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Prevalence and molecular characterization of *Streptococcus pneumoniae* serotype 6C causing invasive disease in Gipuzkoa, northern Spain, 1990–2009

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Abstract The recently discovered pneumococcus serotype 6C was responsible for ten of the 1,530 invasive isolates studied between 1990 and 2009. These ten isolates belonged to seven sequence types (STs) and were isolated only from adult patients: six with bacteremia, three with meningitis, and one with peritonitis. All isolates but one were fully penicillin-susceptible.

Introduction

Streptococcus pneumoniae capsular serotype 6C was first described in 2007 [1] and, to date, there have been no descriptions of invasive pneumococcal disease (IPD) caused by this new serotype in a European region [2, 3]. Invasive disease caused by serotype 6C has previously been reported in North and South America [4–6], Australia [7], and Africa [8]. The importance of this serotype lies in its non-inclusion in all of the current pneumococcal vaccines,

its lack of immunological response to the homologous serotype 6B [9], and the penicillin and multidrug resistance found in some isolates [2, 5, 6]. More recently, isolation of the new serotype 6D from human carriers has also been described [10, 11]. The aim of this study was to identify the presence of these two new serotypes among IPD isolates in a European region (Gipuzkoa, northern Spain) in the last 22 years.

The study

Hospital Donostia is located in San Sebastián, the capital city of the province of Gipuzkoa, northern Spain. Between January 1990 and October 2009, 1,230 *S. pneumoniae* isolates causing IPD were recovered from sterile fluids (blood, cerebrospinal fluid, pleural fluid, etc.) in Hospital Donostia and another 300 invasive pneumococcal isolates sent from other hospitals in the province were serotyped with the Quellung reaction using rabbit polyclonal serogroup and serotype-specific antisera (Staten Serum Institute). Of them, 215 corresponded to isolates from children aged less than 15 years (93 between 1990 and 2001, and 122 between 2002 and 2009) and 1,315 to adults (599 between 1990 and 2001, and 716 between 2002 and 2009). Due to cross-reaction of serotypes 6C and 6D with serotypes 6A and 6B, respectively, all invasive isolates belonging to serogroup 6 were retyped using a recently described serotype-specific polymerase chain reaction (PCR) [10]. Serotype 6C isolates were further studied by multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). Antimicrobial susceptibility testing was performed by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) M100-S19 guidelines. The presence of the *ermB* and *mef*

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genes of macrolide resistance and SXT resistance was studied as previously described [12].

In children less than 15 years old, 17 cases of IPD caused by serotype 6B (11 between 1990 and 2001, and six since 2002) and five by serotype 6A (all of them after 2001) were diagnosed, while neither serotype 6C nor 6D were found among invasive isolates. Although in other studies serotype 6C has been frequently found in young children carriers [2], no serotype 6C invasive infections in this population age group was observed in our area; this low prevalence of serotype 6C IPD in children has also been observed in other studies [9].

In adults (>14 years old) between 1990 and 2001, serotypes 6A, 6B, and 6C caused 11, 29, and three cases of IPD, respectively; since 2002, these serotypes were responsible for 14, 12, and seven episodes, respectively. In adults, no serotype 6D was found throughout the entire study. The clinical picture of the ten patients with serotype 6C invasive infection consisted of three bacteremias, two of abdominal origin (cholangitis and cirrhosis), primary peritonitis in one, meningitis in three, and acute lower respiratory tract infections in three (pneumonia in two and acute exacerbation of chronic obstructive pulmonary disease [COPD] in one) (Table 1). Most serotype 6C isolates were susceptible to all of the antimicrobials tested (Table 1). One isolate showed low-level penicillin resistance (minimum inhibitory concentration [MIC]=0.12 mg/L) and the only erythromycin-resistant isolate harbored the *ermB* gene. The two trimethoprim-sulfamethoxazole-resistant isolates displayed the Ile-100 Leu change in the dihydrofolate reductase associated to trimethoprim resistance.

In our area, the first of the ten serotype 6C invasive isolates was detected in 1996, and five of the ten were isolated between 2007 and 2009 (Table 1). A similar slight increase in the last few years was found among adults in the USA, where 6C became the prevalent serogroup 6 serotype causing IPD in 2007 [5]. Although the heptavalent pneumococcal conjugate vaccine (PCV7) has not been shown to confer protection against pneumococcal serotype 6C or 6D [9, 11], we found no case of IPD caused by these new serotypes in children, despite the 11 cases of serogroup 6 (five 6A and six 6B) invasive infection recorded since the introduction of the vaccine in our country (June 2001). To estimate the presence of these serotypes among non-invasive isolates in our area, we studied 208 pneumococci detected in a carriage study (nasopharyngeal samples) in children aged less than 2 years between 2004 and 2005. Among them, 19 belonged to serogroup 6: three serotype 6A, 15 serotype 6B, and one serotype 6C. No serotype 6D isolates were found.

