Tipus de malaltia _____

     Mesos del diagnòstic (_ _)

6. ☐ CARDIOPATIA CRÒNICA (INSUFICIÈNCIA CARDÍACA, CARDIOPATIA CONGÈNITA CIANÒTICA).

     Mesos del diagnòstic (_ _)

7. ☐ NEUMOPATIA CRÒNICA (INCLÒS ASMA TRACTADA AMB ALTES DOSIS DE CORTICOIDES).

     Mesos del diagnòstic (_ _)

8. ☐ FÍSTULA LÍQUID CEFALORAQUIDI CONGÈNITA I / O FRACTURA CRANIAL.

     Mesos del diagnòstic (_ _)

9. ☐ INSUFICIÈNCIA RENAL CRÒNICA I / O SÍNDROME NEFRÒTIC

     Mesos del diagnòstic (_ _)

10. ☐ MALALTIES ASOCIADES AMB TERÀPIA INMUNOSUPRESORA O RADIOTERÀPIA

- Es considerarà la Radioteràpia si l'ha rebut en els últims tres mesos
- Es considerarà la Quimioteràpia o altres tractaments inmunosupresors: si l'ha rebut a l'últim mes
- Es considerarà la Corticoteràpia: si la dosi> 2 mg/dia /15 dies, per via oral

     Tipus de tractament _____

     Data aproximada d'inici del tractament (_ _ / _ _ / _ _)

11. ☐ NEOPLÀSIA MALIGNA, LEUCÈMIA, LINFOMA, MALALTIA DE HODGKIN

Es considerarà neoplàsia quan ha tingut activitat durant l'últim any (intervenció terapèutica mèdica o quirúrgica durant l'últim any i/o si té metàstasis no tractables)

Localització de la neoplàsia _____

Mesos del diagnòstic (_ _)

12. ☐ TRASPLANTAMENT D'ÒRGANS SÒLIDS

Tipus de trasplantament _____

Mesos del trasplantament (_ _)

13. ☐ DIABETES MELLITUS

Mesos del diagnòstic (_ _)

GRUP 3 (Malalts que no tinguin cap malaltia de base citades al grup 1 o al grup2)

Especificar si tenen alguna malaltia: _____

GRUP DE RISC AL QUE PERTANY: 1. ☐ GRUP D'ALT RISC O GRUP 1

2. ☐ GRUP DE PRESUMIBLE ALT RISC O GRUP 2

3. ☐ GRUP 3

Malaltia seleccionada per aparellar (en els GRUPS 1 i 2) _____

El control s'ha aparellat per a la mateixa malaltia o grup de risc que el cas   1.☐ Sí   2. ☐ No

Observacions : _____

_____

_____

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Dades epidemiològiques

Llar d'infants/ escola:

    1. ☐   Acudeix a Llar d'infants

    2. ☐   Acudeix a escola

    3. ☐   No acudeix a escola/ llar d'infants

Número hores/dia _____

Nom de la guarderia /escola_____

Tractament antibiòtic el mes anterior a l'inici de la malaltia   1. ☐ Sí   2. ☐ No

    Nom antibiòtic:_____

    Pauta:_____   Data finalització: (_ _ / _ _ / _ _)

Infecció respiratòria durant el mes anterior a l'inici de la malaltia   1. ☐ Sí   2. ☐ No

Tipus d'infecció_____

Lactància materna   1. ☐ Sí   2. ☐ No   Temps de lactància_____

Nucli Familiar

Número de persones que viuen en la mateixa vivenda (inclòs el pacient): _____

Té germà/ns?   1. ☐ Sí   2. ☐ No

Edat del/s germà/ns: 1. ☐   2. ☐   3. ☐   4. ☐   5. ☐

    Feina pare: _____

        mare: _____

Hàbit tabac pare 1. ☐ Sí   2. ☐ No   3. ☐ No consta
    Núm. de cigarretes /dia _ _ _ _ _

        mare 1. ☐ Sí   2. ☐ No   3. ☐ No consta
    Núm. de cigarretes /dia _ _ _ _ _

Dades de vacunació antineumocòccica

1. ☐ Sí.   1. ☐ Vacuna Conjugada heptavalent (prevenar®) **Núm de dosis**_____

        2. ☐ Vacuna antineumocòccica 23- valent. **Núm de dosis**_____

2. ☐ No

    Data de vacunació (dd/mm/aa) de cada dosis (només Vacuna conjugada heptavalent prevenar®)

        1ª dosis (_ _ / _ _ / _ _)

        2ª dosis (_ _ / _ _ / _ _)

        3ª dosis (_ _ / _ _ / _ _)

        4ª dosis (_ _ / _ _ / _ _)

Cóm es va conèixer l'estat vacunal de la vacuna **antineumocòccica** :

    1. ☐ Carnet vacunal

    2. ☐ Registre de centre de salut

    3. ☐ Història clínica

    4. ☐ Pediatra privat

Dades obtingudes:

1. ☐ Vacunat + Data d'administració (_ _ / _ _ / _ _)

2. ☐ Vacunat i No consta la data d'administració

3. ☐ No vacunat

4. ☐ No té carnet de vacunacions

5. ☐ No coneixen el seu estat vacunal

Observacions de la dada vacunal (dobles vacunacions, rebuig de la vacuna, etc.)_ _
_ _
_ _ _ _ _ _ _ _ _

LLISTAT CODI-PACIENT. Conexió entre els responsables de seleccionar casos i controls a l'hospital, i els responsables de contactar amb el Centre d' Atenció Primària (o similar) per investigar l'estat vacunal.

Hospital:_____ Data d'enviament del llistat _ _ /_ _/ _ _   INVESTIGACIÓ DE L' ESTAT VACUNAL en el CAP

CODI	COGNOM 1	COGNOM 2	NOM	DATA DE NAIXEMENT	CAP o mutualitat 1* (i municipi)	PEDIATRA	CAP o mutualitat 2** (i municipi)	ESTAT VACUNAL
_ _ _ _ _ _								CONEGUT □ / DESCONEGUT □
_ _ _ _ _ _								CONEGUT □ / DESCONEGUT □
_ _ _ _ _ _								CONEGUT □ / DESCONEGUT □
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* Centre d'Atenció Primària o pediatra privat en el qual el pacient es visita <u>actualment</u>.
**Emplenar quan el cas hagi anat a un altre pediatra d'Atenció Primària o pediatra privat anteriorment.

Fitxa epidemiològica CASOS

DADES MICROBIOLÒGIQUES

Serotipat ☐ Soca   ☐ Mostra directe

☐ Serotip 4	☐ Serotip 6A	☐ Serotip 15/A/B/C/F
☐ Serotip 6B	☐ Serotip 9N	☐ Serotip 24
☐ Serotip 9V	☐ Serotip 23 A/B	☐ No tipable
☐ Serotip 14	☐ Serotip 1	☐ Altres. Especificar_____
☐ Serotip 18C	☐ Serotip 3	
☐ Serotip 19F	☐ Serotip 5	
☐ Serotip 23F	☐ Serotip 10	

Caracterització molecular   ☐ Soca   ☐ Mostra directe

Estudi de sensibilitat antibiòtica   1. ☐ Realitzat   2. ☐ No realitzat

1. Penicil·lina	1. ☐ sensible	2. ☐ intermig	3. ☐ resistent
	CMI ≤0,06	1. ☐ Sí	
	CMI 0,12	1. ☐ Sí	
	CMI 0,25	1. ☐ Sí	
	CMI 0,50	1. ☐ Sí	
	CMI 1	1. ☐ Sí	
	CMI ≥ 2	1. ☐ Sí	

2. Cefotaxima	1. ☐ sensible	2. ☐ intermig	3. ☐ resistent
	CMI ≤0,06	1. ☐ Sí	
	CMI 0,12	1. ☐ Sí	
	CMI 0,25	1. ☐ Sí	
	CMI 0,50	1. ☐ Sí	
	CMI 1	1. ☐ Sí	
	CMI ≥ 2	1. ☐ Sí	

DADES EVOLUTIVES

Seqüeles als 6 mesos: **1.** □ Neurològiques: □ Dèficit motor □ Convulsions □ Retràs psicomotor

□ Altres. Especificar_____

2. □ Pulmonars: □ Paquipleuritis □ Altres. Especificar_____

3. □ Osteoarticular: □ Coixesa □ Altres. Especificar_____

4. □ Pèrdua d'audició

5. □ Altres. Especificar_____

8.2- Informe del Comité Etico

INFORME DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

Don Jesús Pineda Sánchez, Secretario del Comité Ético de Investigación Clínica Fundació Sant Joan de Déu Esplugues de Llobregat (Barcelona)

CERTIFICA

Que en la reunión del Comité Ético de Investigación Clínica de la Fundació Sant Joan de Déu celebrada el día 24 de febrero de 2011 se valoró el proyecto de tesis titulado *"Factores epidemiológicos y microbiológicos asociados a la emergencia de la enfermedad invasiva neumocócica por serotipos no incluidos en la vacuna heptavalente conjugada"* presentado por la **Dra. Mariona Fernández de Sevilla.**

El proyecto está planteado respetando los principios éticos que rigen este tipo de estudios y a la normativa vigente en materia de protección de datos de carácter personal.

Lo que firmo en Esplugues de Llobregat (Barcelona), a 4 de marzo de 2011

Firmado:

Don Jesús Pineda Sánchez
Secretario CEIC Fundación SJD

8.2- Otros trabajos publicados relacionados

Notes

Direct detection of *Streptococcus pneumoniae* in positive blood cultures by real-time polymerase chain reaction

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Susanna Hernandez-Bou[b], Carmen Muñoz-Almagro[a],*

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Abstract

We developed a real-time polymerase chain reaction specific for *Streptococcus pneumoniae* to be applied directly from blood culture bottles without previous DNA extraction step. For the 128 blood culture bottles tested, the assay had 94% and 98.4% sensitivity and specificity, respectively. This assay provides rapid and accurate identification of this pathogen.
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Keywords: Real-time PCR; Blood culture bottles; *Streptococcus pneumoniae*

Streptococcus pneumoniae is one of the major bacterial pathogens worldwide, causing bacteremia and community-acquired pneumonia (Johnson et al., 2008). According to the World Health Organization (WHO) (2008), an estimated 700 000 to 1 million children younger than 5 years die of pneumococcal disease every year, and this represents a major global public health problem.

Detection of the pathogen with automated blood culture systems and subsequent identification with biochemical tests takes a minimum of 48 h (Lakshmi, 2001). Rapid detection of pathogens in blood from septic patients may allow a reduction in the unnecessary use of broad-spectrum antimicrobials and should prevent further emergence of resistance (Gebert et al., 2008; Paule et al., 2005).

The aims of this study were to minimize the waiting time between the detection of a positive blood culture and pathogen identification by microbiologic molecular techniques and to test the sensitivity and specificity of a homemade real-time polymerase chain reaction (PCR) assay for *S. pneumoniae* in blood culture samples without previous DNA extraction step.

We studied a total of 128 blood culture bottle samples (60 positive blood cultures and 68 negative blood cultures) from 128 pediatric patients who attended at the University Hospital Sant Joan de Déu (Esplugues) in Barcelona, Spain, from January to December 2007 and from March to April 2009. This hospital is a tertiary-care children's and maternity hospital with 345 beds and an average referral population of 210 000 children younger than 18 years. Blood samples were inoculated into FAN-aerobic bottles (BioMérieux Laboratories, Boxtel, The Netherlands) containing an antimicrobial-absorbent resin. The bottles were cultured immediately using the automatic Bact-Alert system (BioMérieux Laboratories) for 5 days at 37 °C. All positive blood culture bottles were identified by conventional biochemical and serologic techniques.

Samples were collected directly from the blood culture bottles after incubation (by signal-positive growth or by negative detection after 5 days). An aliquot of 1.5 mL was taken from the bottle, and then the sample was briefly centrifuged (6000 × g for 1 min). Five microliters of the aqueous supernatant was used directly in real-time PCR.

The homemade real-time PCR assay coamplified a specific sequence of *S. pneumoniae* in the pneumolysin gene (*ply*) (GenBank accession number X52474) and RNase P human gene as internal control. The master mixture contained 12.5 μL of TaqMan® Universal PCR Master Mix

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(Applied Biosystems Foster City, CA), 0.9 µmol/L final concentration of *S. pneumoniae* specific primers (*ply*-F: 5′-TGCAGAGCGTCCTTTGGTCTAT-3′ and *ply*-R: 5′-CTCTTACTCGTGGTTTCCAACTTGA-3′), 0.3 µmol/L final concentration of hybridization probe, 1 µL of TaqMan® RNase P Detection Reagents (VIC™) (Applied Biosystems), and 1.20 µL of PCR water. Twenty-microliter aliquots of master mixture were previously prepared and stored at −20 °C for subsequent use. The cost of each PCR reaction was less than 2 Euros.

Real-time PCR assays were carried out in a final 25.5-µL reaction volume, including 20 µL of prepared master mixture and 5 µL of sample. An additional 0.5 µL of Human DNA male (Applied Biosystems) was added to each tube to test potential inhibition.

DNA was amplified with the Applied Biosystems 7300 Real-time PCR System (Applied Biosystems) using the following cycling parameters: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min (total time, 1 h 59 min). Amplification data were analyzed by instrument software (SDS, Applied Biosystems). Negative results were defined as those with cycle threshold (C_T) values above 40.

For testing the lowest limit of detection of the assay, 0.5-McFarland suspension was made from an *S. pneumoniae* strain obtained from our bacteriologic laboratory, and serial dilutions from 1.7 ng/µL (7.107 copies/mL) to 0.6 fg/µL (7 copies/mL) were performed. The real-time PCR assay correctly detected all serial dilutions (range, 7–7.107 copies/mL).

From the total of 60 positive blood culture bottles, 57 types of bacteria were identified by culture: 48 Gram-positive cocci, including 18 *S. pneumoniae*, 3 *Streptococcus agalactiae*, 8 *Streptococcus mitis*, 4 *Streptococcus viridans* group, 3 *Enterococcus faecalis*, 1 *Enterococcus faecium*, 1 *Micrococcus* spp., 1 *Staphylococcus aureus*, 1 *Staphylococcus epidermidis*, 2 *Staphylococcus simulans*, 4 plasma coagulase-negative *Staphylococcus*, and 1 *Corynebacterium* spp.; 8 Gram-negative bacilli, including 4 *Escherichia coli*, 1 *Acinetobacter lwoffii*, 1 *Enterobacter cloacae*, 1 *Klebsiella pneumoniae*, and 1 enteric salmonella; and 1 Gram-negative cocci corresponding to 1 *Neisseria meningitidis*. Any pathogen found was isolated by culture in 2 bottles with Gram-positive cocci and 1 with Gram-negative bacilli.

