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Population genetic structure, serotype distribution and antibiotic resistance of *Streptococcus pneumoniae* causing invasive disease in children in Argentina

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Abstract

Invasive disease caused by *Streptococcus pneumoniae* (IPD) is one of the leading causes of morbidity and mortality in young children worldwide. In Argentina, PCV13 was introduced into the childhood immunization programme nationwide in 2012 and PCV7 was available from 2000, but only in the private market. Since 1993 the National IPD Surveillance Programme, consisting of 150 hospitals, has conducted nationwide pneumococcal surveillance in Argentina in children under 6 years of age, as part of the SIREVA II-OPS network. A total of 1713 pneumococcal isolates characterized by serotype (Quellung) and antimicrobial resistance (agar dilution) to ten antibiotics, belonging to three study periods: pre-PCV7 era 1998–1999 (pre-PCV), before the introduction of PCV13 2010–2011 (PCV7) and after the introduction of PCV13 2012–2013 (PCV13), were available for inclusion. Fifty-four serotypes were identified in the entire collection and serotypes 14, 5 and 1 represented 50% of the isolates. Resistance to penicillin was 34.9%, cefotaxime 10.6%, meropenem 4.9%, cotrimoxazole 45%, erythromycin 21.5%, tetracycline 15.4% and chloramphenicol 0.4%. All the isolates were susceptible to levofloxacin, rifampin and vancomycin. Of 1713 isolates, 1061 (61.9%) were non-susceptible to at least one antibiotic and 235(13.7%) were multidrug resistant. A subset of 413 isolates was randomly selected and whole-genome sequenced as part of Global Pneumococcal Sequencing Project (GPS). The genome data was used to investigate the population structure of *S. pneumoniae* defining pneumococcal lineages using Global Pneumococcal Sequence Clusters (GPSCs), sequence types (STs) and clonal complexes (CCs), prevalent serotypes and their associated pneumococcal lineages and genomic inference of antimicrobial resistance. The collection showed a great diversity of strains. Among the 413 isolates, 73 known and 36 new STs were identified belonging to 38 CCs and 25 singletons, grouped into 52 GPSCs. Important changes were observed among vaccine types when pre-PCV and PCV13 periods were compared; a significant decrease in serotypes 14, 6B and 19F and a significant increase in 7F and 3. Among non-PCV13 types, serogroup 24 increased from 0% in pre-PCV to 3.2% in the PCV13 period. Our analysis showed that 66.1% (273/413) of the isolates were predicted to be non-susceptible to at least one antibiotic and 11.9% (49/413) were multidrug resistant. We found an agreement of 100% when comparing the serotype determined by Quellung and WGS-based serotyping and 98.4% of agreement in antimicrobial resistance. Continued surveillance of the pneumococcal population is needed to reveal the dynamics of pneumococcal isolates in Argentina in post-PCV13. This article contains data hosted by Microreact.

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Keywords: antimicrobial resistance; Global Pneumococcal Sequence Cluster; *Streptococcus pneumoniae*; serotypes; WGS (Whole-genome Sequencing); Argentina.

Abbreviations: CCs, clonal complexes; CDC, centers for disease control and prevention; CLSI, Clinical and Laboratory Standards Institute; DDD, defined daily dose; ENA, European Nucleotide Archive; GPS, Global Pneumococcal Sequencing Project; GPSCs, global pneumococcal sequence clusters; IPD, invasive pneumococcal disease; MDR, multidrug resistance; MIC, minimal inhibitory concentration; MLST, multilocus sequence typing; NS, non-susceptible; PCV7, 7-valent pneumococcal conjugate vaccine; PCV13, 13-valent pneumococcal conjugate vaccine; SNPs, single nucleotide polymorphisms; STs, sequence types; VT, vaccine type; WGS, whole-genome sequencing.

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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Two supplementary tables and two supplementary figures are available with the online version of this article.

