Characterization of *Streptococcus pneumoniae* associated with invasive disease in children in Portugal (2015-2017)

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Abstract

Pneumococcal conjugate vaccines (PCVs) have been available in Portugal since 2001 and in 2015, PCV13 was introduced in the National Immunization Plan. Despite more than a decade of vaccine use, vaccine serotypes remained a major cause of paediatric invasive pneumococcal disease (IPD) in Portugal until 2015. In this work, *S. pneumoniae* associated with paediatric IPD in Portugal between 2015-2017 were characterized by phenotypic and molecular methods. The results were compared with those from the period of 2012-2015 to evaluate the effect of the vaccine in serotype distribution, antimicrobial resistance and clonal composition of the pneumococcal population. Serotype 3 remained the major cause of paediatric IPD (29.1%). Non-vaccine types were responsible for 50.7% of IPD, and the main NVTs found were serotypes 8, 10A and 15B/C. Non-susceptibility to penicillin and resistance to erythromycin were present in 13.2% and 16.5% of isolates, respectively. The MLST analysis of the isolates revealed a diverse population, with the most frequent CCs (CC156, CC63, CC180, CC460, CC393, CC433 and CC1262) accounting for 70.9% of IPD. Routine surveillance should continue to be performed in the future to evaluate the effects of PCV13 vaccination in serotype distribution, antimicrobial resistance and in the genetic population of *S. pneumoniae.*

Introduction

Streptococcus pneumoniae is an important human pathogen causing high mortality and morbidity worldwide [1].

The first pneumococcal conjugate vaccine (PCV7) which targets serotypes 4, 6B, 9V, 14, 18C, 19F and 23F and the 10-valent conjugate vaccine which includes PCV7 serotypes with the addition of serotypes 1, 5 and 7F were introduced in Portugal in 2001 and mid-2009 respectively [2,3]. The 13-valent conjugate vaccine PCV13 (including PCV10 serotypes and serotypes 3, 6A and 19A) has been available in Portugal since early 2010 [3]. However, it was only introduced in the National Immunization Plan (NIP) in 2015.

After the introduction of PCVs, there was a significant decrease on paediatric IPD caused by vaccine serotypes worldwide, including in Portugal [3-5], and an additional indirect (herd) effect has been observed in the older population [1,6].

The introduction of PCVs is known to affect not only serotype distribution, but also genotypes associated with IPD [7-9]. Given this, the study of serotype distribution, antimicrobial resistance and clonal composition of pneumococci is crucial to understand the dynamics of the pneumococcal population and the impact of conjugate vaccines.

National surveillance of IPD has been performed in Portugal since 1999 [2,3,10]. This study aims to evaluate the possible effects of PCV13 on serotype distribution, antimicrobial resistance and clonal composition of *S. pneumoniae* associated with paediatric IPD in Portugal from July 2015 to June 2017.

Materials and Methods

Bacterial isolates

The isolates included in this study were provided by the Portuguese Group for the Study of Streptococcal Infections and the Portuguese Study Group of Invasive Pneumococcal Disease of the Paediatric Infectious Disease Society, involving microbiology laboratories and paediatric departments of 61 hospitals throughout Portugal.

A case of IPD was defined as the isolation of *S. pneumoniae* from a normally sterile body site (only one isolate was considered per patient) or as the detection of pneumococcal DNA in pleural fluid or cerebrospinal fluid. Strains were identified as *S. pneumoniae* through colony morphology, haemolysis in blood agar, optochin susceptibility and bile solubility tests. In cases where the identification was made using molecular methods, two pneumococcal genes (*lytA* and *wzg*) were used for bacterial identification [11].

The isolates were recovered from patients under 18 years, in two epidemiological years, 2015-2016 and 2016-2017 in Portugal. Epidemiological years were defined as starting in week 26 of one year and ending in week 25 of the following year. Four different age groups were considered: infants aged less than 12 months, children aged 12–23 months, children aged from 2 to 4 years and children and adolescents from 5 to less than 18 years.