The results of the comparative microbiologic study of the culture and the *ply* real-time PCR in blood cultures are shown in Table 1. Comparison of real-time PCR showed that 17 of 18 *S. pneumoniae* had a positive result for real-time PCR, with a C_T <28. For the sample that was only identified by culture, the real-time PCR was inhibited with no detection of RNAse P internal control. One of the 8 *S. mitis* isolates had a positive result for the PCR, so we have a false-positive result. From the 2 samples that were Gram-positive cocci but without any pathogen isolated in blood culture, the *ply* real-time PCR was positive in one of them. The rest of the samples with other pathogens and all 68 negative blood

Table 1
Comparative microbiologic study of culture and real-time PCR in blood cultures of 128 patients

Gram stain result	Pathogen isolated in blood culture	No. of patients	Real-time PCR result	
			Positive	Negative
GPC	*S. pneumoniae*	18	17	1
GPC	*S. agalactiae*	3	0	3
GPC	*S. mitis*	8	1	7
GPC	*S. viridans* group	4	0	4
GPC	*E. faecalis*	3	0	3
GPC	*E. faecium*	1	0	1
GPC	*Micrococcus* spp.	1	0	1
GPC	*S. aureus*	1	0	1
GPC	*Staphylococcus auricularis*	1	0	1
GPC	*S. epidermidis*	1	0	1
GPC	*S. simulans*	2	0	2
GPC	Coagulase-negative staphylococci	4	0	4
GPC	*Corynebacterium* spp.	1	0	1
GPC	Non detected	2	1	1
GNB	*E. coli*	4	0	4
GNB	*A. lwoffii*	1	0	1
GNB	*E. cloacae*	1	0	1
GNB	*K. pneumoniae*	1	0	1
GNB	Enteric salmonella	1	0	1
GNB	Non detected	1	0	1
GNC	*N. meningitidis* group B	1	0	1
Negative	Nondetected	68	0	68
	Total	128	19	109

Sensitivity and specificity of *pneumolysin* real-time PCR according to culture results: 94% and 98.4%, respectively.
GPC = Gram-positive cocci; GNB = Gram-negative bacilli; GNC = Gram-negative cocci.

cultures were negative by real-time PCR. The sensitivity and specificity of real-time PCR, with culture as the gold standard, were 94% and 98.4%, respectively.

S. pneumoniae, as one of the major bacterial pathogens worldwide, requires a fast early diagnostic test (Johannes, 2008; WHO, 2008). Like several authors (Gebert et al., 2008; Gröbner and Kempf, 2007; Hogg et al., 2008; Kurupati et al., 2004; Paule et al., 2005; Selvarangan et al., 2003), we found high sensitivity and specificity for the rapid identification of 1 invasive pathogen using molecular technology in comparison with conventional identification from blood cultures.

Of note, some of these authors (Gebert et al., 2008; Hogg et al., 2008) evaluated and compared different extraction methods and highlighted the importance of evaluating specific DNA extraction methods. However, we tested the technology without previous DNA extraction. The result is a rapid, specific, and sensitive *S. pneumoniae* detection test (it takes less than 2 1/2 h) because the master mixture is previously prepared and no extraction method is required. The bottles used for bacterial culture have FAN medium that contains adsorbent material called Ecosorb (containing adsorbent charcoal, fuller's earth, and other components). Activated charcoal is an extremely porous carbonaceous

adsorbent material with a very large surface area available for adsorption, which allows the removal of PCR inhibitors from the sample. Abolmaaty et al. (2007) reported the use of activated charcoal as a promising and convenient technology for removal of PCR inhibitors from biologic samples.

We used *pneumolysin* gene as the target for *S. pneumoniae* detection, which has been successfully used for the diagnosis of pneumococcal infection (Lahti et al., 2006). However, the results of the present study show that 1 nonpneumococcal *Streptococcus*, *S. mitis*, had a positive result for the real-time PCR. According to several authors (Carvalho et al., 2007; Kee et al., 2008), the presence of *ply* gene in other streptococci, in particular, *S. mitis*, could be a limitation of this assay, and other primers for detecting *S. pneumoniae* by PCR, such as *autolysin* (*lytA*) gene, could have higher specificity.

In this study, we examined the useful contribution of molecular techniques to the microbiologic identification of *S. pneumoniae*, but in the future, it may become possible to detect more pathogens, not only for pathogen identification but also for resistance, serogrouping (Munoz-Almagro et al., 2008), and serotyping assays (Tarrago et al., 2008). This method may prove to be suitable for implementation in routine emergency diagnostic laboratories.

In conclusion, real-time PCR can be used for rapid, accurate detection of *S. pneumoniae* bacteremia, which can permit optimal therapeutic treatment at the earliest time. It offers the benefit of yielding reliable results in a few hours' time as opposed to the days required with conventional methods. PCR technology has also shown its great potential in routine molecular diagnostic screening for the identification of several pathogens.

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PsrP, a Protective Pneumococcal Antigen, Is Highly Prevalent in Children with Pneumonia and Is Strongly Associated with Clonal Type[▽]

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Invasive pneumococcal disease (IPD) is a major health problem worldwide. Due to ongoing serotype replacement, current efforts are focused in an attempt to identify the pneumococcal antigens that could be used in a next-generation multivalent protein vaccine. The objective of our study was to use real-time PCR to determine the distribution and clonal type variability of PsrP, a protective pneumococcal antigen, among pneumococcal isolates from children with IPD or healthy nasopharyngeal carriers. *psrP* was detected in 52.4% of the 441 strains tested. While no differences were determined when the prevalence of *psrP* in colonizing strains (*n* = 89) versus that in all invasive strains (*n* = 352) was compared, a strong trend was observed when the prevalence of *psrP* in all pneumonia isolates (*n* = 209) and colonizing isolates (*P* = 0.067) was compared, and a significant difference was observed when the prevalence in all pneumonia isolates and those causing bacteremia (*n* = 76) was compared (*P* = 0.001). An age-dependent distribution of *psrP* was also observed, with the incidence of *psrP* being the greatest in strains isolated from children >2 years of age (*P* = 0.02). Strikingly, the presence of *psrP* within a serotype was highly dependent on the clonotype, with all isolates of invasive clones such as clonal complex 306 carrying *psrP* (*n* = 88), whereas for sequence type 304, only 1 of 19 isolates carried *psrP*; moreover, this was inversely correlated with antibiotic susceptibility. This finding suggests that inclusion of *psrP* in a vaccine formulation would not target resistant strains. We conclude that *psrP* is highly prevalent in strains that cause IPD but is most prevalent in strains isolated from older children with pneumonia. These data support the potential use of PsrP as one component in a multivalent protein-based vaccine.

Invasive pneumococcal disease (IPD), defined herein as the isolation of *Streptococcus pneumoniae* from normally sterile sites during a clinical syndrome of infection such as bacteremia/sepsis, pneumonia, or meningitis, is an important health problem worldwide. In the year 2000, it is estimated that there were 11 million to 18 million episodes/cases of IPD and 0.7 million to 1 million deaths in children younger than 5 years of age as a result (17). *Streptococcus pneumoniae* is a Gram-positive commensal that colonizes the nasopharynx of healthy children and, less frequently, adults. From the upper respiratory tract, the bacteria can be aspirated into the lungs and can translocate through mucosal cell barriers to the bloodstream and lead to development of IPD (18). This primarily occurs in young children, elderly individuals, and those who are immunocompromised.

The ability of *S. pneumoniae* to cause IPD is dependent on the presence of a polysaccharide capsule that prevents phagocytosis (1). At least 92 chemically and immunologically distinct capsular types (i.e., serotypes) can be produced by the pneumococcus, with certain serotypes more frequently being associated with invasive disease (23). Importantly, while the capsule is requisite for IPD, it is insufficient alone to confer virulence; and an assortment of additional determinants such as adhesins, proteases, toxins, transport systems, and enzymes that modify the extracellular milieu are also required (25). This requirement for noncapsular virulence determinants is proven by human epidemiological studies that show that invasive and noninvasive clonotypes exist within the most invasive serotypes, comparative genomic analyses that find an unequal distribution of noncapsular genes between invasive and noninvasive isolates within the same serotype, and scores of studies that show that deletion of noncapsular genes impact pneumococcal virulence in animal models of pneumonia, sepsis, and meningitis (7, 11, 19, 22).

One recently identified pneumococcal virulence determinant is the pneumococcal serine-rich repeat protein (PsrP), a lung cell and intraspecies bacterial adhesin that is encoded within the 37-kb pathogenicity island called *psrP-secY2A2* (16). PsrP is an extremely large glycosylated cell surface protein that belongs to the serine-rich repeat protein (SRRP) family of Gram-positive bacteria (22). For the pneumococcus, the presence of PsrP has been positively correlated with strains that cause human disease, and PsrP has been shown to mediate adhesion to keratin 10 on lung cells and to mediate the formation of bacterial aggregates in the nasopharynges and lungs of infected mice (21, 22). Antibodies against PsrP neutralize

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bacterial adhesion to cells *in vitro* and inhibit biofilm formation (20, 21). Furthermore, passive immunization of mice with PsrP antiserum or active immunization with recombinant protein protected mice against pneumococcal challenge (20). Thus, PsrP is an important virulence factor by which *S. pneumoniae* is able to cause IPD and is potentially a vaccine candidate.

At this time, considerable resources are being spent in an attempt to identify the pneumococcal antigens that would be used in a next-generation multivalent protein vaccine designed against the pneumococcus. The advantage of such a vaccine is that it would have a lower cost and potentially expanded global coverage compared with the cost and coverage of existing conjugate vaccines. It is generally accepted that multiple antigens will be necessary due to the fact that not all protein determinants are conserved or found within all pneumococcal strains and on their own are not able to confer sufficient protection. To this end, knowledge of the real prevalence of a protein in different clones and serotypes of *Streptococcus pneumoniae* is necessary to consider any protein as a candidate vaccine antigen. Therefore, the objective of our study was to determine the distribution and clonal type variability of PsrP among pneumococcal isolates from children with IPD or healthy nasopharyngeal carriers.

MATERIALS AND METHODS

Strain collection. We analyzed all invasive pneumococcal isolates collected at the Molecular Microbiology Department of the University Hospital Sant Joan de Deu, Barcelona, Spain, from January 2004 to November 2009 ($n = 358$). We have also included 89 strains isolated from the nasopharynges of healthy children during 2004 and 2005 ($n = 89$). A detailed description of our institution and the geographic area was reported elsewhere (13). IPD was defined as the presence of clinical findings of infection, including pneumonia, together with isolation of *S. pneumoniae* in blood, cerebrospinal fluid, or any other normally sterile fluid. The clinical syndrome was classified according to the International Classification of Disease, ninth revision (ICD-9), specific for diseases caused by *S. pneumoniae*, including sepsis, occult bacteremia, meningitis, pneumonia, parapneumonic empyema, peritonitis, arthritis, and endophthalmitis.

Serotyping and antimicrobial susceptibility. All isolates were serotyped by the Quellung reaction at the National Pneumococcus Reference Centre (Majadahonda, Madrid, Spain). In addition, all isolates identified during 2009 were also tested in our laboratory with a rapid specific real-time PCR of the main invasive serotypes according to the methods described for a published assay (24). The agar dilution technique was used to determine the MICs of penicillin and other antibiotics; antibiotic susceptibility was defined according to the 2008 meningeal breakpoints of the Clinical and Laboratory Standards Institute (formerly NCCLS) (14). Isolates with intermediate or high-level resistance were defined as nonsusceptible.

MLST. Genetic characterization was performed using multilocus sequence typing (MLST). In brief, internal fragments of the *aroE, gdh, gki, recP, spi, xpt,* and *ddl* genes were amplified by PCR from chromosomal DNA of pneumococci using the primer pairs described by Enright and Spratt (5). The sequences of both DNA strands were obtained by use of an ABI 3730xl DNA analyzer (Applied Biosystems). The sequences at each of the seven loci were then compared with the sequences of all of the known alleles at that locus. Sequences that are identical to a known allele were assigned the same allele number, whereas those that differ from any known allele were assigned new allele numbers. The assignment of alleles at each locus was carried out using the software at the pneumococcal web page (www.mlst.net). The alleles at each of the seven loci define the allelic profile of each isolate and their sequence type (ST). Allelic profiles are shown as the combination of 7 alleles in the order *aroE, gdh, gki, recP, spi, xpt,* and *ddl*. A clone is defined as a group of isolates with identical allelic profiles or STs.

CC. Isolates with genotypes with allelic profiles that differ at only one of the seven loci were called single-locus variants (SLVs). SLVs are sufficiently related to be considered members of a cluster of closely related genotypes, referred to as a clonal complex (CC). Analysis of sequence types and assignment to a clonal complex was performed with the eBURST (based upon related sequence types)

program. This program compares a data set of sequence types and groups them into related genotypes and clonal complexes (6).

Extraction of DNA. Genomic DNA was extracted using Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA). Four to 5 CFU/ml was suspended in 100 μl of phosphate-buffered saline (PBS) buffer; 50 μl was transferred to a new microcentrifuge tube and vigorously vortexed with 150 μl of 20% (wt/vol) Chelex-100 in PBS. The bacterium/resin suspensions were incubated for 20 min at 56°C, followed by a 10-min incubation at 99°C. After cooling and centrifugation of the suspensions, the supernatant was used as a template in real-time PCR experiments. Free water and genomic DNA from the *psrP*-carrying TIGR4 and an isogenic mutant deficient in *psrP* (T4 Δ*psrP*) were used as positive and negative controls, respectively (16).

Real-time PCR assay. We analyzed the nucleotide sequence of *psrP* in TIGR4 and all other publically available *S. pneumoniae* genomes available through the United States National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) for primers and probe design. The primers and probe selected were as follows: the forward primer was 5′-CTTTACATTTACCCCTTACGCTGCTA, the reverse primer was 3′-CTGAGAGTGACTTAGACTGTG AAAGTG, and the probe was FAM-CTGGTCGTGCTAGATTC (where FAM is 6-carboxyfluorescein; the quencher was the minor groove binder [MGB] moiety). These primers identified a conserved region within the basic region domain of *psrP* (16).

The reaction volume of 25 μl contained 5 μl of DNA extract from samples or controls and 12.5 μl 2× TaqMan universal master mix (Applied Biosystems), which includes dUTP and uracil-*N*-glycosylase; each primer was used at a final concentration of 300 nM. The TaqMan probe was used at a final concentration of 150 nM. Amplification was done under universal amplification conditions: incubation for 2 min at 50°C (uracil-*N*-glycosylase digestion), 10 min denaturation at 95°C, and 45 cycles of a two-step amplification (15 s at 95°C, 60 s at 60°C). Amplification data were analyzed by SDS software (Applied Biosystems). The reporter dye signal was measured relative to the internal reference dye (carboxy-X-rhodamine) signal to normalize for non-PCR-related fluorescence fluctuations occurring from well to well. The cycle threshold (C_T) value was defined as the cycle at which the reporting dye fluorescence first exceeds the calculated background level.

Statistical analysis. Statistical analysis was performed with the PASW software package (version 17.0). Continuous variables were compared using the *t* test (for approximately normally distributed data) or the Mann-Whitney U test (for skewed data) and were described as mean values and standard deviations or medians and interquartile ranges (IQRs; 25 to 75th percentiles), according to the presence of a normal distribution. The chi-square test or Fisher's exact test (two-tailed) was used to compare categorical variables. Comparison between groups was performed by the Kruskal-Wallis test. Statistical significance was set at a *P* value of <0.05.

