Decline and Rise of the Antimicrobial Susceptibility of *Streptococcus pneumoniae* Isolated from Middle Ear Fluid in Children: Influence of Changes in Circulating Serotypes

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Changes in the antimicrobial susceptibility of *Streptococcus pneumoniae* causing otitis media were studied in 916 isolates from children <5 years old between 1999 and 2010 in a region of northern Spain. The rate of antimicrobial resistance decreased between the period before the introduction of the heptavalent pneumococcal conjugate vaccine (from 1999 to 2001) and the period from 2005 to 2007. However, in 2008 to 2010, resistance rates increased again due to the spread of serotype 19A, especially the multidrug-resistant ST320 and ST276 clones.

Acute otitis media (AOM) is one of the most common infections during childhood, and nearly all children have experienced an episode of AOM by the age of 5 years (4, 10). *Streptococcus pneumoniae* is the most important bacterial agent causing AOM.

The aims of this work were to analyze changes in the antimicrobial susceptibility of *S. pneumoniae* causing AOM in San Sebastián (Basque Country, northern Spain) before the introduction of the 13-valent pneumococcal conjugate vaccine (PCV13) and to assess the potential impact of the introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in 2001.

This study included all *S. pneumoniae* isolates obtained from children <5 years old with a diagnosis of AOM. Samples were collected from children after spontaneous middle ear drainage (>85% of samples) or after tympanic membrane puncture. In total, 28.9% episodes were diagnosed in children younger than 1 year old and 5.5% were diagnosed in children older than 3 years. The study was carried out between 1999 and 2010 and was divided into four periods: one prevaccine period, from 1999 to 2001, and three post-PCV7 periods (the early, intermediate, and late post-PCV7 periods). Pneumococci were identified by their colony morphology, optochin susceptibility, and bile solubility. Serotyping was performed with the Quellung reaction, a microarray technique using the PneumoArray system (Abyntek, Spain), and/or multiplex PCR.

MICs were determined by the broth microdilution assay according to Clinical and Laboratory Standards Institute guidelines (3). *S. pneumoniae* ATTC 49619 was used as a control. Tetracycline and macrolide resistance genes were detected as reported previously (8).

An isolate was considered multidrug resistant if it was nonsusceptible to oral penicillin (MIC > 0.06 µg/ml) and nonsusceptible to a further two or more antimicrobial classes. When a second episode was produced by an isolate with the same serotype and genotype in a period of more than 10 and fewer than 90 days, the episode was considered a relapse and the second isolate was not included in the present series. Genotyping was performed by pulsed-field gel electrophoresis (PFGE) according to previously described protocols (7) and by multilocus sequence typing

(MLST) according to the methodology described on the pneumococcal MLST website (www.mlst.net).

Between 1999 and 2010, a total of 916 pneumococci from 775 children were collected: 132, 234, 294, and 256 isolates were obtained in the periods from 1999 to 2001, 2002 to 2004, 2005 to 2007, and 2008 to 2010, respectively (Table 1).

The rate of intermediate or low-level penicillin resistance (penicillin MIC, 0.12 to 1 µg/ml) progressively decreased throughout the study period, from 35.6% in 1999 to 2001 to 16.0% in 2008 to 2010 ($P < 0.001$) (Table 1). The proportion of isolates for which the penicillin MIC was >1 µg/ml (indicating moderate and high-level resistance) also decreased between 1999 to 2001 and 2005 to 2007, from 15.2% to 5.1% ($P < 0.001$). However, this trend changed in the last period (2008 to 2010), when the rate of moderate and high-level penicillin resistance increased to 9.8% ($P = 0.04$). This increase was due to the presence of resistant isolates of non-PCV7 serotypes, especially of 19A isolates (Table 2).

Overall, there were 46 amoxicillin-resistant isolates (amoxicillin MIC, ≥ 4 µg/ml), of which 30 were serotype 19A. All serotype 19A amoxicillin-resistant isolates were multidrug-resistant sequence type 320 (ST320), for which MICs (in micrograms per milliliter) were as follows: penicillin, >1; erythromycin, >16; clindamycin, >0.25; tetracycline, >2; and trimethoprim-sulfamethoxazole, >1. As shown in Tables 1 and 2, MICs of cefotaxime were >1 for 21 isolates, of which 12 were serotype 19A. The remaining nine isolates were serotype 14 ($n = 6$), 19F ($n = 1$), 6A ($n = 1$), or 6B ($n = 1$).