Antimicrobial susceptibility testing and MLST were performed in cases where an isolate was available.

Serotyping and antimicrobial susceptibility testing

Serotyping was performed by the Quellung capsular reaction using the chessboard system [12] and specific sera (Statens Serum Institut, Copenhagen, Denmark). In the cases where identification was made by molecular methods, serotyping was performed by rPCR (real time PCR) targeting 21 serotypes [11].

Antimicrobial susceptibility testing was performed using Etest strips (bioMérieux, Marcy-l'Étoile, France) to determine the minimal inhibitory concentration (MIC) to penicillin, cefotaxime, ceftriaxone, meropenem and levofloxacin (the last one was only performed when the isolate was resistant to norfloxacin using the Kirby-Bauer disk diffusion technique).

The recommended breakpoints for interpretation of MIC values for penicillin were changed in 2008, however in this case, the Clinical and Laboratory Standards Institute (CLSI) guidelines from 2007 were used to interpret the MIC values to allow comparison with the previous studies. Susceptibility to levofloxacin, norfloxacin, erythromycin, clindamycin, telithromycin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, vancomycin, linezolid and rifampicin was determined by the Kirby-Bauer disk diffusion technique using commercial disks (Oxoid, Hampshire, United Kindgom), according to the CLSI recommendations [13].

Macrolide resistance phenotypes were identified using a double disc test with erythromycin and clindamycin. The MLS_B phenotype is defined by resistance to both erythromycin and clindamycin (resistance to macrolides, lincosamides and streptogramin B) while the M phenotype is defined by resistance to erythromycin.

DNA extraction and sequencing

Pneumococcal DNA was extracted using the PureLink Genomic DNA Mini Kit

(ThermoFisher Scientific, Massachusetts, USA), according to the manufacturer's instructions. The purity of the DNA was evaluated using Nanodrop 2000 (ThermoFisher Scientific, Massachusetts, USA).

To check the quality of the pneumococcal DNA, an agarose gel was run and Qubit (Invitrogen by ThermoFisher Scientific, Massachusetts, USA) was used to check the DNA concentration.

Whole genome sequencing was performed at Instituto Gulbenkian de Ciência, Gene Express Unit (Oeiras, Portugal). Whole genome sequencing libraries were prepared using paired-end Nextera XT DNA Library Prep Kit, Index Kit v2 (Illumina, San Diego, CA, USA) and sequenced on Illumina NextSeq 500 system (Illumina) using NextSeq 500/550 Mid-Output v2 Kit (300 cycles). The quality of the 151 bps paired-end reads obtained was assessed with INNUca pipeline (https://github.com/B-UMMI/INNUca). INNUca v3.1 was run using Docker image "ummidock/innuca:3.1" (https://hub.docker.com/r/ummidock/innuca/) providing Nextera XT adapter sequences for adapters removal using a predicted genome size of 2.1 Mb. Briefly, the quality of the reads was checked with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and they were cleaned using Trimmomatic [14]. De novo assembly was performed using SPAdes [15] and subsequently, contigs were polished using Pilon [16]. The MLST type was determined for the final draft assembly through MLST software (https://github.com/tseemann/mlst).

Lineages were assigned using the goeBURST algorithm and the online pneumococcal MLST database (http://pubmlst.org/spneumoniae/). Results were visualized at PHYLOViZ [17]. Clonal complexes were defined at the single-locus-variant (SLV) level.

Statistical Analysis

Simpson's Index of Diversity (SID) was used to evaluate the population diversity.

The Odds ratio (OR) was calculated to evaluate possible associations between variables and the obtained p-values were corrected using the false discovery rate (FDR) correction for multiple testing [18]. The Cochran-Armitage test was used for trends. A p-value < 0.05 was considered significant for all tests.