RESULTS

Prevalence of *psrP* in clinical isolates from healthy carriers and individuals with IPD. Of the 358 invasive pneumococcal isolates in our library, 6 of them were not viable and were therefore excluded from the study. As such, we studied a total of 352 invasive pneumococcal isolates and 89 nasopharyngeal pneumococcal isolates (total of 441 strains).

Table 1 shows the prevalence of *psrP* in pneumococcal isolates according to clinical diagnosis, serotype, and clonal type. Overall, we detected *psrP* in 231 (52.4%) of pneumococcal strains tested. No significant differences were found when the prevalence of *psrP* in colonizing strains (43 of 89 isolates; 48.3%) was compared with that in all invasive strains (188 of 352 isolates; 53.4%) ($P = 0.4$). However, a strong trend was observed when the prevalence of *psrP* in all pneumonia isolates (125 of 209; 59.8%) was compared with that in colonizing isolates ($P = 0.067$). The lowest prevalence of *psrP* was found in strains isolated from children with bacteremia (29 of 76 strains; 38.2%). In a breakdown of those causing pneumonia, *psrP* was detected in 62 of 104 (59.6%) isolates causing uncomplicated pneumonia (versus colonizing strains, $P = 0.1$) and 63 of 105 (60%) of isolates from individuals with para-

TABLE 1. Prevalence of positive *psrP* in pneumococcal isolates in children with IPD and healthy carriers by clinical diagnosis, serotype, and clonal type

Subject group and clinical diagnosis	No. of strains *psrP* positive/total no. of strains (%)	Serotype		Clonal type	
		Main serotype	No. of strains *psrP* positive/total no. of strains (%)	Main clonal type	No. of strains *psrP* positive/total no. of strains (%)
IPD patients	188/352 (53.4)	1	84/104 (80.8)	CC306	82/82 (100)
Pneumonia	125/209 (59.8)			ST304	1/18 (5.5)
		19A	15/36 (41.7)	CC230	2/9 (22.2)
				ST2013	2/7 (28.5)
				ST1201	5/5 (100)
		5	15/20 (75)	CC289	15/20 (75)
		7F	2/13 (15.3)	ST191	2/12 (16.6)
		Others[a]	9/36 (25)		
Bacteremia	29/76 (38.2)	19A	12/20 (60)	ST1201	7/7 (100)
				ST320	0/4 (0)
		7F	0/9 (0)	ST191	0/9 (0)
		14	0/5 (0)	CC156	0/2 (0)
		19F	3/5 (60)	CC177	2/2 (100)
		Others[b]	14/37 (37.8)		
Meningitis	25/49 (51)	19A	5/8 (62.5)	CC199	2/2 (100)
				CC230	0/2 (0)
		19F	4/5 (80)	CC177	2/2 (100)
		6A	2/4 (50)	ST1692	1/2 (50)
		7F	1/3 (33.3)	ST191	1/3 (33.3)
		Others[c]	13/29 (44.8)		
Other clinical syndromes[d]	9/18 (50)	19A	2/4 (50)	CC230	0/1 (0)
				ST320	0/1 (0)
		1	2/2 (100)	CC306	2/2 (100)
		19F	1/2 (50)	ST2948	1/1 (100)
		23F	0/2 (0)	ST109	0/1 (0)
		Others[e]	4/8 (50)		
Healthy carriers	43/89 (48.3)	19A	6/9 (66.6)	CC199	2/2 (100)
				CC202	1/2 (50)
		6A	6/9 (66.6)	CC97	1/1 (100)
				ST1143	1/1 (100)
		19F	5/7 (71.4)	CC177	2/2 (100)
		15B	4/6 (66.7)	ST101	1/2 (50)
		23B	1/6 (16.7)	ST2372	0/1 (0)
		6B	2/5 (40)	ST386	1/1 (100)
		9V	1/5 (20)	CC156	0/1 (0)
		Others[f]	18/42 (42.8)		

[a] Other serotypes in pneumonia: S14 (*n* = 9); S3 (*n* = 6); S24F/B (*n* = 6); S6A (*n* = 3); S6B (*n* = 3); S9V (*n* = 3); and S10A, S15B, S18C, S2, S38, and S4 (*n* = 1 each).

[b] Other serotypes in bacteremia: S1 (*n* = 4); S3 (*n* = 4); S5 (*n* = 4); 23B (*n* = 3); S38 (*n* = 3); S6A (*n* = 3); S10A (*n* = 2); S15B (*n* = 2); S23F (*n* = 2); S34 (*n* = 2); and S12F, S18C, S21, S22F, S24F, S27, S35B, and S4 (*n* = 1 each).

[c] Other serotypes in meningitis: S15C, S18C, S22, S23B, S23F, S3, S5, S6BS, and 24F (*n* = 2 each) and S1, S10A, S12F, S13, S14, S15A, S16, S16F, S27, S31, and S9N (*n* = 1 each).

[d] Other clinical syndromes: arthritis (*n* = 11), appendicitis (*n* = 5), pericarditis (*n* = 1), and peritonitis (*n* = 1).

[e] Other serotypes in other clinical syndromes: S14, S28, S35B, S38, S4, S5, S6A, and S7F (*n* = 1 each).

[f] Other serotypes in healthy carriers: S21 (*n* = 4); S23F (*n* = 4); S10A (*n* = 3); S15A (*n* = 3); S23A (*n* = 3); S3 (*n* = 3); S1 (*n* = 2); S15C (*n* = 2); S24 (*n* = 2); S29 (*n* = 2); S35B (*n* = 2); S37 (*n* = 2); and S10, S11, S16, S17, S28, S31, S38, S37, 7F, and 9N (*n* = 1 each).

pneumonic empyema (versus colonizing strains, *P* = 0.19). Thus, consistent with its role as a lung cell adhesin, *psrP* was more frequently present in pneumonia isolates than in colonizing isolates, albeit not to a significant level, and was present at a significantly higher rate in pneumonia isolates than in those causing bacteremia (*P* = 0.001). Surprisingly, we also observed an age-dependent distribution for *psrP* in clinical isolates from children with IPD. *psrP* was detected in 76 of 175 (46.1%) strains isolated from children with IPD less than 24

months old, while in older children, this rate was significantly higher: 112 of 187 (59.9%) (*P* = 0.01).

Specifically, the prevalence of *psrP* in pneumonia isolates from children less than 24 months was 49.3% (36 of 73 strains), and that in isolates from older children was 65.4% (89 of 136 strains) (*P* = 0.02). Moreover, for isolates from older children, this 65.4% prevalence of *psrP* was significantly higher than that for nonpneumonia IPD isolates (56 of 116; 48.3%; *P* = 0.006) and that for colonizing isolates (33 of 65; 50.8%; *P* = 0.04).
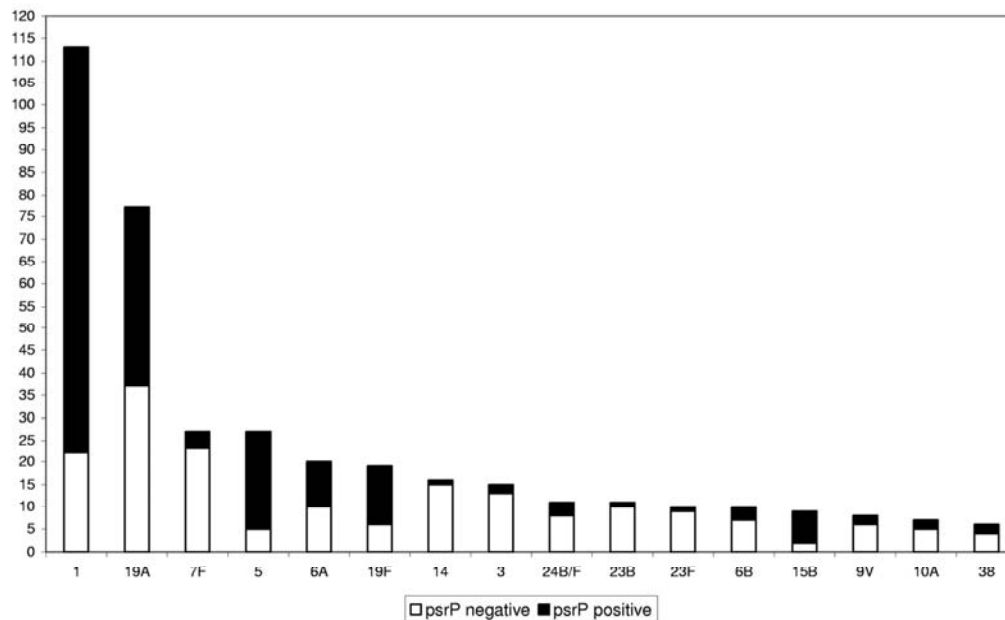
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FIG. 1. Prevalence of *psrP* according to serotype of *Streptococcus pneumoniae* in groups of more than five isolates.

Prevalence of *psrP* according to serotype of isolates. A total of 37 different serotypes were detected among the isolates causing IPD and 29 were detected among those from carriers. Significant differences in the prevalence of *psrP* were observed according to the serotype of the strains ($P < 0.0001$). Figure 1 show the distribution of *psrP* according to serotypes with more than 5 isolates. *psrP* was observed at very high frequencies in some epidemic serotypes, such as serotype 1 (91 of 113 isolates; 80.5%) and serotype 5 (22 of 27 isolates; 81.5%). In contrast, positivity for *psrP* was rarely detected in other serotypes, such as serotype 3 (2 of 15 isolates; 13.3%), serotype 7F (4 of 27 isolates; 14.8%), and serotype 14 (1 of 16 strains; 6.3%). The prevalence of *psrP* in serotype 19A, which is an emergent serotype in the geographic area where the isolates for this study were collected (12), was 51.9% (40 of 77 strains).

Importantly, the prevalence of *psrP* in serotypes included in the 7-valent conjugate vaccine (PCV-7; 26 of 70 strains; 37.1%) was significantly lower than that in nonvaccine serotypes (205 of 371; 55.3%) ($P = 0.006$). In contrast, in the context of the newly approved 10-valent pneumococcal conjugate vaccine (PCV-10) and 13-valent pneumococcal conjugate vaccine (PCV-13), the difference between vaccine and nonvaccine strains was switched. For PCV-10, a higher rate of positivity for *psrP* in the vaccine serotypes was observed: 143 of 237 (60.3%) among PCV-10 serotypes versus 88 of 204 (43.1%) among non-PCV-10 serotypes ($P < 0.0001$). For PCV-13, *psrP* was detected in 195 of 349 (55.9%) strains of PCV-13 serotypes and 36 of 92 (39.1%) strains of non-PCV-13 serotypes ($P = 0.004$).

Prevalence of *psrP* according to antibiotic susceptibility of strains. Antibiotic susceptibility information was obtained for 432 of 441 total isolates (98%). In general, a positive correlation between the presence of *psrP* and antibiotic susceptibility was observed. The prevalence of *psrP* in penicillin-susceptible

strains from children with IPD was 64.5% (171 of 265 strains), whereas it was only 15.2% (12 of 79 strains) in nonsusceptible strains ($P < 0.0001$). The same pattern was observed for cefotaxime, tetracycline, and erythromycin (Table 2). Strikingly, *psrP* was absent in almost all strains with high-level resistance to penicillin or cefotaxime: only 2 (10%) of 20 strains with penicillin MICs of ≥ 2 µg/ml and none of 6 strains with cefotaxime MICs of ≥ 2 µg/ml were positive for *psrP*. No significant differences in *psrP* prevalence were found according to the susceptibilities of nasopharyngeal strains in healthy carriers.

Prevalence of *psrP* according to clonal type. Sequence and clonal type analyses were performed for 372 of 441 strains (84% of the collection). A total of 94 different sequence types were found, and these were grouped into 17 clonal complexes and 52 SLVs. The overall prevalence of *psrP* in this collection was 54.8% (204 of 372).

A significant difference in the prevalence of *psrP* was observed according to clonal type ($P < 0.0001$). Moreover, the presence or absence of *psrP* was closely related to specific genotypes but not to specific serotypes. Figure 2 shows the relative frequency of *psrP* among genotypes with more than 5 isolates. In brief, *psrP* was present in all 89 isolates (100%) belonging to CC306 (all of them serotype 1) and, in contrast, was absent in 18 of 19 isolates (5.3%) of serotype 1 belonging to ST304. Similarly, all 15 isolates belonging to ST1201 and all 11 strains belonging to CC199 (all of them serotype 19A) were positive for *psrP*. In contrast, 100% of 9 strains of serotype 19A belonging to multiresistant clone ST320 and 87% of 23 strains belonging to multiresistant clone CC230 (16 serotype 19A isolates and 7 serogroup 24 isolates) were negative for *psrP*. Other clones with a high prevalence of *psrP* were CC177 (7 of 7 isolates) and CC289 (21 of 26 isolates).

TABLE 2. Prevalence of positivity for *psrP* according to antimicrobial resistance (meningeal breakpoints) in pneumococcal isolates in children with IPD and healthy carriers

Subject group, antimicrobial, and MIC	No. of strains *psrP* positive/total no. of strains (%)	*P*
Patients with IPD		
Penicillin		
MIC ≤ 0.06 μg/ml	171/265 (64.5)	<0.000
MIC ≥ 0.12 μg/ml	12/79 (15.2)	
Cefotaxime		
MIC < 0.5 μg/ml	180/307 (58.6)	<0.000
MIC = 1 μg/ml	3/31 (9.7)	
MIC ≥ 2 μg/ml	0/6 (0)	
Erythromycin		
MIC ≤ 0.25 μg/ml	162/267 (60.7)	<0.000
MIC = 0.5 μg/ml	0/0	
MIC ≥ 1 μg/ml	21/77 (27.3)	
Tetracycline		
MIC ≤ 2 μg/ml	160/262 (61.1)	<0.000
MIC = 4 μg/ml	1/1	
MIC ≥ 8 μg/ml	21/78 (26.9)	
Healthy carriers		
Penicillin		
MIC ≤ 0.06 μg/ml	33/61 (54.1)	0.10
MIC ≥ 0.12 μg/ml	9/27 (33.3)	
Cefotaxime		
MIC ≤ 0.5 μg/ml	38/76 (50)	0.49
MIC = 1 μg/ml	3/10 (30)	
MIC ≥ 2 μg/ml	1/2 (50)	
Erythromycin		
MIC ≤ 0.25 μg/ml	30/62 (48.4)	1
MIC = 0.5 μg/ml	0/0	
MIC ≥ 1 μg/ml	12/26 (46.2)	
Tetracycline		
MIC ≤ 2 μg/ml	34/65 (52.3)	0.22
MIC = 4 μg/ml	0/0	
MIC ≥ 8 μg/ml	8/23 (34.8)	

DISCUSSION

The current conjugate pneumococcal vaccines, in which capsular polysaccharides are bound to either diphtheria or tetanus toxoid, are immunogenic and efficacious in children and prevent disease caused by the serotypes whose capsule types are in the vaccine (2, 4, 26). However, as these vaccines do not cover the full spectrum of invasive pneumococcal serotypes, temporal and geographic changes in serotype frequency associated with IPD exist. The pneumococcus is able to replace its capsule type through natural transformation, and children remain at risk of infection by nonvaccine serotypes. Moreover, the possibility of serotype shift, where the nonvaccine serotypes acquire an ecological advantage for increased carriage prevalence and, concomitantly, disease, remains real (8).