The trend in erythromycin resistance was similar to that in moderate and high-level penicillin resistance. Overall, erythromycin resistance decreased from 49.2% in 1999 to 2001 to 27.9% in 2005 to 2007 ($P < 0.001$). However, from 2008 to 2010, erythromycin resistance

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TABLE 1 Numbers and percentages of pneumococcal isolates with diminished susceptibilities to antimicrobial agents

Agent or phenotype	MIC (μg/ml)	No. (%) of isolates ^a in period:				Total no. (%) of isolates ^a (n = 916)
		1999-2001 (n = 132)	2002-2004 (n = 234)	2005-2007 (n = 294)	2008-2010 (n = 256)	
Penicillin	0.12-1	47 (35.6)	46 (19.7)	51 (17.3)	41 (16.0)	185 (20.2)
	>1	20 (15.2)	24 (10.3)	15 (5.1)	25 (9.8)	84 (9.2)
	>2	3 (2.3)	1 (0.4)	2 (0.7)	5 (2.0)	11 (1.2)
Amoxicillin	>2	9 (6.8)	5 (2.1)	11 (3.7)	21 (8.2)	46 (5.0)
Cefotaxime	>1	7 (5.3)	1 (0.4)	3 (1.0)	10 (3.9)	21 (2.3)
Erythromycin	>0.25	65 (49.2)	96 (41.0)	82 (27.9)	93 (36.3)	336 (36.7)
Clindamycin	>0.25	58 (43.9)	93 (39.7)	73 (24.8)	86 (33.6)	310 (33.8)
Tetracycline	>2	57 (43.2)	89 (38.0)	68 (23.1)	88 (34.4)	302 (33.0)
SXT ^b	>0.5	68 (51.5)	77 (32.9)	75 (25.5)	61 (23.8)	281 (30.7)
	>1	53 (40.2)	49 (20.9)	52 (17.7)	45 (17.6)	199 (21.7)
Cloramphenicol	>4	9 (6.8)	7 (3.0)	0 (0.0)	1 (0.4)	17 (1.9)
Ciprofloxacin	>2	3 (2.3)	1 (0.4)	8 (2.7)	9 (3.5)	21 (2.3)
Multiresistance ^c		43 (32.6)	56 (23.9)	45 (15.3)	54 (21.1)	198 (21.6)

^a Number and percentage of isolates corresponding to the indicated MIC or phenotype among the number of isolates tested (n).

^b SXT, sulfamethoxazole-trimethoprim.

^c Multiresistance was defined as nonsusceptibility to penicillin and a further two or more antimicrobials.

increased again, reaching 36.3% ($P = 0.03$). This increase was due mainly to the increase in erythromycin-resistant non-PCV7 serotypes (accounting for >95% of erythromycin-resistant isolates) and especially to serotype 19A (68 [73.1%] of 93 erythromycin-resistant

isolates). The trend in tetracycline resistance was similar because more than 90% of isolates that were erythromycin resistant in the late post-PCV7 period carried the *ermB*, *tetM*, *int*, and *xis* genes and were tetracycline and clindamycin resistant.

TABLE 2 Changes in the susceptibility (nonsusceptibility) of isolates included in PCV7 and of serotype 19A isolates in one pre-PCV7 period (1999 to 2001) and three post-PCV7 periods

Isolate group	Agent or phenotype	MIC (μg/ml)	No. (%) of isolates ^b in period:				Total no. (%) of isolates ^b (n = 916)
			1999-2001 (n = 132)	2002-2004 (n = 234)	2005-2007 (n = 294)	2008-2010 (n = 256)	
Serotypes included in PCV7 ^a	Penicillin	0.12-1	34 (25.8)	26 (11.1)	14 (4.8)	1 (0.4)	75 (8.2)
	Penicillin	>1	17 (12.9)	18 (7.7)	5 (1.7)	0 (0.0)	40 (4.4)
	Penicillin	>2	2 (1.5)	1 (0.4)	0 (0.0)	0 (0.0)	3 (0.3)
	Amoxicillin	>2	8 (6.1)	3 (1.3)	2 (0.7)	0 (0.0)	13 (1.4)
	Cefotaxime	>1	6 (4.5)	1 (0.4)	1 (0.3)	0 (0.0)	8 (0.9)
	Erythromycin	>0.25	40 (30.3)	44 (18.8)	15 (5.1)	4 (1.6)	103 (11.2)
	Clindamycin	>0.25	37 (28.0)	41 (17.5)	13 (4.4)	4 (1.6)	95 (10.4)
	Tetracycline	>2	38 (28.8)	42 (17.9)	15 (5.1)	4 (1.6)	99 (10.8)
	SXT ^c	>0.5	48 (36.4)	35 (15.0)	22 (7.5)	1 (0.4)	106 (11.6)
	SXT	>1	43 (32.6)	27 (11.5)	20 (6.8)	0 (0.0)	90 (9.8)
	Chloramphenicol	>4	8 (6.1)	5 (2.1)	0 (0.0)	0 (0.0)	13 (1.4)
	Ciprofloxacin	>2	1 (0.8)	0 (0.0)	3 (1.0)	0 (0.0)	4 (0.4)
	Multiresistance ^d		28 (21.2)	33 (14.1)	7 (2.4)	1 (0.4)	69 (7.5)
Serotype 19A	Penicillin	0.12-1	9 (6.8)	14 (6.0)	21 (7.1)	27 (10.5)	71 (7.8)
	Penicillin	>1	2 (1.5)	5 (2.1)	10 (3.4)	24 (9.4)	41 (4.5)
	Penicillin	>2	0 (0.0)	0 (0.0)	2 (0.7)	5 (2.0)	7 (0.8)
	Amoxicillin	>2	0 (0.0)	0 (0.0)	9 (3.1)	21 (8.2)	30 (3.3)
	Cefotaxime	>1	0 (0.0)	0 (0.0)	2 (0.7)	10 (3.9)	12 (1.3)
	Erythromycin	>0.25	20 (15.2)	39 (16.7)	52 (17.7)	68 (26.6)	179 (19.5)
	Clindamycin	>0.25	18 (13.6)	39 (16.7)	48 (16.3)	67 (26.2)	172 (18.8)
	Tetracycline	>2	16 (12.1)	36 (15.4)	47 (16.0)	67 (26.2)	166 (18.1)
	SXT	>0.5	13 (9.8)	19 (8.1)	33 (11.2)	44 (17.2)	109 (11.9)
	SXT	>1	4 (3.0)	3 (1.3)	15 (5.1)	29 (11.3)	51 (5.6)
	Chloramphenicol	>4	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Ciprofloxacin	>2	1 (0.8)	1 (0.4)	2 (0.7)	3 (1.2)	7 (0.8)
	Multiresistance ^d		11 (8.3)	18 (7.7)	31 (10.5)	49 (19.1)	109 (11.9)