Results

Bacterial Isolates

A total of 134 cases of paediatric invasive pneumococcal disease were reported between July 2015 and June 2017, with 91 isolates (67.9%) and 43 samples (32.1%). Regarding the source of the isolates, 70 were recovered from blood (76.9%), 13 from CSF (14.3%), 6 from pleural fluid (6.6%), 1 from peritoneal fluid (1.1%) and 1 from synovial fluid (1.1%). Among the cases detected by molecular methods, the majority were isolated from pleural fluid (n=40) and 3 were from CSF. The number of cases per epidemiological year remained constant with 64 from 2015-2016 and 70 from 2016-2017.

Among this collection, 27.6% (n=37) of the cases were from infants below 12 months of age, 15.7% (n=21) from children between 12 and 23 months, 30.6% (n=41) from children aged 2-4 years and 26.1% (n=35) from individuals aged 5-17 years.

Serotyping

Among this collection, 25 different capsular types were detected (SID=0.950, CI95%:

0.936-0.965). The most frequent serotype was serotype 3 (n=39), representing 29.1% of all cases.

When a serotype could not be determined or when the isolate was non-typable, the case was considered caused by a non-vaccine type (NVT). Non-vaccine types represented a significant proportion of cases (50.7%; n=68), of which 8 and 10A (n=10, 7.5% each), 15B/C (n=6, 4.5%), 22F (n=5, 3.7%), 11A, 29/35B and 33F (n=4, 3.0% each) were the most frequent (Figure 1).

Serotypes included in the PCV7 (4, 6B, 9V, 14, 18C, 19F and 23F) and PCV13 (PCV7 serotypes plus serotypes 1, 3, 5, 6A, 7F and 19A) constituted respectively 12.7% and 49.3% of the isolates and samples responsible for invasive pneumococcal disease in patients aged <18 years, in Portugal during the study period. Some serotypes included in the vaccines were absent from this collection (6A, 7F and 18C) when compared with the previous time period. Furthermore, serotype 14 which is included in PCV7, was present in 5 cases in 2015-2016 but absent from 2016-2017.

To evaluate possible trends in serotype distribution over time, the Cochran-Armitage test was used. When considering serotype variation from 2010 (year in which PCV13 was introduced in the private market in Portugal) to 2017 [3, unpublished data], only serotypes 1, 3, 7F and 19A showed a trend after FDR correction. More specifically, serotype 1 ($p < 0.001$), 7F ($p=0.008$) and 19A ($p=0.015$) decreased in incidence, while serotype 3 ($p < 0.001$) increased.

Antimicrobial susceptibility testing

Susceptibility to antimicrobials was tested among the 91 available isolates and it is resumed in Figure 2. No resistance to cefotaxime, ceftriaxone, meropenem, levofloxacin, telithromycin, vancomycin, linezolid and rifampicin was detected.

Overall, there were 12/91 (13.2%) isolates non-susceptible to penicillin, of which 7/12 (58.3%) were also resistant to erythromycin (EPNSP). If the current CLSI breakpoints for parenteral penicillin were considered [19], only 2/13 isolates from CSF would have been considered resistant to penicillin.

Resistance to erythromycin was found in 15/91 isolates (16.5%), of which 13 isolates (86.7%) expressed the $cMLS_B$ phenotype and 2/15 of the isolates (13.3%) the M phenotype. Resistance to norfloxacin was found in 3 isolates (3.3%) and resistance to tetracycline in 7 isolates (7.7%). All the tetracycline resistant isolates were resistant to both erythromycin and clindamycin ($cMLS_B$ phenotype).

Together, serotypes 6B and 14 contribute significantly to EPNSP (5/7, 58.3%) and serotypes 6B,14 and 19F to resistance to erythromycin (10/15, 66.7%). PCV13 serotypes represent 8/12 (66.7%), 10/15 (66.7%) and 6/7 (85.7%) of the penicillin non-susceptible, erythromycin resistant and EPNSP isolates, respectively.