A possible solution to this problem is either replacement of the carrier toxoid with a conserved and highly antigenic single pneumococcal protein, thus providing serotype-independent protection, or alternatively, if a single antigen is insufficient, creation of a multivalent protein vaccine that eschews the capsular polysaccharide entirely. At this time, it is not clear which approach is best or which pneumococcal protein(s) should be included in any revised vaccine formulations. To address these issues, detailed molecular epidemiology is required to assess the frequency and distribution of various pneumococcal determinants in invasive clinical isolates. This is the first large study analyzing the prevalence of *psrP* in pneumococcal isolates from children with IPD and healthy nasopharyngeal carriers. The results of our study are in agreement with published data regarding the function of this new pneumococcal virulence factor and provide clues to the forces responsible for the spread of the pathogenicity island encoding PsrP, *psrP-secY2A2*, among different serotypes and clones.

The increased frequency of the gene encoding PsrP in clinical isolates from individuals with pneumonia compared with the frequency in those isolated from the nasopharynges of healthy carriers or children with bacteremia is consistent with published findings showing that PsrP is exclusively a lung cell adhesin and that it does not play a role in the nasopharynx during colonization or in the bloodstream in an intraperitoneal model of sepsis. These data also suggest that PsrP alone would protect against only 60% of strains that are capable of causing pneumonia. Thus, these findings indicate that, at best, PsrP could be a single component of a multicomponent vaccine formulation. The inclusion of a second or third protein that protects against bacteremia and whose coverage helps to cover the ~40% of invasive isolates that lack PsrP would be required.

Given that *psrP* was found to be predominantly associated with antimicrobial-susceptible isolates, it can be inferred that its inclusion within any vaccine would not serve as a mechanism to decrease the incidence of existing multidrug-resistant pneumococcal isolates. It also suggests that the extensive use of antimicrobials within the community is not responsible for promoting the preponderance of clonotypes that carry *psrP*. Counter to the latter view, we have recently shown that PsrP mediates the formation of bacterial aggregates within the lungs and the formation of more dense biofilms *in vitro* (20). As *psrP* is predominantly found in antimicrobial-susceptible isolates and bacterial aggregates and biofilms are considered to be more resistant to antimicrobials, it is a distinct possibility that in the absence of a dedicated antimicrobial resistance mechanism, PsrP confers resistance to susceptible isolates *in vivo* through enhanced biofilm formation. Thus, antimicrobial pressures may be serving to maintain *psrP* within susceptible clonotypes. To test this hypothesis, ongoing experiments are testing the resistance of these PsrP-mediated aggregates to antimicrobials.

When we stratified the incidence of *psrP* in children with pneumonia by age, we found that 65.4% of pneumococcal strains that caused disease in children greater than 2 years of age carry this virulence gene. This rate was significantly higher that the rate found in strains that cause disease in young children. One possible explanation for this may be the serotype distribution of *psrP*. *psrP* was found to be predominant in serotypes not covered by the 7-valent conjugate vaccine. In the prevaccine era, the 7 serotypes included in the vaccine were most prominent in Europe. Thus, the discrepancy in age might be explained by the fact that the infant nasopharynx is first
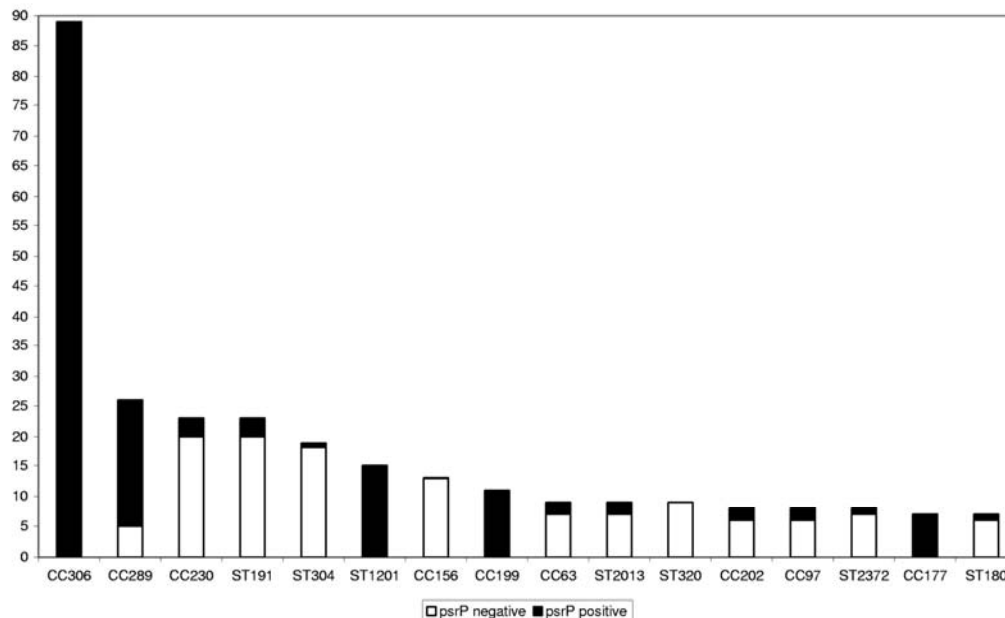
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FIG. 2. Prevalence of *psrP* according to clonal type of *Streptococcus pneumoniae* in groups of more than five isolates.

occupied by these 7 serotypes and is then filled with those from the nonvaccine serotypes at a later age. Studies by Melegaro and colleagues would support this explanation (10). However, in our study, this is not a valid explanation because PCV-7 serotypes were not found to be predominant in either younger children or older children (36 of 189 [19%] in children less than 2 years of age versus 34 of 252 [13.5%] in children ≥2 years of age; $P = 0.1$). Thus, other reasons, which are not yet clear, must explain why strains that carry *psrP* cause IPD at a later age than those that do not. Importantly, the fact that *psrP* is found predominantly in the PCV-7 nonvaccine serotypes suggests that its inclusion would expand coverage beyond that of the current vaccine. However, this is less so for the PCV-10 and PCV-13 formulations due to the inclusion of serotypes 1 and 19A, the strains of which have a high frequency of *psrP*. Of note, the reported prevalence of *psrP* among PCV-7 serotypes may be skewed by the fact that relatively few PCV-7 serotype strains were isolated in our study. It would therefore be interesting to test a collection of clinical isolates archived prior to the introduction of the vaccine. Such a study would determine if the current 60% incidence of *psrP* in pneumonia clinical isolates was due to serotype replacement (i.e., positive selection of nonvaccine serotypes with clones that carry *psrP*) or if *psrP* has been prevalent all along among serotypes and clones that frequently cause pneumonia.

Finally, we observed the presence of *psrP* in certain clonotypes and its absence in others. For example, all strains of serotype 1 with CC306 were positive for *psrP*, while only 1 of 18 strains with ST304 was positive. ST306, together with ST304, ST228, and others, belongs to lineage A of serotype 1, which is the major lineage detected in North America and Europe (3). According to some epidemiological studies ST306 has been related to several outbreaks of invasive pneumococcal disease

(9) and the emergence of pleuropneumonia in the vaccine era (15), while ST304 has not. The high prevalence of PsrP, a lung cell bacterial adhesin, in ST306 strains could be associated with this fact. It is not clear why *psrP* would be present in some isolates but not others; however, this suggests that other pneumococcal virulence determinants compensate for the absence of PsrP. Thus, detailed comparative genomic analyses of invasive clonotypes within the same serotype containing *psrP* and not containing *psrP* may be warranted to identify the compensatory determinants that are responsible for disease and, moreover, to determine if their inclusion along with PsrP in a multicomponent vaccine would enhance coverage.

In conclusion, *psrP* is highly prevalent in our clinical collection and is mainly present in strains isolated from older children with pneumonia. The distribution of *psrP* seemed to be strongly associated with antimicrobial sensitivity, non-PCV-7 serotypes, and specific clonotypes of pneumococci. These data support the potential use of PsrP as a protective antigen in the design of a next generation of protein-based combination vaccines. However, the data also indicate that additional components that fill the bacteremia and serotype niche not covered by PsrP are required.

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ARTICLE

Serotype 3 is a common serotype causing invasive pneumococcal disease in children less than 5 years old, as identified by real-time PCR

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Abstract Serotype 3 is one of the most often detected pneumococcal serotypes in adults and it is associated with serious disease. In contrast, the isolation of serotype 3 by bacterial culture is unusual in children with invasive pneumococcal disease (IPD). The purpose of this study was to learn the serotype distribution of IPD, including culture-negative episodes, by using molecular methods in normal sterile samples. We studied all children <5 years of age with IPD admitted to two paediatric hospitals in Catalonia, Spain, from 2007 to 2009. A sequential real-time polymerase chain reaction (PCR) approach was added to routine methods for the detection and serotyping of pneumococcal infection. Among 257 episodes (219 pneumonia, 27 meningitis, six bacteraemia and five others), 33.5% were identified by culture and the rest, 66.5%, were detected exclusively by real-time PCR. The most common serotypes detected by culture were serotypes 1 (26.7%) and 19A (25.6%), and by real-time PCR, serotypes 1 (19.8%) and 3 (18.1%). Theoretical coverage rates by the PCV7, PCV10 and PCV13 vaccines were 10.5, 52.3 and 87.2%, respectively, for those episodes identified by culture, compared to 5.3, 31.6 and 60.2% for those identified only by real-time PCR. Multiplex real-time PCR has been shown to be useful for surveillance studies of IPD. Serotype 3 is under-diagnosed by culture and is important in paediatric IPD.

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Introduction

Streptococcus pneumoniae is an important pathogen responsible for high mortality and morbidity worldwide, despite the availability of antibiotic treatment and vaccines [1]. Knowledge of pneumococci serotype distribution causing invasive pneumococcal disease (IPD) remains of primary importance in order to assess the effectiveness of new conjugate vaccines and closely monitor the emergence of non-vaccine serotypes.

Classic diagnosis of the disease is by microbiological bacterial culture, which has high specificity but low sensitivity, particularly in paediatric patients who have undergone prior antibiotic therapy; this increases the number of false-negative results [2]. Microbiological culture requires the isolation and identification of *S. pneumoniae* from normally sterile clinical specimens; it requires 48–72 h to confirm the results, which may have low sensitivity. Studies of serotype distribution based on

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culture-proved IPD have the same limitation. Therefore, antibiotic-susceptible pneumococcal serotypes may be misdiagnosed and, consequently, the rates of IPD can be underestimated. In addition, technical difficulties inherent in conventional serotyping limit its use to a few specialised laboratories. Different authors have reported that the sensitivity of molecular methods was significantly higher than culture methods, and that molecular methods can be used directly on sterile biological samples, improving the ability to diagnose IPD [3–8]. In addition, real-time PCR methods that specifically identify the capsular type in a direct sample offer a sensitive, rapid and simple approach for the surveillance of pneumococcal disease.

The objective of this study was to learn the serotype distribution, including culture-negative episodes of IPD, among young children before the introduction of 10-valent and 13-valent pneumococcal conjugate vaccines (PCV10 and PCV13) in Catalonia, Spain.

Materials and methods

Patients and setting

We studied all children <5 years of age with IPD who had been admitted to two tertiary-care paediatric hospitals in Barcelona from January 2007 to December 2009. Children included in the study have been analysed by routine microbiological methods and a sequential real-time PCR approach applied in normal sterile samples for the diagnosis and serotyping of IPD.

In Spain, the 7-valent pneumococcal conjugate vaccine (PCV7) was introduced in June 2001. However, it was not included in the routine childhood vaccination schedule because it was not subsidised by the Spanish Health Service. The recommendations of the Spanish Paediatric Academy for the use of PCV7 were to cover all children aged <23 months and children aged 24–59 months who were at high risk for pneumococcal infection. The Academy recommends PCV7 vaccination for children aged ≤2 years, scheduled at 2, 4 and 6 months of age, with a booster in the second year of life, and for older children at high risk of IPD. During the study period, the use of PCV7 in the community was around 50% [9]. PCV10 and PCV13 were not introduced in our country during the study period.

IPD was defined as the presence of clinical findings of infection (which were used for the classification of disease), together with the isolation of S. pneumoniae and/or DNA detection of the *pneumolysin* (*ply*) gene and an additional capsular gene of S. pneumoniae by real-time PCR in any sterile fluid (plasma, cerebrospinal fluid or any other sterile fluid). DNA detection of the *pneumolysin* (*ply*) gene by real-time PCR was performed according to a published

assay [3] and was performed in the first 48 h after admission. IPD was classified according to the International Classification of Disease, Ninth Revision (ICD-9-CM) specific for diseases caused by S. pneumoniae, including: meningitis, pneumonia, parapneumonic empyaema, occult bacteraemia, sepsis and arthritis. Meningitis was considered by a compatible clinical syndrome and biochemical cerebrospinal fluid (CSF) test. Pneumonia was considered by the increase in respiratory rate, difficulty in breathing and pathological breath sounds. Complicated pneumonia was considered when a lobar or segmental lung consolidation with pleural effusion was detected. Occult bacteraemia/sepsis was considered among admitted patients with fever (>37.5°C axillary temperature), with or without clinical signs of sepsis. Osteomyelitis or arthritis were considered by the presence of local signs and confirmed by X-ray.

We registered the demographic and clinical variables, including: age, sex, date of admission, clinical manifestations, outcomes, vaccination status and previous antibiotic therapy (defined as exposure to an antibiotic treatment in the preceding 30 days before the diagnosis of IPD).

Data were recorded following the guidelines of the Hospital's Ethical Committee.

Microbiological culture and antimicrobial susceptibility studies of S. pneumoniae isolates

All pneumococcal isolates were identified by standard microbiological methods. The agar dilution technique was used to determine the minimum inhibitory concentration (MIC) of several antibiotics. Penicillin and other antibiotic susceptibilities were defined according to the breakpoints of the Clinical Laboratory Standards Institute (CLSI, M100-S20, 2010) [10].

Molecular diagnosis of S. pneumoniae

DNA detection of the *pneumolysin* (*ply*) gene by real-time PCR in normal sterile samples was carried out according to a published assay [3] and was performed in the first 48 h after admission. The presence of S. pneumoniae DNA was confirmed by the amplification of the *wzg* (*CpsA*) gene by real-time PCR, as previously reported [11]. Only positive samples for both the *ply* and *wzg* genes in real-time PCR were included in the sequential serotyping analysis.

Serotype identification from direct clinical samples and S. pneumoniae isolates

The detection of pneumococcal serotypes from direct samples and from S. pneumoniae strains was performed at our laboratory, according to a published multiplex real-time PCR methodology [11]. This sequential PCR approach

detected 24 serotypes (1, 3, 4, 5, 6A/B, 7F/A, 8, 9V/A/N/L, 14, 15B/C, 18C/B, 19A, 19F/B/C, 23A/F).

In addition, strains isolated by culture were also serotyped using the Quellung reaction or dot blot. MICs and serotyping of the strains was performed at the National Center for Microbiology (Majadahonda, Spain).