^a PCV7 serotypes: 4, 6B, 9V, 14, 18C, 19F, and 23F.

^b Number and percentage of isolates corresponding to the indicated MIC or phenotype among the number of isolates tested (n).

^c SXT, sulfamethoxazole-trimethoprim.

^d Multiresistance was defined as nonsusceptibility to penicillin and a further two or more antimicrobials.

All chloramphenicol-resistant isolates except three serotype 6A isolates and one serotype 5 isolate were PCV7 serotypes, and this resistance decreased throughout the study and has been absent since 2005. Most isolates (17 of 21) for which the MIC of ciprofloxacin was >2 $\mu\text{g/ml}$ were serotypes not included in PCV7, the most common serotypes being 19A (7 of 21 isolates) and serotype 3 (5 of 21 isolates).

Overall, there were 198 multiresistant isolates, of which 192 were erythromycin nonsusceptible. In the pre-PCV7 period (1999 to 2001), most multiresistant isolates (28 of 43; 65.1%) belonged to serotypes included in PCV7. However, beginning in 2005 to 2007, more than 90% of multidrug-resistant isolates were not serotypes included in PCV7, and 90.7% of multiresistant isolates (49 of 54) found in 2008 to 2010 were serotype 19A (Table 2). All but one serotype 19A multiresistant isolate found in 2008 to 2010 belonged to ST276 ($n = 24$) or ST320 ($n = 24$), and all carried the *ermB*, *tetM*, *int*, and *xis* genes. In addition, ST320 but not ST276 isolates carried the *mefE* gene. Isolates from only 15 children were serotype 6C, of which 11 isolates were detected in the 2008-to-2010 period. Ten isolates (66.7%) showed full susceptibility to all antibiotics tested. Erythromycin and tetracycline MICs for two isolates were >16 and 4 $\mu\text{g/ml}$, respectively, and one of them also showed diminished susceptibility to penicillin (MIC of 0.12 $\mu\text{g/ml}$). The MIC of penicillin for another 3 isolates was 0.12 $\mu\text{g/ml}$.

Since the introduction of PCV7 for the prevention of invasive pneumococcal disease, many researchers have reported a reduction in AOM cases in vaccinated and nonvaccinated children (2, 4) and an increase in the proportion of AOM caused by non-vaccine serotypes (2, 9).

In our study, we observed a dramatic reduction in the rates of PCV7 serotypes causing AOM, from 59.8% in the prevaccine period to 2.7% in 2008 to 2010. A Spanish study conducted in a similar time period found a comparable reduction: from 70.7% in 1997 to 2000 to 10.6% in 2009 (5).

The overall increase in antibiotic resistance in the last period, 2008 to 2010, was due mainly to the dissemination of two multidrug-resistant serotype 19A clones: ST276 and ST320. Of these two clones, only ST276 was already present in the pre-PCV7 period in the present series. Contrarily, ST320, a clone with worldwide distribution that is highly prevalent in Asia and the United States, was detected for the first time in this series in 2005, showing rapid and broad spreading among children. To date, ST320 has had limited diffusion among the Spanish adult population (1, 6),

but it will probably be highly prevalent soon unless the new vaccine PCV13 is able to contain its dissemination as the previous vaccine PCV7 did with other multidrug-resistant isolates.

No other clone showing high percentages of antimicrobial resistance was present in our region in the last years of this study. However, the threat of new resistant serotypes/clones arising after the introduction of PCV13 highlights the need for continued surveillance studies on pneumococcal serotypes causing AOM.

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