When evaluating the association between serotype and penicillin non-susceptibility, no significant associations were observed. However, serotypes 6B, 14 and 23F presented a significant p-value before FDR correction ($p=0.045$, $p=0.007$, $p=0.045$ respectively). Regarding erythromycin resistance, there was a significant association with serotypes 6B ($p=0.048$ after FDR correction) and 14 ($p=0.013$ after FDR correction).

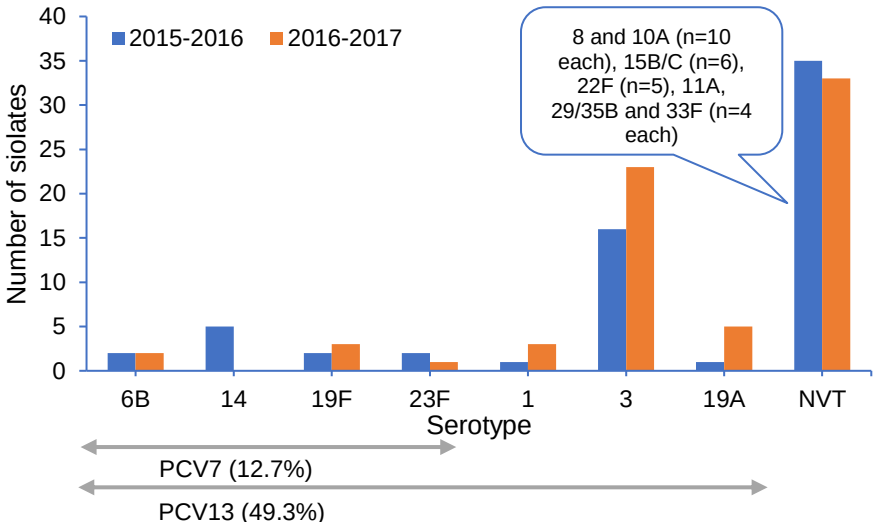

Figure 1 - Graphic representation of serotype distribution of *S. pneumoniae* isolates and patient samples causing children IPD in Portugal from July 2015 to June 2017. Serotypes included in PCV13 are indicated by an arrow and percentage of the number of isolates and samples expressing the serotypes included in the PCV13 vaccine is indicated. Among serotypes included in PCV13, serotypes 4, 5, 6A, 7F, 9V and 18C were not detected in this collection.

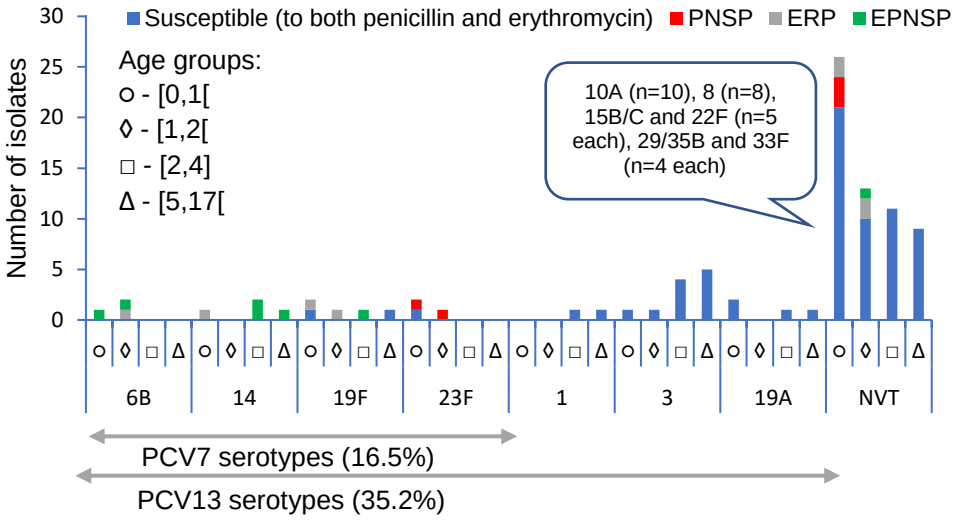

Figure 2 - Antimicrobial resistance of *S. pneumoniae* serotypes responsible for invasive pneumococcal disease in patients aged < 18 years, in Portugal from July 2015 to June 2017. PNSP: penicillin non-susceptible isolates; ERP: erythromycin resistant isolates; EPNSP: isolates presenting both erythromycin resistance and penicillin non-susceptibility; NVT- non-vaccine types.