Statistical analysis

We used the Chi-square test or Fisher's exact test, when appropriate, to compare proportions. Statistical analyses were performed using SPSS for Windows, version 17.0. We calculated 95% confidence intervals (CIs), and two-sided p-values ≤ 0.05 were considered to be statistically significant.

Results

During the study period, there were 319 patients with IPD, including 170 male patients (53.3%) and 149 female patients (46.7%), with a mean age of 29.6 months $\pm$ 15.7 months. Ninety-one (28.5%) patients had received antibiotic treatment in the month before the diagnosis of IPD and 168 (52.8%) children had received at least one dose of PCV7, although only 141 (44.3%) had been correctly vaccinated according to their age.

We included in the present study data of 257 serotyped episodes (80.6%) which had undergone both bacterial cultures and real-time PCR (we excluded 35 patients studied only by culture, nine studied only by real-time PCR and 18 without serotype study). One hundred and forty children (54.5%) were male and 117 (45.5%) were female, with a mean age of 30.95 months$\pm$15 months (age range from 20 days to 59 months). The distribution of patients by age group was as follows: 10 patients (3.8%) <6 months, 19 patients (7.4%) between 6–11 months, 64 patients (25%) between 12–23 months and 164 patients (63.8%) between 24–59 months. Eighty-three patients (32%) had received antibiotic treatment in the month before the diagnosis of IPD. One hundred and forty (54.5%) patients had received one dose or more of PCV7 and 122 (47.5%) were correctly vaccinated according their age.

Seventy-one (27.6%) patients had both positive culture and real-time PCR, 15 (5.8%) had positive culture and negative real-time PCR, and 171 (66.5%) had positive real-time PCR and negative culture. The proportion of cases diagnosed by real-time PCR only was 66.5% (95% CI 60.9–72.5), in contrast to 33.4% (95% CI 27.9–39.4) diagnosed by bacterial culture. The 257 episodes were detected in 64 (24.9%) positive blood specimens, 163 (63.4%) positive pleural fluid specimens, 28 (10.9%) positive CSF specimens and 2 (0.8%) positive joint fluid specimens. Table 1 shows the distribution of positive samples detected by culture and by real-time PCR only.

Overall, the clinical diagnosis of patients included in this study was pneumonia 219 (85.2%), meningitis 27 (10.5%), bacteraemia 6 (2.3%), arthritis 2 (0.8%), sepsis 2 (0.8%) and cellulitis 1 (0.4%). One hundred and eighty-eight (85.8%) of 219 patients with pneumonia had complicated pneumonia with empyaema. There were 4 (1.5%) deaths, comprising three patients with meningitis and one with sepsis. These episodes were caused by serotypes 7F, 27, 6A and 23F (this last one occurring in an unvaccinated child).

The major increase of microbiological diagnosis by using real-time PCR was in patients with pneumonia; 74.0% (95% CI 68.4–80.0) were only diagnosed by real-time PCR, while 26% (95% CI 20.4–32.0) were diagnosed by bacterial culture. Statistically significant differences were also observed in meningitis and bacteraemia (Table 2).

Serotyping study was done in 86 (33.5%) strains isolated from culture and the remaining 171 (66.5%) episodes directly by real-time PCR from the biological sample.

Only two of the 86 strains (2.3%) with available antimicrobial susceptibility study were penicillin intermediate-resistant (MIC 4 µg/mL) and none (0%) penicillin fully-resistant according to non-meningeal breakpoints. The percentage of penicillin non-susceptible isolates was 33.7% and cefotaxime 16.3% according to meningeal breakpoints, and the serotypes that caused the most penicillin non-susceptible-related IPDs were serotype 19A (51.7% of non-susceptible isolates), serotype 23B (10.3%) and serotype 24B/F (10.3%).

Differences in serotype distribution among patients with positive culture versus patients with negative culture

Fifty percent of patients identified by culture and 48% of patients identified only by real-time PCR had been well vaccinated with PCV7. No patients were vaccinated with either PCV10 or PCV13. We found significant differences ($p<0.002$) in the rank order of the five main serotypes in IPD episodes identified by culture versus. those identified only by real-time PCR. The three most frequent serotypes in the group of 86 episodes identified by culture were serotype 1 (28%; $n=23$), serotype 19A (26%; $n=22$) and serotype 7F (9%; $n=8$), while in the group of 171 patients diagnosed only by real-time PCR, they were serotype 1 (20%; $n=34$), serotype 3 (18%; $n=31$) and serotype 19A (9%; $n=16$). Of note, the rate of serotype 3 detected by real-time PCR was significantly higher than the rate of this serotype detected by culture ($p=0.01$). Figure 1 shows the different serotype distributions according to diagnosis by culture or only real-time PCR.

As expected for the routine use of PCV7 during the study period, IPD caused by serotypes included in PCV7 was a rare event. PCV7 serotypes were found in 10.5% of patients identified by culture versus 5.3% of patients identified by real-time PCR ($p=0.12$). Among the PCV7

Table 1 Distribution of positive samples detected by culture and only by real-time polymerase chain reaction (PCR)

Type of sample	Positive samples by culture, n (%[a]; 95% CI)	Positive samples only by real-time PCR, n (%[a]; 95% CI)	p-value[b]
Plasma	21 (32.8; 22.1–44.9)	43 (67.2; 55–77.8)	<0.001
Pleural fluid	42 (25.8; 19.5–32.9)	121 (74.2; 67.1–80.5)	<0.001
CSF	21 (75; 56.7–88.3)	7 (25; 11.6–43.3)	<0.001
Joint fluid	2 (100; 22.4–100)	0 (0; 0–77.6)	0.3

[a] Percentage with respect to all positive samples by type of sample

[b] Pearson's Chi-square or Fisher's exact test comparing percentage by type of sample

cases by culture ($n=9$ patients; 10.5%), only one child (11%) had received three doses of PCV7, and among the PCV7 cases by PCR ($n=9$ patients; 5.3%), three children (33.3%) had received one, two or three doses of PCV7. The proportion of serotypes included in the PCV10 rose to 52.3% in patients identified by culture versus 31.6% in patients detected only by real-time PCR ($p<0.002$). Serotypes included in PCV13 were detected in 87.2% of patients diagnosed by positive culture and in 60.2% of patients diagnosed by real-time PCR ($p<0.000$). Figure 2 shows the potential coverage of these three conjugate vaccines according to diagnosis by culture or only by real-time PCR.

Differences in serotype distribution according to age and microbiological diagnosis technique

Overall, patients identified by culture were significantly younger than patients identified only by real-time PCR (mean age 25.9 months ±15 months vs. 33.4 months ±14.1 months).

Of the total number of children studied, 93 (36.2%) were younger than 2 years of age; of these, 46 (49.5%; 95% CI 38.7–60.2) were identified by culture and 47 (50.5%; 95% CI 39.8–61.2) were identified only by real-time PCR. In the group of patients identified by culture, the main serotype

detected was serotype 19A (16 patients; 34.8%), while in those identified only by real-time PCR, serotype 3 was the strain most often detected (14 patients; 29.8%).

Among children over 2 and less than 5 years of age ($n=164$), 40 were identified by culture (24.4%; 95% CI 17.5–31.3) and 124 (75.6%; 95% CI 68.7–82.5) were identified only by real-time PCR. Serotype 1 was the main serotype detected in patients identified by culture (20 patients; 50%) and by real-time PCR (32 patients; 25.8%). The second most prevalent serotype was serotype 19A among patients identified by culture (6 patients; 15%) and serotype 3 among patients identified by real-time PCR (17 patients; 13.7%).

Differences in serotype distribution among patients with pneumonia according to microbiological diagnosis technique

Among episodes of pneumonia, we found significant differences ($p<0.001$) in the serotype distribution of isolates when comparing episodes identified by culture versus those identified only by real-time PCR. Twenty-three of 57 episodes (68.4%) identified by culture were caused by serotypes 1 (40.3%) and 19A (28%). In this group, serotype 3 was found in only 5 patients (8.7%). However, in 162 episodes identified only by real-time PCR, the second most

Table 2 Clinical forms of invasive pneumococcal disease (IPD) in children diagnosed by bacterial culture versus those diagnosed only by real-time PCR

Clinical form	Patients diagnosed by culture, n (%[a]; 95% CI)	Patients diagnosed only by real-time PCR, n (%[a]; 95% CI)	p-value[b]
Pneumonia	57 (26.0; 20.4–32.0)	162 (74.0; 68.4–80.0)	<0.001
Meningitis	20 (74.1; 53.7–88.9)	7 (25.9; 11.1–46.3)	0.09
Bacteraemia	6 (100)	0	0.001
Sepsis	0	2 (100)	0.55
Arthritis	2 (100)	0	0.11
Cellulitis	1	0	0.33
Total	86 (33.4; 27.9–39.4)	171 (66.5; 60.9–72.5)	<0.001

[a] Percentage with respect to all microbiological diagnoses by clinical form

[b] Pearson's Chi-square or Fisher's exact test comparing the distribution of microbiological diagnoses in each clinical form with respect to all clinical forms
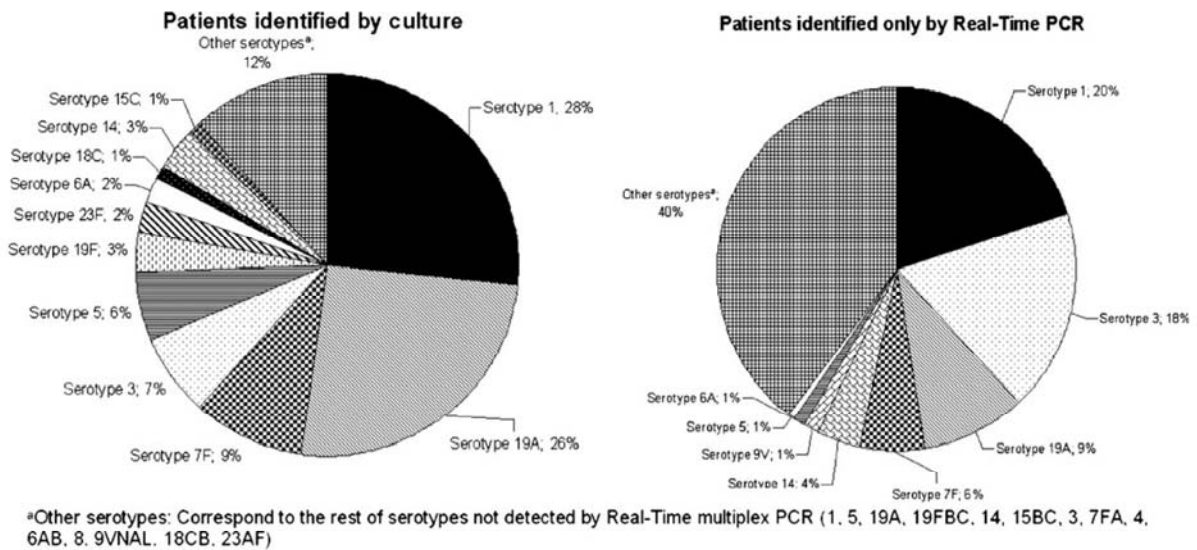
Patients identified by culture

Patients identified only by Real-Time PCR

ᵃOther serotypes: Correspond to the rest of serotypes not detected by Real-Time multiplex PCR (1, 5, 19A, 19FBC, 14, 15BC, 3, 7FA, 4, 6AB, 8, 9VNAL, 18CB, 23AF)

Fig. 1 Serotype distribution according to diagnosis by culture or only real-time polymerase chain reaction (PCR)

prevalent serotype causing IPD was serotype 3 (19.1%, 31 patients). Serotypes 1 and 19A were detected in 34 (20.9%) and 15 (9.2%) episodes, respectively.

Differences in serotype distribution according to previous antibiotic therapy and microbiological diagnosis technique

The microbiological study was performed after previous antibiotic therapy in 83 (32.3%) children. In this group of patients, only 13 (15.3%) showed positive results of bacterial

culture, while in the group of 172 (66.9%) patients without previous antibiotic therapy, bacterial culture was positive in 72 (41.9%). This difference is statistically significant ($p<0.000$). For two patients, information about their previous antibiotic therapy was not available.

We detected important and significant differences ($p=0.02$) of the serotype distribution in patients exposed to previous antibiotic therapy according to the microbiological diagnosis technique (Table 3). Of the total patients with previous antibiotic therapy and a positive culture ($n=13$),

Fig. 2 Coverage of different pneumococcal conjugate vaccines (PCVs)
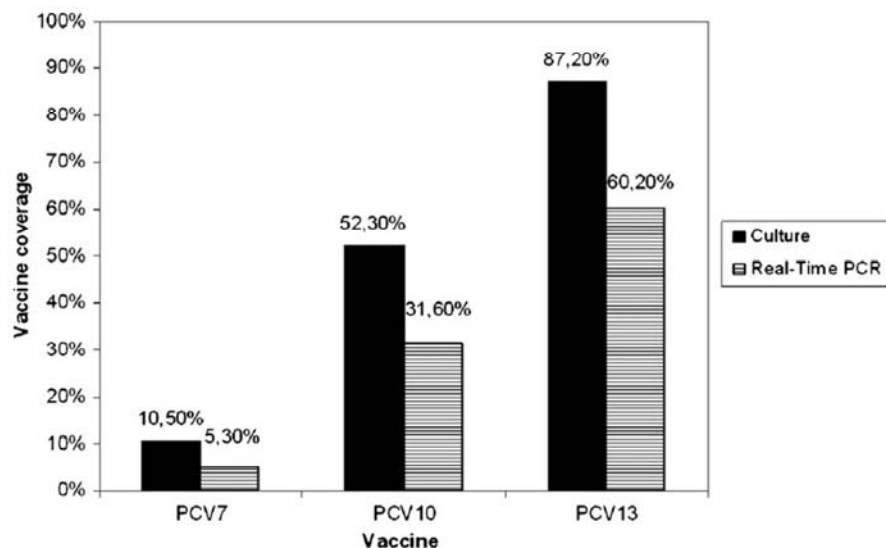
PCV7: Pneumococcal conjugate vaccine 7-valent, PCV10: Pneumococcal conjugate vaccine 10-valent, PCV13: Pneumococcal conjugate vaccine 13-valent

Table 3 Serotype distribution according to previous antibiotic therapy and microbiological diagnosis technique in 255 children[a] with IPD

Serotypes	Patients exposed to previous antibiotic therapy		Patients not exposed to previous antibiotic therapy	
	n (%)		n (%)	
	Patients diagnosed by culture	Patients diagnosed only by real-time PCR	Patients diagnosed by culture	Patients diagnosed only by real-time PCR
1	2 (15.4)	14 (20)	21 (29.2)	20 (20)
19A	6 (46.2)	7 (10)	16 (22.2)	9 (9)
5	2 (15.4)	1 (1.4)	3 (4.2)	0 (0.0)
3	0 (0.0)	12 (17.1)	6 (8.3)	19 (19)
7F	1 (7.6)	5 (7.1)	7 (9.7)	5 (5)
14	0 (0.0)	2 (2.9)	3 (4.2)	5 (5)
6A	0 (0.0)	1 (1.4)	2 (2.8)	1 (1)
19F	0 (0.0)	0 (0.0)	3 (4.2)	0 (0.0)
23F	0 (0.0)	0 (0.0)	2 (2.8)	0 (0.0)
9V	0 (0.0)	0 (0.0)	0 (0.0)	2 (2)
15C	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)
18C	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)
Other serotypes[b]	2 (15.4)	28 (40)	7 (9.45)	39 (39)
Overall	13 (100)	70 (100)	72 (100)	100 (100)

[a] For two patients, information about their previous antibiotic therapy was not available

[b] Correspond to the rest of the serotypes not detected by real-time multiplex PCR (non-serotypes 1, 5, 19A, 19F/B/C, 14, 15B/C, 3, 7F/A, 4, 6A/B, 8, 9V/A/N/L, 18C/B, 23A/F)

the main serotype detected was serotype 19A (6 isolates; 46.2%). However, among the total group of patients exposed to antibiotic therapy and diagnosed by real-time PCR (n=70), the main serotype detected was serotype 1 (14 isolates, 20.0%).