One study found the over-representation of meningitis caused by serotype 6C in IPD [8]; in the present small series, three meningitis cases were recorded. In contrast, three of the bacteremic cases were related to intra-

Table 1 Microbiological characteristics and clinical pictures of the ten serotype 6C isolates from invasive diseases

Code	Date	Sex	Age (years)	Specimen	Diagnosis	MLST	Clonal complex ^a	PFGE pattern ^b	Pen	Amo	Ctx	Tet	Eri	Cli	Cip	Lev	SXT
1	C96128	17-12-96	M	50	Peritoneal fluid	Primary peritonitis	ST2440	Singleton A	≤0.06 ^c	≤0.25	≤0.06	≤2	≤0.25	≤0.25	0.5	0.5	≥4/76
2	L736J	28-10-97	M	53	CSF	Meningitis	ST2440	Singleton A	≤0.06	≤0.25	≤0.06	≤2	≤0.25	≤0.25	2	1	≥4/76
3	H228104	20-06-00	M	70	Blood	Hepatic cirrhosis	ST1150	1	≤0.06	≤0.25	≤0.06	≤2	≤0.25	≤0.25	1	2	≤0.5/9.5
4	H229489	30-10-03	F	54	Blood	COPD exacerbation	ST1150	1	0.12	0.5	0.12	≤2	≤0.25	≤0.25	0.5	0.5	≤0.5/9.5
5	H225082	01-06-05	F	33	Blood	Cholangitis	ST1624	7	≤0.06	≤0.25	≤0.06	≤2	≤0.25	≤0.25	2	1	≤0.5/9.5
6	H228468	29-03-07	F	42	Blood	Septic shock non-Hodgkin's lymphoma	ST395	27	≤0.06	≤0.25	≤0.06	≤2	≤0.25	≤0.25	1	1	≤0.5/9.5
7	807536	18-05-07	F	87	Blood	Meningitis	ST1150	1	≤0.06	≤0.25	≤0.06	≤2	≤0.25	≤0.25	1	1	≤0.5/9.5
8	H224227	24-05-07	F	48	Blood	Pneumonia in non-Hodgkin's lymphoma	ST2667	1	≤0.06	≤0.25	≤0.06	≤2	≤0.25	≤0.25	1	1	≤0.5/9.5
9	H229777	27-03-09	M	66	CSF	Meningitis	ST4534	1	≤0.06	≤0.25	≤0.06	≤2	>32	>32	1	1	≤0.5/9.5
10	H229931	01-04-09	M	47	Blood	Pneumonia	ST4535	1	≤0.06	≤0.25	≤0.06	≤2	≤0.25	≤0.25	1	1	≤0.5/9.5

MLST multi-locus sequence typing; CSF cerebrospinal fluid; COPD chronic obstructive pulmonary disease; Pen penicillin; Amo amoxicillin; Ctx cefotaxime; Tet tetracycline; Eri erythromycin; Cip clindamycin; Cip ciprofloxacin; Lev levofloxacin; SXT trimethoprim-sulfamethoxazole

^a As determined by eBURST (<http://spneumoniae.mlst.net/eburst/>)

^b Isolates with a pulsed-field gel electrophoresis (PFGE) similarity pattern >80% but <100% were arbitrarily named with the same letter but different numbers

^c Minimum inhibitory concentration (MIC) in mg/L

abdominal diseases, an unusual situation in bacteremic *S. pneumoniae* infections.

The ancestral origin of the new serotype 6C is still unknown. Serotypes 6A and 6B capsular loci only differ from those of serotypes 6C and 6D in that serotypes 6A and 6B have the *wciN* gene and serotypes 6C and 6D the *wciN_β* gene [1, 10]. In addition, serotypes 6A and 6B capsular loci only differ consistently in one nucleotide of the *wciP* gene. In our area, the first serotype 6C causing IPD was detected years after the isolation of serotypes 6A and 6B, and two of the three predicted primary founders of serotype 6C clonal complexes belonged to serotype 6B. Jacobs et al. [4] also found two clusters of serotype 6C isolates closely related to serotype 6B strains, suggesting a capsular switching from serotype 6B to serotype 6C. If serotype 6B was the ancestral origin of serotype 6C, apart from gaining the *wciN_β* gene, another sequential or concomitant mutation in the *wciP* gene must have taken place, as, otherwise, serotype 6D would have been the serotype isolated. Like other authors [3, 5], we did not find the *wciN_β* gene among the 58 invasive serotype 6B isolates studied.

Overall, seven different sequence types (STs) were found in the ten serotype 6C isolates, including the two new ST4534 and ST4535, described for the first time in this study. All STs except one singleton (ST2440) could be grouped into clonal complexes 1, 7, and 27, whose predicted founders are ST176, ST90, and ST395 of serotypes 6B, 6B, and 6A, respectively. Only two of the STs found in our study have been previously described among serotype 6C isolates: ST1150 and ST395 from nasal carriers from Portugal [2] and ST395 among invasive isolates from the USA [5]. Indeed, most serotype 6C invasive isolates from North [4, 5] and South America [6] showed no similarity among each other or to our isolates, which is in the line of thinking that this newly discovered serotype probably emerged by shuttling of the *wciN_β* element within a pre-existing diverse genetic background, whether followed or not by additional changes.

By PFGE, five different clusters were defined among serotype 6C invasive isolates (Table 1). Curiously, eBURST revealed that ST2667 and ST1624 isolates, which share no common allele, belonged to the same clonal complex and were grouped by PFGE in the same arbitrarily named clone B (PFGE pattern similarity 96%).

Conclusion

Our study describes, for the first time, the presence and characteristics of *Streptococcus pneumoniae* serotype 6C isolates causing invasive disease in a European region, with a trend toward a slight increase in its incidence in the adult population. No serotype 6D was found among invasive

pneumococcal disease (IPD) isolates after 1990. Genotyping techniques showed two new sequence types (STs) within serotype 6C related to serotype 6B isolates included in the MLST database. Like other authors, we found that some of our isolates previously serotyped as 6A were, in fact, 6C [3, 4, 6, 8]. Whether serotype 6A strains included in the MLST database are the newly described serotype 6C or not should be determined in order to gain understanding on the ancestral origin of *S. pneumoniae* serogroup 6.

Conflict of interest All authors: no conflict.

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