Molecular characterization

The molecular characterization of the isolates by MLST revealed 47 different STs (SID=0.971, CI95%: 0.957-0.985) that grouped into 23 CCs (SID=0.908, CI95%: 0.875-0.940), after goeBURST analysis using all STs deposited in the database (http://pubmlst.org/spneumoniae/). The most frequent STs were ST180 (n=9, 10.5%), ST53 (n=8, 9.3%), ST97 (n=6, 7.0%), ST393 and

ST433 (n=4, 4.7% each) which together accounted for 36.0% of all isolates analysed.

Four new STs were detected and submitted to the *S. pneumoniae* MLST database (http://pubmlst.org/spneumoniae/). In two cases, there were new allele sequences, of which one was in *rec*P (427) and one in *xpt* (841) originating ST13864 and ST13863 respectively, while the remaining two cases were new allelic combinations, ST13669 and ST13811.

However, it should be mentioned that 3 isolates could not be analysed because they didn't grow in liquid media and 1 isolate with a new ST was sent to the curator of the MLST database for number attribution, but the ST hasn't been attributed yet.

The most prevalent CCs were CC156 (n=19, 22.1%), CC63 (n=12, 13.9%), CC180 and CC460 (n=9, 10.5 % each), CC393, CC433 and CC1262 (n=4, 4.7% each), which together represent 70.9% (n=61) of all isolates studied. Table 1 presents the serotypes of the STs in each CC.

The Cochran-Armitage test was used to evaluate ST variation from 2010 to 2017 (unpublished data). It was observed that ST53 (p=0.042), ST180 (p=0.033) and ST306 (p=0.044) which are associated to serotypes 8, 3 and 1, respectively, showed a trend after FDR correction. More specifically, ST53 and ST180 increased in prevalence, while ST306 decreased. Additionally, some STs presented a significant p-value before FDR correction: ST97 (p=0.006), ST191 (p=0.013), ST276 (p=0.015) and ST338 (p=0.033) that are associated to serotypes 10A, 7F, 19A and 23F respectively. More specifically, ST97 increased in prevalence and ST191, ST276 and ST338 decreased overtime.

Discussion

During the study period, the most frequent serotypes causing disease were serotypes 3 (n=39, 29.1%), 8 and 10A (n=10, 7.5% each) and 19A and 15B/C (n=6, 4.5% each), together accounting for 52.9% of IPD. When comparing with the 3-year period prior to the introduction of PCV13 in the NIP (2012-2015), the similarities were the persistence of serotype 3 as the major cause of IPD and the presence of vaccine serotype 19A and NVTs 10A and 15B/C among the most frequent serotypes. As for differences, PCV13 serotypes 14, 1, and 6B were also present in 2015-2017, however in lower prevalence when compared to 2012-2015, not being among the most frequent serotypes.

Certain serotypes that were expressed in Portugal between 2012-2015 were absent in 2015-2017, such as vaccine serotypes 6A, 7F and 18C and NVT 12B. The cases of serotype 7F and 12B are the most surprising, since both serotypes were among the most frequent causes of IPD in 2012-2015, but were absent in 2015-2017. The case of serotype 7F could be related to the vaccine effect since this serotype is included PCV13, therefore a decrease in incidence of IPD cases due to this serotype is to be expected. As for NVT 12B, it may be a serotype that had no success in dissemination. It should also be mentioned, that vaccine serotypes 1 and 19A, which were among the most frequent serotypes in 2012-2015, decreased in incidence when compared to 2015-2017, from 9.9% to 3.0% and from 5.6% to 4.5%, respectively. Furthermore, serotype 14, included in PCV7, was present in 5 cases in 2015-2016, but absent in 2016-2017. Routine surveillance should be performed in the future to ascertain if this trend is permanent or if this was just a temporary decrease.