In the group of patients not exposed to previous antibiotic therapy with positive culture (n=72 patients), serotype 1 was the most frequently detected serotype (21 isolates, 29.2%), followed by serotype 19A (16 isolates, 22.2%) and serotype 7F (7 isolates, 9.7%). In contrast, in the 100 episodes of patients without antibiotic exposure and diagnosed only by real-time PCR, the main serotypes detected were serotype 1 (20 isolates; 20%), followed by serotype 3 (19 isolates, 19%) and serotype 19A (9 isolates, 9%) (p=0.02).

Discussion

In the present study, performed during the routine use of PCV7 vaccine, we found that the proportion of microbiological diagnoses of IPD carried out only by real-time PCR (with negative culture) is twice the proportion of diagnoses carried out by culture. The diagnosis of *S. pneumoniae* infections may be problematic, mainly in paediatric children, who present the peculiarity that it is not always

possible to collect an adequate volume of the sample on which to perform blood culture. Moreover, many of these patients received treatment with antibiotics previous to sample collection and, therefore, cultures are frequently negative. In this study, comparing the patients with previous antibiotic therapy, only 15.3% showed positive results of bacterial culture, in contrast to patients without previous antibiotic therapy, among whom 41.9% of the cases showed positive results to bacterial culture. For this reason, new sensitive diagnostic methods are needed not only for diagnosis, but also to monitor the epidemiology of pneumococcal disease and the impact of vaccines.

This study has value for epidemiologic surveillance and also as a further evaluation of the potential impact of new conjugate vaccines. Immunisation with PCV7 has changed the distribution of the main serotypes causing IPD [9, 12, 13]. In Spain, Fenoll et al. [14] reported the temporal trends of invasive *S. pneumoniae* serotypes and antimicrobial resistance over a period of 30 years; serotypes 1 and 19A have become more prevalent since the introduction of PCV7, while other serotypes, such as 3, 4 and 8, have maintained their steady secular trend over the three decades. Temporal trends of pneumococcal serotype distribution have been reported and observed during different periods of time [14–16] and have been associated with antibiotic treatment or/and vaccines.

Changes in serotype distribution may be an important factor to explain changes in the epidemiological characteristics of IPD comparing the pre-vaccine and vaccine eras. In the pre-vaccine era, the risk of IPD was usually highest in those <2 years old and then tapers off after 2 through 5 years of age. In the present study, children >2 years old are the main group with IPD. This fact was also detected in previous studies of our group, where bacterial culture was the only microbiological criterion for the definition of IPD [9]. The emergence of non-vaccine serotypes, such as serotype 1, which is mainly detected in older children, and the good results of PCV7 against serotypes mainly detected in children younger than 2 years of age, are partial explanations for this event.

Therefore, it is important to learn the distribution of serotypes in our population in order to analyse the impact of PCV7 before the introduction of the new conjugate vaccines (10-valent and 13-valent, PCV10 and PCV13, respectively). According to our results, the coverage of the current conjugate vaccines PCV7, PCV10 and PCV13 is lower than that expected in those patients diagnosed only by real-time PCR, especially in PCV7 and PCV10. For this reason, further molecular epidemiology studies are needed after vaccination in order to predict the trends in particular serotypes and to detect a possible replacement phenomenon for non-vaccine serotypes similar to that detected in PCV7.

The serotype distribution of patients identified by culture reported in this study is similar to what other authors have found [17, 18]. The most prevalent serotype in patients identified by culture is serotype 1, as in other regions of Spain and in Portugal. Marimon et al. [19] reported an increase in the number of IPD cases in children caused by serotype 1 in the Basque region of Spain following the introduction of PCV7. Nunes et al. [20] reported an emergence of serotype 1 lineage of pneumococci among healthy carriers in Portugal after 2003. The other most prevalent serotypes detected by culture were serotypes 19A and 7F. Serotype 19A has been reported by many authors as the most common serotype causing invasive pneumococcal infections in children [12, 21, 22], and many 19A isolates have been associated with multidrug resistance [23, 24]. A surveillance study conducted in Germany [25] beginning in 1992 reported that serotype 7F was statistically more prevalent among children less than 4 months old than among individuals in other age groups.

Prevalent serotypes found by culture have also been common among patients detected only by real-time PCR, but we have found additional serotypes identified only by real-time PCR that are not so commonly seen among culture episodes. In the rank order of serotypes in patients with negative culture, we found serotype 3 to be the second most frequent serotype. Serotype 3 has been associated with invasive disease in older children and adults [26] and

with higher case–fatality ratios compared to other serotypes [27]. In Spain, a study reported that serotype 3 was one of the most prevalent serotypes causing paediatric parapneumonic empyaema (PPE) and was associated with significantly more complications than PPE caused by other serotypes [28]. Recently, Bender et al. [29] identified an increasing incidence of *S. pneumoniae*-related haemolytic uraemic syndrome in children in Utah, associated with serotype 3. We have found that serotype 3 is mainly detected by molecular methods and is less frequent in those patients identified by culture. According to this data, multiplex real-time PCR has the potential to reveal a different distribution of serotypes circulating in the population compared to culture-positive cases. A recent study comparing conventional and molecular microbiology in detecting differences in pneumococcal colonisation among healthy carriers and ill children showed that real-time PCR was superior to bacterial culture in identifying a great number of pneumococcal serotypes in both groups of patients, healthy nasopharyngeal carriers and children with upper respiratory illness [30].

Our study has several limitations. First, the real-time multiplex PCR [11] assay used in this study does not differentiate between certain serotypes, such as 6A/C or 19F/B/C, although it detects all serotypes included in the three conjugate vaccines. Second, we found that 44% of pneumococci in patients diagnosed by real-time PCR corresponded to serotypes other than those detected by real-time multiplex PCR. This could be explained by the fact that the number of serotypes detected in the assay was limited to 24 of the 93 serotypes currently known. Another putative explanation is that these pneumococci may be other species closely related to *S. pneumoniae*, rather than *S. pneumoniae* itself. Recently, new species such us *Streptococcus pseudopneumoniae* [31, 32] and closely related streptococci [33] have been described in the literature. In our laboratory, we perform real-time PCR of the *pneumolysin* (*ply*) gene as screening and a second real-time PCR assay to detect the capsular *wzg* gene before performing the serotyping study. Therefore, the detection of a virulence gene of pneumococci and an additional capsular gene in a sterile sample of a patient with clinical symptoms of bacterial infection may have significant clinical value. Although few data are available, it has been reported [33] that these closely related streptococci are critical in pneumococcal colonisation studies because they inhabit the same niche and can be highly resistant to antibiotics. The clinical role of these closely related pneumococcal strains isolated in sterile samples needs to be clarified.

The results of this study may be different from those obtained in other geographical areas, with different use of PCV7, different antibiotic political use or different use of

blood cultures. The present study is performed with a intermediate introduction of PCV7 (around 50%), intermediate use of previous antibiotics (32% of children were exposed to antibiotics) and only including hospitalised patients. In addition, the presence of a specific clone such as ST306 of serotype 1 in our community may be related with the high prevalence of pneumonia [34] and disease observed in older healthy children, while in other communities with other predominant clones (i.e multiresistant PCV7 serotypes), the picture of disease may be different.

To conclude, multiplex real-time PCR has been shown to be very useful for surveillance studies of IPD and it is a good complement for classical microbiological methods. Serotype 3 is underdiagnosed by culture and it is important in paediatric IPD.

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Effectiveness of 7-valent pneumococcal conjugate vaccine in the prevention of invasive pneumococcal disease in children aged 7–59 months. A matched case-control study

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ABSTRACT

The aim of this study was to evaluate the effectiveness of the administration of the 7-valent pneumococcal conjugate vaccine in a region with an intermediate vaccination coverage.

A matched case-control study was carried out in children aged 7–59 months with invasive pneumococcal disease (IPD) admitted to two university hospitals in Catalonia. Three controls matched for hospital, age, sex, date of hospitalization and underlying disease were selected for each case. Information on the vaccination status of cases and controls was obtained from the vaccination card, the child's health card, the hospital medical record or the vaccination register of the primary healthcare center where the child was attended for non-severe conditions. A conditional logistic regression analysis was made to control for the effect of possible confounding variables.

The adjusted vaccination effectiveness of the complete vaccination schedule (3 doses at 2, 4 and 6 months and a fourth dose at 15 months, 2 doses at least two months apart in children aged 12–23 months or a single dose in children aged >24 months) in preventing IPD caused by vaccine serotypes was 93.7% (95% CI 51.8–99.2). It was not effective in preventing cases caused by non-vaccine serotypes.

The results of this study carried out in a population with intermediate vaccination coverage confirm those of other observational studies showing high levels of effectiveness of routine 7-valent pneumococcal conjugate vaccination.

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1. Introduction

The importance of pneumococcal infection in children aged <2 years [1–3] and growing pneumococcal resistance to antibiotics [4] stimulated the search for conjugated pneumococcal vaccines, of which the first, the 7-valent vaccine (PCV7) was licensed in 2000 in the United States, after a controlled clinical trial demonstrated protective efficacy against invasive pneumococcal disease (IPD) in small children [5,6]. The vaccine was licensed in Spain in 2001 [2].

In the 1990s, the PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) were mainly responsible for cases of IPD in children in the United States [7] and 71% of cases of IPD in children aged <2 years in Catalonia [8], a region in Northeastern Spain with more than 7 million inhabitants.

In Spain, all routine vaccines included in the official vaccination calendar are administered free of charge. Except for the Madrid region, the PCV7 has not been included in the official vaccination calendar, but is recommended by the Spanish Association of Pediatrics [9]. In Catalonia (Spain), many private and public pediatric practices recommend the vaccine, which is paid for by parents. The recommended schedule is three doses at 2, 4 and 6 months, with a fourth dose at 15 months. Two doses at least 2 months apart are recommended in children aged 12–23 months and a single dose in children aged ≥2 years. A 2004 Catalan study found a vaccination

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coverage of 27% [10] and later studies found coverages of 34% and 50% [11,12].

This vaccination strategy has achieved only intermediate vaccination coverages and has had a limited impact [13]. Unlike the Unites States [14], in Catalonia the global incidence of IPD has hardly diminished after licensing of the vaccine [13]. The overall incidence of IPD in Catalonia in children aged < 2 years was 53.7 per 100,000 persons-year in 2001 and 56.4 in 2008 [15]. The proportion of vaccine serotypes causing IPD has diminished due to vaccination, but the proportion of non-vaccine serotypes has increased, explaining the limited impact [11,12,16–19]. Some authors [20–24] have shown that the PCV7 is effective in preventing IPD caused by vaccine serotypes, but it is important to investigate the effectiveness of the vaccine in different age groups in communities where vaccination coverages are not very high.

The purpose of this study was to evaluate the effectiveness of the PCV7 (protective value of vaccination in usual circumstances in the field) in children aged 7–59 months in a population with intermediate vaccination coverages.

2. Methods

2.1. Study design

A matched case-control study was carried out in patients with IPD admitted to two university hospitals in Barcelona (Hospital Sant Joan de Déu and Hospital Vall d' Hebron) with 339 and 210 pediatric beds, respectively. There are about 400,000 children aged <5 years in Catalonia and the two study hospitals attend more than 30% of this population. Both hospitals form part of the Network of Public Hospitals of Catalonia and all care are free at the point of use. All children from the assigned population requiring hospitalization are referred by private or public health pediatricians to these hospitals. Therefore, the study population can be considered as representative of the population aged <5 years in Catalonia. The study period was January 1, 2007 to December 31, 2009.

2.2. Case selection

All patients aged 7–59 months hospitalized for IPD were initially studied. Children aged 0–6 months were excluded as they had not had the opportunity to receive primary immunization according to the schedule recommended in the technical data sheet, i.e., three doses of vaccine administered during the six first months of age. IPD was defined as isolation of *Streptococcus pneumoniae* or detection of DNA of the pneumolysin (*ply*) gene and an additional capsular gene of *S. pneumoniae* by real-time PCR in any normally sterile site. Children who presented IPD in whom serotyping of *S. pneumoniae* was not possible because the sample was not available were excluded.

Cases of IPD were clinically classified as: pneumonia, pneumonia complicated by empyema, meningitis, occult bacteremia or sepsis and other clinical forms. Pneumonia and pneumonia with empyema were diagnosed clinically and radiologically (pneumonia alone or pneumonia plus pleural effusion).

Strains of *S. pneumoniae* isolated by culture were identified by identical standard microbiological methods throughout the study period. Detection of the *ply* gene of *S. pneumoniae* was performed by real-time PCR according to a previously reported method [25].

Strains isolated by culture were serotyped using the Quellung reaction or dot blot by the National Centre for Microbiology, Majadahonda, Madrid. Serotypes in patients with negative cultures were detected at the Sant Joan de Déu Hospital by multiplex real-time PCR [26], which detects the conserved *wzg* capsule gene and other genes selected to differentiate the 24 serotypes most frequently implicated in IPD (1, 3, 4, 5, 6A, 6B, 7F/A, 8, 9V/A/N/L, 14,

15B/C, 18C/B, 19A, 19F/B/C, 23A and 23F). Serotypes were classified into 2 groups: vaccine serotypes (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F), and non-vaccine serotypes (all others). Since PCR does not differentiate between serotypes 9V, 9A, 9N and 9L, between serotypes 18C and 18B or between serotypes 19F, 19B and 19C these serotypes were all considered as vaccine serotypes 9V, 18C or 19F, respectively.

2.3. Selection of controls

Three controls for each case were selected from patients aged 7–59 months treated in the same hospitals. Controls were selected prospectively in wards and outpatient visits for non-infectious diseases and matched by age (±3 months if aged 7–11 months, but always older than 6 months, and ± 6 months if aged 12–59 months), sex, date of hospitalization or outpatient visit at the same center (±30 days) and underlying risk condition when present. Risk conditions are detailed in the clinical variables section.

Study investigators were blinded to the vaccination status of all controls during selection.

2.4. Vaccination status of cases and controls

A case was considered vaccinated if they had received the last dose (or only dose if this was the schedule corresponding to their age) of the PCV7 ≥ 15 days before symptom onset. Controls were considered vaccinated if they had received the last or only dose ≥15 days before hospital admission or outpatient visit. A child was considered completely vaccinated when they had received the recommended doses corresponding to age. A case or control was considered vaccinated when confirmed by records. For both cases and controls, the vaccination card or health card, where pediatricians (both public and private) record the vaccines administered and the date, were asked for. If neither was available, hospital medical records were consulted and, finally, if there was no record of vaccination, registers of the primary healthcare center where the child was attended for non-severe conditions were consulted.

The number of doses received and the date of administration were recorded. Cases and controls whose vaccination status could not be determined were excluded.

2.5. Sociodemographic, clinical and epidemiological variables

The demographic and clinical variables recorded for each case were: age, sex, date of hospitalization, clinical form of IPD, risk medical conditions [27] (sickle cell disease, congenital or acquired asplenia, HIV infection, cochlear implants, congenital immune deficiency, chronic heart disease, chronic pulmonary disease including asthma if treated with high risk-dose oral corticosteroid therapy, cerebrospinal fluid leaks, chronic renal failure including nephrotic syndrome, immunosuppressive or radiation therapy, solid organ transplantation and diabetes mellitus), and antibiotic therapy and history of respiratory infection in the 30 days before symptom onset. Other epidemiological variables recorded were: day care or school attendance, parental smoking, number of cohabitants, age of siblings and parental socioeconomic level, classified into six groups according to parental occupation using the British Classification of Occupations [28]. Two levels were considered: high (classes I–III) and low (classes IV–V).

The same variables were recorded for controls except for those relating to IPD. All variables were collected using a single questionnaire for cases and controls and there was an instruction manual to facilitate compliance.

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Table 1
Distribution of serotypes causing invasive pneumococcal disease according to age.

	7–23 Months		24–59 Months		7–59 Months	
	No.	%	No.	%	No.	%
Vaccine serotypes	14	15.1%	10	5.6%	24	8.9%
9V	0	0%	1	0.6%	1	0.4%
14	6	6.5%	6	3.4%	12	4.4%
18C	1	1.1%	0	0%	1	0.4%
19F	5	5.4%	2	1.1%	7	2.6%
23F	2	2.2%	1	0.6%	3	1.1%
Non-vaccine serotypes	79	84.9%	168	94.4%	247	91.1%
1	5	5.4%	57	32.0%	62	22.9%
3	16	17.2%	21	11.8%	37	13.7%
5	0	0%	7	3.9%	7	2.6%
6A	3	3.2%	1	0.6%	4	1.5%
7F	6	6.5%	10	5.6%	16	5.9%
10A	1	1.1%	0	0%	1	0.4%
15A	1	1.1%	1	0.6%	2	0.7%
19A	27	29.0%	14	7.9%	41	15.1%
22F	0	0%	1	0.6%	1	0.4%
23B	2	2.2%	0	0%	2	0.7%
24	1	1.1%	0	0%	1	0.4%
24F	1	1.1%	0	0%	1	0.4%
38	1	1.1%	0	0%	1	0.4%
Non-typeable	15	16.1%	56	31.5%	71	26.2%
Total	93	34.3%	178	65.7%	271	100%

2.6. Sample size

The sample size required was calculated using Schlesselman's criteria [29]. Assuming a prevalence of history of vaccination in controls of 25% (data from Catalonia before the start of the study), a vaccination effectiveness of 80%, a bilateral α error of 0.05 (two-tailed), a β error of 0.2, and supposing that 20% of cases would be caused by vaccine serotypes (preliminary data), that three controls would be sought per case, and that children aged <2 years and those aged 24–59 months would be analyzed separately, the minimum number of cases required was estimated at 270 and the number of controls as 810.

2.7. Statistical analysis

Differences in demographic and epidemiological variables between cases and controls were analyzed using Pearson's chi-square test for categorical variables and the Student t-test for continuous variables. A two-tailed distribution was assumed for all p-values. The crude odds ratio (OR) and their 95% confidence intervals (CI) taking into account the distribution of completely vaccinated children and unvaccinated children and IPD were estimated using McNemar's chi-square test.

To avoid the effect of possible confounding variables, multivariate analysis was performed using conditional logistic regression and including independent variables found to be associated with both the disease and vaccination with a cut-off point of $p < 0.1$ in the bivariate analysis. The variable age was also included due to its relevance.

Vaccination effectiveness (VE) was calculated using the formula $VE = (1 - OR) \times 100$. Analyses were performed for vaccine and non-vaccine serotypes and age group. The statistical power of the analyses made was calculated using Schlesselman's formula [29].

The analysis was performed using the SPSS v18 statistical package.

2.8. Data confidentiality and ethical aspects

All data was treated as confidential in accordance with legislation on observational studies. Because there was no intervention, and the health care provided to cases and controls was the same whether parents agreed to participate or not, cases and controls were enrolled if a parent or guardian provided oral informed consent. The Institute of Health Studies, Generalitat of Catalonia and the Ethics Committee, Fundació Hospital Sant Joan de Déu approved the study.

3. Results

During the study period, 293 cases of IPD were detected in patients aged 7–59 months, of which 271 were included. The remaining cases were excluded because the vaccination history was not determined (2 cases), no controls meeting the study criteria were found (1 case) or the sample for serotyping was not available (19 cases).

Of the 271 cases, 98 (36.2%) were diagnosed by culture and real-time PCR and 173 (63.8%) by real-time PCR only. 8.9% were caused by vaccine serotypes and 91.1% by non-vaccine serotypes (Table 1). The most-frequent serotypes were 1 (22.9%), 19A (15.1%) and 3 (13.7%). No changes occurred in the real-time PCR methodology during the study period.

A total of 65.7% of cases were aged 24–59 months and 34.3% were aged <24 months (Table 1). The most-frequent serotypes identified were 1 (32.0%), 3 (11.8%) and 19A (7.9%) in the 24–59 months age group and 19A (29.0%), 3 (17.2%) and 14 (6.5%) in the 7–23 months age group.

The most frequent clinical forms were pneumonia with empyema (59% of cases), pneumonia without empyema (26.2%), meningitis (7.4%) and non-focal bacteremia or sepsis (5.9%) (Table 2). Other clinical forms were cellulitis (2 cases) and osteoarticular infection (2 cases). Pneumonia with empyema increased from 37 cases in 2007 to 65 in 2009. No increase was observed in the remaining clinical forms. The most-frequent serotypes were 1 (47 cases), 3 (28 cases) and 19A (27 cases) in cases of pneumonia with empyema and serotype1 (15 cases) and serotype 3 (8 cases) in cases of pneumonia without empyema. In cases of meningitis and non-focal bacteremia or sepsis the most frequent serotype was 19A (3 cases and 4 cases, respectively).

Of the 751 possible controls aged 7–59 months selected, 747 were finally included. The remaining 4 children were excluded because the vaccination history was not determined. Cases presented similar characteristics to controls, with the exception of

Table 2
Distribution of invasive pneumococcal disease-causing serotypes according to age and clinical form.

	Pneumonia without empyema		Pneumonia with empyema		Meningitis		Non-focal bacteremia/sepsis	
	7–23 months No. (%)	24–59 months No. (%)	7–23 months No. (%)	24–59 months No. (%)	7–23 months No. (%)	24–59 months No. (%)	7–23 months No. (%)	24–59 months No. (%)
Vaccine serotypes	2 (10.0%)	6 (11.8%)	3 (7.5%)	2 (1.7%)	4 (25.0%)	1 (25.0%)	5 (38.5%)	1 (33.3%)
9V	0 (0.0%)	1 (2.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
14	2 (10.0%)	4 (7.8%)	3 (7.5%)	2 (1.7%)	0 (0.0%)	0 (0.0%)	1 (7.7%)	0 (0.0%)
18C	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
19F	0 (0.0%)	1 (2.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	1 (25.0%)	4 (30.8%)	0 (0.0%)
23F	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (12.5%)	0 (0.0%)	0 (0.0%)	1 (33.3%)
Non-vaccine serotypes	18 (90.0%)	45 (88.2%)	37 (92.5%)	118 (98.3%)	12 (75.0%)	3 (75.0%)	8 (61.5%)	2 (66.7%)
1	1 (5.0%)	14 (27.4%)	4 (10.0%)	43 (35.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
3	6 (30.0%)	2 (3.9%)	10 (25.0%)	18 (15.0%)	0 (0.0%)	1 (25.0%)	0 (0.0%)	0 (0.0%)
5	0 (0.0%)	2 (3.9%)	0 (0.0%)	4 (3.3%)	0 (0.0%)	1 (25.0%)	0 (0.0%)	0 (0.0%)
6A	0 (0.0%)	1 (2.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	1 (7.7%)	0 (0.0%)
7F	1 (5.0%)	4 (7.8%)	2 (5.0%)	5 (4.1%)	2 (12.5%)	0 (0.0%)	1 (7.7%)	1 (33.3%)
10A	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
15A	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	1 (25.0%)	0 (0.0%)	0 (0.0%)
19A	4 (20.0%)	1 (2.0%)	14 (35.0%)	13 (10.8%)	3 (18.7%)	0 (0.0%)	4 (30.8%)	0 (0.0%)
22F	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (33.3%)
23B	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	1 (7.7%)	0 (0.0%)
24	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
24F	0 (0.0%)	0 (0.0%)	1 (2.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Non-typeable	6 (30.0%)	21 (41.2%)	6 (15.0%)	35 (29.2%)	2 (12.5%)	0 (0.0%)	1 (7.7%)	0 (0.0%)
Total	20 (7.5%)	51 (19.1%)	40 (15.0%)	120 (44.9%)	16 (6.0%)	4 (1.5%)	13 (4.9%)	3 (1.1%)

greater day care or school attendance in cases compared with controls ($p < 0.01$) and more cohabitants in cases than in controls ($p = 0.003$). Complete vaccination was lower in cases aged 7–23 months than in children aged 24–59 months (41.9% and 47.8%, respectively; $p = 0.035$) (Table 3).

After adjusting for age, vaccination effectiveness (complete vaccination schedule) was 93.7% (95% CI 51.8–99.2) for vaccine serotypes. There was no protection against IPD caused by non-vaccine serotypes (Table 4).

Vaccination effectiveness was higher in the 7–23 months age group than in the whole study group. In the 24–59 months age group, no statistically significant differences were found (Table 4).

4. Discussion

As in most countries [30], only a few of the 93 known *S. pneumoniae* serotypes cause IPD in children aged <5 years in Catalonia. In our study, 67.3% of all cases were caused by 7 serotypes (14, 19 F, 19 A, 1, 3, 5, and 7F) (Table 1). However, after the PCV7 was marketed in Catalonia, the proportion of cases caused by PCV 7serotypes has fallen substantially, from 76.2% in 1999 [8] to 8.9% during the study period.

Although only 47% of controls were completely vaccinated, the marked reduction found in cases of IPD due to vaccine serotypes suggests that herd protection has played some role in Catalonia.

The predominant clinical form was pneumonia with empyema which is mostly caused by non-vaccine serotypes and is increasingly frequent in Spain, with Calbo et al. [11] finding that the rate rose from 1.7 per 100,000 in the prevaccination era (1999–2001) to 8.5 in the postvaccination era (2002–2004), a five-fold increase, and Muñoz-Almagro et al. [12] finding that cases rose seven-fold between 1997–2001 and 2002–2006. Our results show that, in the 36-month study period, cases of pneumonia with empyema rose from 37 in 2007 to 65 in 2009, a phenomenon that has occurred to a lesser degree in the United States [31], but not in England [32]. Because no changes in diagnostic methods occurred during the study period, this increase can be considered as real and not a consequence of more diagnoses made by PCR.

The fact that three doses of PCV7 in infants aged <1-year old were required to consider a case or control as completely

Table 3
Characteristics of cases and controls.

Characteristic	Cases N = 271	Controls N = 747	p-Value
Months, median (range)	33.0 (7–59)	31.0 (7–59)	0.69[*]
Gender			0.57[†]
Male	145 (53.5%)	414 (55.5%)	
Female	126 (46.5%)	332 (44.5%)	
Period of hospitalization			0.16[†]
January–March	86 (31.7%)	263 (35.3%)	
April–June	55 (20.3%)	180 (24.1%)	
July–September	21 (7.7%)	59 (7.9%)	
October–December	109 (40.3%)	244 (32.7%)	
Underlying disease			0.81[†]
Yes	3 (1.1%)	7 (0.9%)	
No	268 (98.9%)	739 (99.1%)	
Social class			
I– III	143 (59.6%)	333 (57.7%)	0.62[†]
IV–V	97 (40.4%)	244 (42.3%)	
Attendance at day care or school			<0.01[†]
Yes	226 (86.3%)	550 (73.8%)	
No	36 (13.7%)	195 (26.2%)	
Antibiotic treatment in previous month			0.11[†]
Yes	41 (15.5%)	148 (19.9%)	
No	224 (84.5%)	596 (80.1%)	
Infection in previous month			0.71[†]
Yes	127 (48.1%)	348 (46.8%)	
No	137 (51.9%)	396 (53.2%)	
Breastfeeding			0.28[†]
Yes	197 (75.5%)	523 (72.0%)	
No	64 (24.5%)	203 (28.0%)	
Exposure to tobacco in the home			0.32[†]
Yes	106 (40.6%)	313 (44.1%)	
No	155 (59.4%)	396 (55.9%)	
Cohabitants, mean (range)	4.02 (2–10)	3.81 (2–9)	0.003[*]
Siblings			0.29[†]
Yes	139 (53.3%)	421 (57.0%)	
No	122 (46.7%)	317 (43.0%)	
Siblings < 5 years of age			0.12[†]
Yes	76 (29.1%)	191 (25.9%)	
No	185 (70.9%)	547 (74.1%)	
Complete vaccination			
7–23 months	39 (41.9%)	150 (55.1%)	0.03[†]
24–59 months	85 (47.8%)	201 (42.3%)	0.22[†]
7–59 months	124 (45.8%)	351 (47.0%)	0.71[†]

[*] Student *t*-test.
[†] Pearson's chi-square test.

Table 4
Adjusted effectiveness of the 7-valent pneumococcal conjugate vaccination in the prevention of invasive pneumococcal disease in completely vaccinated children aged 7–59 months.[a]

Serotype	Cases	Controls	Crude vaccination effectiveness			Adjusted vaccination effectiveness[b]			
	vaccinated/N (%)	vaccinated/N (%)	%	95% CI	p value	%	95% CI	p value	Power
All serotypes	124/251 (49.4%)	351/658 (53.3%)	16.1%	−13.5–37.9	0.25	13.2%	−20.7–37.6	0.40	12.6%
Vaccine serotypes									
7–23 Months	1/14 (7.1%)	24/40 (60.0%)	92.3%	38.1–99.0	0.02	92.5%	39.3–99.1	0.02	98.5%
24–59 Months	3/9 (33.3%)	12/21 (57.1%)	79.2%	−84.7–97.7	0.16	79.4%	−84.0–97.7	0.16	40.5%
7–59 Months	4/23 (17.4%)	36/61 (59.0%)	93.8%	51.9–99.2	0.01	93.7%	51.8–99.2	0.01	99.8%
Non-vaccine serotypes	120/228 (52.6%)	315/597 (52.8%)	−8.0%	−56.4–25.4	0.68	−10.9%	−65.6–25.7	0.61	18.9%

[a] Incomplete vaccinated children were excluded from the analysis.
[b] Adjusted using conditional logistic regression for attendance at day care or school (all serotypes and non-vaccine serotypes), cohabitants (all serotypes and non-vaccine serotypes) and age (all serotypes, vaccine serotypes in all age groups and non-vaccine serotypes).

vaccinated is important, because the benefits of this schedule against a reduced-dose regimen have been reported recently [33].