Table 1 – Serotypes of the STs found among the 23 CCs identified by goeBURST.

CC (n)	ST	Total	Dominant serotype (n)	Other serotypes
CC156 (19)	**72**	2	24F (2)	
	143	2	14 (2)	-
	338	2	23A (1), 23F (1)	-
	8126	2	23F (2)	-
	66	1	9N (1)	-
	138	1	6B (1)	-
	162	1	24F (1)	-
	177	1	19F (1)	-
	271	1	19F (1)	-
	469	1	19F (1)	-
	1877	1	21 (1)	-
	2372	1	23B (1)	-
	4948	1	11A (1)	-
	13863	1	19F (1)	-
	13864	1	19F (1)	-
CC63 (12)	**53**	8	8 (8)	-
	62	1	11A (1)	-
	445	1	22F (1)	-
	673	1	33F (1)	-
	1012	1	33F (1)	-
CC180 (9)	**180**	9	3 (7)	10A (1) ,11A (1)
CC460 (9)	**97**	6	10A (4)	14 (1), 19A (1)
	461	1	10A (1)	-
	1551	1	10A (1)	-
	1635	1	35F (1)	-
CC393 (4)	**393**	4	25A/38 (3)	NT (1)
CC433 (4)	**433**	4	22F (4)	-
CC1262 (4)	**1262**	2	15B/C (2)	-
	8711	1	15B/C (1)	-
	9975	1	15B/C (1)	-
CC198 (3)	**198**	3	29/35B (3)	-
CC994 (3)	**994**	3	19A (3)	-
CC30 (2)	**30**	1	3 (1)	-
	12069	1	10A (1)	-
CC306 (2)	**306**	2	1 (2)	-
CC558 (2)	**558**	2	29/35B (1), NT (1)	-
CC717 (2)	**717**	2	33F (2)	-
CC1368 (2)	**4083**	2	10A (1), 34 (1)	-
CC15 (1)	**9**	1	14 (1)	-
CC199 (1)	**199**	1	15B/C (1)	-
CC315 (1)	**13669**	1	6B (1)	-
CC378 (1)	**232**	1	3 (1)	-
CC439 (1)	**9579**	1	23B (1)	-
CC896 (1)	**896**	1	15A (1)	-
CC1046 (1)	**1046**	1	34 (1)	-
CC1475 (1)	**1475**	1	27 (1)	-
CC13811 (1)	**13811**	1	16F (1)	-

The decrease in vaccine serotypes should be associated with the vaccination effect, however it should always be taken into account that the natural fluctuations of serotypes can also play a part in the prevalence of serotypes.

The number of IPD cases detected solely by molecular methods in 2015-2017 (n=43/134, 32.1%) was higher when compared to 2012-2015 (n=47/259, 18.1%) (unpublished data). Serotype 3 was mainly detected in pleural fluid samples by molecular methods (n=33/39, 84.6%). This serotype was previously associated with cases of vaccine failure [11,20], which emphasizes the importance of using molecular methods which in this case

contributed to the identification of a significant proportion of paediatric IPD cases.

Cases of IPD related with PCV7 types were mostly due to serotypes 6B, 14, 19F and 23F that are associated with antibiotic resistance [21,22] which could explain why they were still responsible for a fraction of IPD.

In the case of PCV13 types, the most problematic was serotype 3. One of the factors that may have contributed to the persistence of this serotype might be vaccine uptake. Vaccine uptake in Portugal around 2008 reached 75% [3], but declined to between 58% and 65% in 2009-2014 (IMS and INE data). Additionally, there could be IPD cases in children that were not vaccinated, because when they were at the age when the vaccine is administered, PCV13 wasn't still included in the NIP. Furthermore, some studies suggested that since the synthesis of serotype 3 capsule is different from other serotypes and the polysaccharide is not covalently linked to the peptidoglycan, it can be released and potentially reduce opsonophagocytosis and interfere with antibody-mediated clearance, which may result in a higher anti-capsular antibody concentration being required for protection against serotype 3 [11,20].

Among NVTs, serotypes 10A and 15B/C remained in the most frequent serotypes both in 2012-2015 and 2015-2017. In fact, between 2008-2012, serotype 10A was already reported to have increased significantly in Portugal [3]. A portuguese study on carriage revealed that serotype 15B/C was among the most frequently carried NVTs in 2009-2010, therefore this could be related to the prevalence of this serotype. However, serotype 10A was not associated with carriage [23], but since there is no carriage data from the same time period (2015-2017), it is possible that 10A is actually an important NVT in carriage in Portugal. Furthermore, a study regarding invasiveness of pneumococcal serotypes reported that serotype 3 and NVT 8 have an enhanced propensity to cause invasive disease [24], which can also explain how they were among the more frequent serotypes in 2015-2017. A previous study reported that serotypes 3, 8 and 19A were also among the major serotypes responsible for invasive disease in adults in Portugal from 2012-2014, which makes sense since children are the main carriers of *S. pneumoniae* and transmission to adults can occur [6].

It is known that serotype distribution varies according to geographic location. Nevertheless, the most frequent serotypes found in this study were also found among other regions. A study in England and Wales reported that in children < 5 years, serotypes 12F, 8, 10A, 15B/C and 3 were among the most frequent [25], however serotype 12F was not observed in Portugal. Furthermore, in a study from Denmark, NVTs 24F, 12F and 23B were the most prevalent non-vaccine types [26]. This situation is different from what was observed in Portugal between 2015-2017, where none of those NVTs were among the most frequent.

Between 2015-2017, antimicrobial resistance in vaccine serotypes was associated with serotypes 6B, 14, 19F and 23F, which are all included in PCV7, and were already related to antimicrobial resistance in Portugal [3]. In the case of NVTs, antimicrobial resistance was observed in serotypes 10A, 23A, 23B, 29/35B and 33F.

When considering antibiotic resistance, penicillin non-susceptibility decreased from 23.2% (43/185) in 2012-2015 to 13.2% (12/91) in 2015-2017. Erythromycin resistance

decreased from 22.7% (42/185) in 2012-2015 to 16.5% (15/91) in 2015-2017. Isolates presenting both resistance to erythromycin and non-susceptibility to penicillin decreased from 13.5% (25/185) to 7.7% (7/91) in 2015-2017 (unpublished data).

The overall decrease in resistance must be due to the effect of vaccination in decreasing the incidence of vaccine serotypes associated with resistance, as seen with serotype 6B that decreased from 5.2% to 3.0% and serotype 14 from 9.9% to 3.7% when comparing 2012-2015 to 2015-2017.

A study in France revealed a different situation from what was observed in Portugal. The French study reported that in children < 2 years, vaccine serotypes 14, 19A and 19F were associated with resistance, together with NVTs 15A, 24F and 35B [27]. The similarities were that serotypes 14 and 19F are related to antimicrobial resistance in both countries, however in Portugal none of the 19A isolates presented antimicrobial resistance. Another study in the UK reported serotypes 15A, 23B, 23A, 19F, 19A and 10A as being associated with resistance [28]. In Portugal, antimicrobial resistance was also detected in serotypes 23B, 23A, 19F and 10A. As observed in France, serotypes 15A and 19A were also associated with antimicrobial resistance in the UK [28], however the same as not observed in Portugal.