The adjusted effectiveness of the complete vaccination schedule was high against IPD caused by vaccine serotypes (93.7%, 95% CI 51.8–99.2) and was not effective against non-vaccine serotypes (−10.9%, 95% CI −65.6–25.7).

The point effectiveness of the complete vaccination schedule against cases caused by vaccine serotypes was similar to that observed in case-control studies by Whitney et al. [20] (96%; 95% CI 93–98), Deceuninck et al. [21] (92%; 95% CI 83–96), and Barricarte et al. [22] (88%; 95% CI 9–98), and in indirect cohort studies by Mahon et al. [23] (90.5%; 95% CI 17.7–98.9), and Rückinger et al. [24] (94.6%; 95% CI 69.7–99.5). The confidence intervals of these studies overlap with ours.

The study of effectiveness according to age showed that, in children aged 7–23 months, the vaccine was very effective in preventing cases due to vaccine serotypes when the complete vaccination schedule was administered (92.5%; 95% CI 39.3–99.1). In the 24–59 months age group, there was a non-significant trend to protection (79.4%; 95% CI −84.0–97.7). However, the statistical power of the study was only 40.5% in this age group due to the small sample size (only 10 cases of IPD due to vaccine serotypes). The fact that a lower proportion of cases due to vaccine serotypes was found in children aged > 24 months is probably related to the higher vaccine coverage found in this age group with respect to the 7–23 months age group (47.8% and 41.9, respectively). Another possible reason is the higher proportion of cases due to serotype1 reported in children aged > 24 months in Catalonia [34].

The small number of vaccine serotype cases included in the study did not confer enough power to analyze vaccination effectiveness according to clinical form, number of doses received, single serotype or risk medical conditions.

The PCV7 reduces the prevalence of carriers of vaccine serotypes and, possibly, of serotype GA [35]. Since nasopharyngeal carriers of S. pneumoniae are the main source of IPD, this reduction results in herd immunity, indirectly protecting unvaccinated people. In case-control studies matched by hospital and date of admission, such as our study, patients and controls probably have the same probability of benefiting from indirect protection. In fact, this type of study primarily measures direct protection, and other designs are necessary to estimate indirect protection [36]. The Centers for Disease Control and Prevention made this estimate in the United States using epidemiological disease surveillance and concluded that indirect protection prevented more cases (20,459) than direct protection (9149) in the four years after introduction of the vaccine [37].

Our study, like all observational studies, has strengths and weaknesses. One strength is that the study design and methodology were intended to minimize potential selection and information biases. Selection bias was minimized by matching cases and controls and by the diagnostic techniques used for cases. The age, sex, risk medical conditions, hospital and the date of admission or outpatient visit were used for matching. Social class was not used for matching, but no differences were observed between cases and controls.

Diagnosis using cultures and PCR increases the capacity to detect IPD [12,38]. If PCR had not been used, two-thirds of cases would not have been detected, and the cases included would not have been representative of hospitalized cases of IPD and could have skewed the study results.

The possibility of information bias was minimized collecting information on vaccination status from personal health records (vaccination card, health card, medical history or the primary healthcare center register). Although investigators collecting this information were not blinded to the status of each case or control, vaccination histories were always collected according to this recorded information.

Likewise, adjustment of age and other variables that differed between cases and controls by conditional logistic regression helped minimize potential confounding.

The sample size is another strength. Only the study by Whitney et al. [20] included a greater number of cases and controls.

The main weakness of our study is the low proportion of cases (8.9%) caused by vaccine serotypes, which made it difficult to obtain significant results in the analysis by specific subgroups. However, this variable could not be controlled.

Our results suggest that the recently licensed conjugate vaccines will increase coverage of IPD-causing serotypes in Catalonia from the 8.9% covered by the PCV7 to 40.3% for the 10-valent vaccine (incorporating serotypes 1, 5 and 7F) and 70.6% for the 13-valent vaccine (additionally incorporating serotypes 3, 6A, and 19A).

In summary, our results confirm those of other observational studies showing high levels of effectiveness of complete vaccination with PCV7 in the prevention of IPD caused by vaccine serotypes in children aged 7–59 months in a population with intermediate vaccination coverages. The small number of cases due to vaccine serotypes in children aged 24–59 months substantially reduced the power of the study in this age group, and explains why the protection observed was not statistically significant.

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lates, based on the meropenem susceptibility test result and the use of meropenem disks supplemented with APB, cloxacillin or DPA. These tests will enable routine laboratories to identify, with high confidence levels, those *P. aeruginosa* isolates suspected of producing either KPC or MBL carbapenemases.

Transparency Declaration

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Streptococcus pneumoniae serotype 1 causing invasive disease among children in Barcelona over a 20-year period (1989–2008)

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Abstract

Fifty-six isolates of serotype 1 were identified during a 20-year prospective study (1989–2008), including all children with culture-proven invasive pneumococcal disease (IPD) admitted to a children's hospital in Barcelona. Forty-eight of them (85.7%) were in children aged >2 years. Complicated pneumonia ($n = 28$) and non-complicated pneumonia ($n = 20$) were the main clinical presentations. The frequency of serotype 1 IPD increased from 1999–2003 to 2004–2008: 1.2 to 4.4 episodes/100 000 children (p <0.001). The ST306 clone were identified in 70.4% of isolates. As IPD caused by serotype 1 is mainly detected in older children, a vaccination programme for children >2 years should be considered.

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Invasive pneumococcal disease (IPD) is associated with high rates of morbidity and mortality in children. Serotype 1 is one of the most prevalent invasive serotypes of *Streptococcus pneumoniae*. In recent years, several studies have found an increase in IPD caused by serotype 1 in different countries [1–5].

In this study, we analysed changes in the epidemiology of serotype 1 among children in Barcelona, and determined the clonal composition of invasive strains of serotype 1.

We carried out a 20-year prospective study that included all children and adolescents aged <18 years with IPD who were admitted to Sant Joan de Déu Hospital.

This is a 345-bed children's teaching hospital located in Barcelona that was responsible for 18.5% of all paediatric hospitalizations in Catalonia, Spain in1999–2003 and 18.3% in 2004–2008. A detailed description of the methodology used in this study has been reported elsewhere [6].

IPD was defined as the presence of clinical findings of infection together with isolation of *S. pneumoniae* in a blood sample, cerebrospinal fluid sample, or any other sterile fluid sample. Pneumococcal strains were serotyped by the Quellung reaction, and clonal analysis was performed by multilocus sequence typing (MLST).

For statistical analysis, we used the chi-square test or Fisher's exact test, when appropriate, to compare proportions, and Student's *t*-test to compare means. Rates of IPD (episodes/100 000 population) were calculated from the annual estimates of the paediatric population obtained from the Department of Statistics in Catalonia [7].

A total of 347 episodes of IPD were identified. Fifty-six of 344 episodes (16.3%) with the serotype available were caused by serotype 1. According to age group, eight episodes (14.3%) caused by serotype 1 were in children aged <2 years, 19 episodes (33.9%) were in children aged 2–4 years, and 29 episodes (51.8%) were in children and adolescents aged 5–17 years. Table 1 shows the characteristics of patients with IPD caused by serotype 1 vs. other serotypes.

On comparison of the proportion of serotype 1 with respect to the total episodes of IPD, there was a statistically significant increase in serotype 1 throughout the study period: one of 51 episodes (2%) in 1989–1993; five of 58 episodes (8.6%) in 1994–1998; 11 of 74 episodes (14.9%) in 1999–2003; and 39 of 164 episodes (23.8%) in 2004–2008 (chi-square test for trend, p <0.001).

Fig. 1 shows the increase in incidence of IPD among children throughout the study by age group. In addition, a statistically significant increase was observed on comparison of the rates of serotype 1 per 1000 hospital admissions between 1999–2003 and 2004–2008 (13 vs. 45.1 per 1000

TABLE 1. Clinical manifestations of invasive pneumoccocal disease (IPD) in children

	Episodes (%), serotype 1 (n = 56)	Episodes (%), other serotypes (n = 288)	p-value
Age (months), mean (±SD)	62.3 (42.0)	34.5 (39.2)	p <0.001[d]
Sex			
Female	27 (48.2)	115 (39.9)	NS; p 0.44[e]
Male	29 (51.8)	173 (60.1)	
PCV7 vaccination[a]	5 (9.4)	25 (9.4)	NS; p 0.78[e]
Clinical manifestations of IPD[b]			
Meningitis	1 (1.8)	70 (24.5)	p <0.001[e]
Pneumonia (overall)	48 (85.7)	77 (26.9)	
With empyema	28	40	
Without empyema	20	37	
Bacteraemia/sepsis	5 (8.9)	100 (34.9)	
Appendicitis	2 (3.6)	9 (3.1)	
Arthritis	0	28 (9.8)	
Others	0	2 (0.7)	
PICU admission[c]	3 (5.3)	72 (27.6)	p <0.001[f]
Mortality	0	13 (4.5)	NS; p 0.1[f]

NS, not significant; PCV7, heptavalent pneumococcal conjugate vaccine; PICU, paediatric intensive care unit; SD, standard deviation.
[a]PCV7 vaccination status with was available in 319 patients (53 with serotype 1 and 266 with other serotypes).
[b]Clinical manifestations of IPD were known in 342 patients (56 with serotype 1 and 286 with other serotypes).
[c]PICU admission status was available in 316 patients (56 with serotype 1 and 260 with other serotypes).
For statistical analysis, we used the chi-square test or Fisher's exact test, when appropriate, to compare proportions, and Student's *t*-test to compare means: [d]Student's *t*-test, [e]chi-square test, [f]Fisher's exact test.

admissions; Fisher's-exact test, p <0.001) and comparison of the rates of serotype 1 per 1000 blood cultures between the two periods (12.8 vs. 79.2 per 1000 blood cultures; Fisher's-exact test, p <0.001).

According to the meningeal breakpoints of the CLSI [8], all serotype 1 isolates were susceptible to penicillin and cefotaxime during the study period, but three strains (7.7%) detected in 2004–2008 were resistant to erythromycin, and two (5.1%) were also resistant to tetracycline and chloramphenicol.

Molecular analysis by MLST was performed in 55 of 56 (98.2%) serotype 1 isolates. Overall, there were five different sequence types (STs) expressing serotype 1, including three PMEN clones: ST306 (Sweden [1]-28) in 39 isolates (71%), ST304 (Sweden [1]-40) in 11 isolates (20%), and ST217 (Sweden [1]-27) in one isolate (1.8%). In addition, ST228, which is a double-locus variant of Sweden [1]-28, was detected in three isolates (5.5%), and a new MLST profile, ST2376, which is a single-locus variant of Sweden [1]-28, was detected in one isolate. Serotype 1 was detected for the first time in 1994–1998, and the first STs detected were ST304 and ST228. All ST306 isolates were detected after January 2000.

Some studies have suggested that certain surface or subsurface proteins of pneumococci may contribute significantly to the pathogenesis and virulence of some strains [9]. One recently identified pneumococcal virulence determinant is the pneumococcal serine-rich repeat protein (PsrP). Animal models
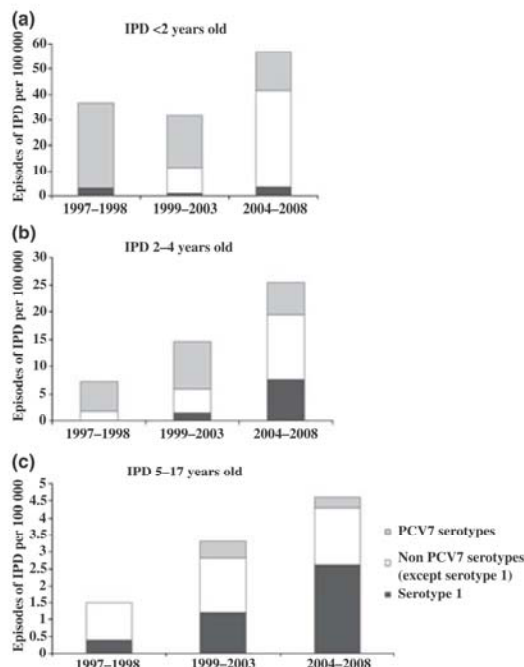
FIG. 1. Incidence of invasive pneumococcal disease (IPD) among children throughout the study period by age group. On comparison of the pre-vaccine period, the early heptavalent pneumococcal conjugate vaccine (PCV7) period (1999–2003) and the late PCV7 period (2004–2008), there was no statistically significant increase in serotype 1 in children aged <2 years (1 vs. 3.5 per 100 000 population; Fisher's exact test, p 0.1), a statistically significant increase in serotype 1 was observed in children aged 2–4 years (1.5 vs. 7.5 per 100 000 population; Fisher's exact test, p 0.010), and there was a trend for an increase in children and adolescents aged 5–17 years (1.2 vs. 2.6 per 100 000 population; Fisher's exact test, p 0.050).

have shown that PsrP mediates attachment to lung cells [10,11]. In addition, we recently reported that PsrP is highly prevalent in children with pneumonia and is strongly associated with clonal type. In that study, PsrP was detected in all ST306 isolates studied (n = 88) [12]. Therefore, the introduction of ST306 in our geographical area was associated with the introduction of this virulence factor that could be related, in part, to an increased ability of pneumococci to cause pneumonia.

Overall, younger children are a higher risk for IPD, owing to the high rate of pneumococcal nasopharyngeal colonization. However, serotype 1 is rarely found in the nasopharynx, and it is a serotype with a low rate of colonization [13]. Nevertheless, in our series, the incidence of serotype 1 was significantly higher in older children than in younger children (<2 years). This observation has also been reported by other authors [14]. The low prevalence of serotype 1 in carriers

could also be an explanation for the low rate of antibiotic resistance and the relative stability of clonal composition. We have detected five different STs, which confirms the high homogeneity of this serotype described by other authors [13] in comparison with the high diversity of other serotypes, such as serotype 19A [15].

The presence of some clones in a specific geographical area depends on several factors, such as the characteristics of the people and social factors in that community and/or the virulence of the clonal types [16]. Other authors have reported that ST306 is a predominant clone in continental Europe [5,17,18], whereas other STs, such as ST217, are more common in North America, England, Canada, and Gambia [14,19].

In conclusion, our study shows an increase in IPD caused by serotype 1 among children in Barcelona. Implementation of the new conjugate vaccines that include serotype 1 is urgently needed to reduce the burden of IPD. As IPD caused by serotype 1 is mainly detected in older children, vaccination programmes for children aged >2 years should also be considered.

References

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