After molecular characterization of the isolates, the most frequent clonal complex was CC156 (n=19, 22.1%), which expressed mainly PCV7 serotypes (n=11, 57.9%) therefore it could be expected that CC156 would lose its dominance. However, even after years of administration of conjugate vaccines, CC156, mainly expressing vaccine serotypes, was still the most frequent CC. This persistence can be related to a few hypotheses such as antibiotic resistance, due to serotypes such as 6B, 14, 19F and 23F. In fact, 11/19 (57.9%) of the CC156 isolates were resistant to at least one antimicrobial. Furthermore, even though there was a decrease in vaccine serotypes responsible for IPD, PCV7 and PCV13 serotypes still remained important causes of paediatric IPD, with 78.6% (n=11/14) of PCV7 and 37.9% (n=11/29) of PCV13 isolates belonging to CC156. Additionally, CC156 has high genomic diversity which may facilitate adaptation to selective pressures [29].

After CC156, the most frequent CCs were CC63 (n=12, 13.9%), CC180 and CC460 (n=9, 10.5% each), CC393, CC433 and CC1262 (n=4, 4.7% each). In a previous study in Spain, CC156, CC180 and CC433 were also reported to be among the most frequent CCs found in IPD, along with CC191 (associated with serotype 7F), CC230 and CC320 (associated with serotype 19A) [8], which were absent in Portugal in 2015-2017.

When considering the clonal composition of the six most frequent serotypes (3, 10A, 8, 15B/C, 19F and 22F), both differences and similarities were found with other regions. Serotype 3 was mainly represented by ST180, which was already reported to have a wide geographic dissemination, being present in countries such as Spain, Japan and Sweden [7,8,30]. However, in Spain the second most frequent ST observed in serotype 3 isolates was ST260 [8], not found in Portugal. As for serotype 8, ST53 dominated as previously seen in Spain. However, in contrast to what was reported in Spain, where a higher genetic diversity was associated with serotype 8 strains [8], in Portugal all the serotype 8 isolates were included in the same genetic lineage, defined by

ST53 (CC63). Furthermore, a study in Canada revealed that ST1480 was the most common ST found among serotype 8 isolates [9]. Among serotype 10A isolates, a higher genetic diversity was noted. In spite of this, six of the nine isolates belonged to the same genetic lineage (CC460), which included ST97 (n=4) as well as ST461 and ST1551 (n=1 each). Similarly to what was reported here, serotype 10A was also reported to be an important cause of IPD among NVT, also associated with ST97 in Spain [8]. Another study in Japan reported that ST97 was absent and ST5236 was the most prevalent [7]. In the case of serotype 19F, although grouped in the same clonal complex, CC156, these isolates presented high genetic diversity (which is frequent among isolates included in CC156). In a study in Japan, ST236 was the most prevalent [7]. Even though this ST was not observed in Portugal, ST13863 present in one isolate was identified as a DLV of ST236. In the case of serotype 15B/C, CC1262 is the most prevalent and there is no dominant ST. In Spain, ST1262 was the most frequent, however in Japan ST1262 was absent and ST199 was the most prevalent [7,8]. ST199 was also observed in Portugal, however only in one isolate. As for serotype 22F, ST433 was the most commonly found ST, as observed in a previous study in Spain [8]. The same was also detected in Sweden and Canada in the post PCV13 period [9,30].

Overall, after the introduction of PCVs, there was an increase in incidence of non-vaccine types [4,5]. An increase in non-vaccine serotypes can be the result of capsular switching, when a successful clonal lineage expresses a different serotype not covered by conjugate vaccines and is able to persist in the population. However, in this collection, the capsular switching events seemed to have occurred at a low frequency, involving single isolates without clonal expansion and dissemination. Nevertheless, it is important to keep track of those cases since they may disseminate in the future as successful lineages.

It is important to continue routine surveillance to keep track of serotype changes in the context of universal vaccination, allowing the identification of vaccinal serotypes still persisting in the population and the possible emergence of NVT as important causes of IPD. Moreover, it is also important to monitor antimicrobial resistance rates, as they can be affected by the use of PCVs and to study the genetic relatedness of *S. pneumoniae* isolates, in order to gain a better understanding of the dynamics of the population causing paediatric IPD.

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