DATA SUMMARY

Genome sequences are deposited at ENA and the accession number is available in the metadata. Phylogenetic snapshot is available at https://microreact.org/project/GPS_Argentina. The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

Impact Statement

This study is an in-depth genetic structure analysis of the *S. pneumoniae* population in Argentina based on whole-genome sequencing (WGS). In this work, we analysed genomic data from pneumococcal isolates causing invasive pneumococcal diseases (IPD) among children in three study periods: pre-PCV7 era 1998–1999 (pre-PCV), before the introduction of PCV13 2010–2011 (PCV7) and after the introduction of PCV13 2012–2013 (PCV13). The use of standardized genome definition of strain typing (GPSC) in this study showed a high strain diversity consisting of 52 GPSCs, 10 of which are found only in Argentina. The eight previously recognized global-spreading strains were also present in the current collection with a prevalence of 28.3%. Considering that PCV13 was introduced into the vaccination calendar in January 2012 in Argentina, this study provides the early effect of vaccination at a national level with global context and highlights the usefulness of genomic surveillance.

INTRODUCTION

In Latin America and the Caribbean, pneumococcal infections were estimated to account for 12000–18000 deaths, 327000 cases of pneumonia, 4000 cases of meningitis and more than 1200 cases of sepsis each year in children aged under 5 years before vaccine introduction [1], on a general population of approximately 600 million inhabitants.

There are ~100 known serotypes but only a limited number cause the majority of invasive pneumococcal disease (IPD) [2]. To reduce the burden of pneumococcal disease, different formulations of pneumococcal conjugate vaccines have been introduced in many countries [3]. In 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. The introduction of PCV7 in many countries has substantially decreased the incidence of IPD caused by pneumococci expressing vaccine serotypes [4]. PCV7 was available in Argentina from 2000, but only in the private market. A 13-valent formulation (PCV13) that included serotypes 1, 3, 5, 6A, 7F and 19A in addition to the serotypes contained in PCV7, was included in the National Immunization Programme in January 2012 for children less than 1 year of age in a 2+1 schedule and catch-up between 12–24 months. Vaccine coverage with the third dose of PCV13 was 41 and 86% in 2012 and 2013, respectively [5].

Since 1993, the National IPD Surveillance Programme has been conducted nationwide in Argentina in children less than 6 years old, as part of SIREVA II-PAHO (Pan American Health Organization) network [6]. Through the surveillance programme, we collected 1713 *S. pneumoniae* isolates from children under 6 years old with IPD from Argentina and 413 (24%) were randomly selected for WGS. The aim of this study was to determine the serotype distribution, antimicrobial resistance and population genetic structure of this pneumococcal collection before and after the introduction of PCVs. We also evaluated the correlation between the serotype and the resistance profile determined phenotypically and those inferred by WGS.

METHODS

Bacterial isolates

Pneumococcal isolates included in the present study were collected from children under 6 years old with IPD at 150 hospitals from 23 provinces and Buenos Aires city as part of the national laboratory-based surveillance carried out from 1993 in Argentina (SIREVA II). An invasive pneumococcal disease case was defined as pneumococci recovered from a normally sterile site. The commonest manifestations of IPD are pneumonia with bacteraemia, empyema and/or bacteraemia, and meningitis. The 1713 isolates were collected during three periods: 424 in pre-PCV7 era 1998–1999 (pre-PCV), 726 before the introduction of PCV13 2010–2011 (PCV7) and 563 after the introduction of PCV13 2012–2013 (PCV13). The majority of the samples were isolated from blood (62%), cerebrospinal fluid (19%) or pleural fluid (6%) and the most common clinical manifestations were pneumonia (52%) meningitis (21%) and sepsis (10%).

All the isolates were serotyped by Quellung using serotype-specific antisera (Statens Serum Institut, Denmark). Of these 1713 isolates, a randomly selected collection of 413 was whole-genome sequenced at the Wellcome Sanger Institute as part of the Global Pneumococcal Sequencing (GPS) Project (https://www.pneumogen.net/gps/): 29% (122/424) isolates collected in pre-PCV, 18% (134/726) collected in PCV7 and 28% (157/563) collected in PCV13. Fig. S1 (available in the online version of this article) shows the geographical distribution of the 413 pneumococcal genomes from Argentina in pre-PCV (1998–1999), PCV7 period (2010–2011) and PCV13 period (2012–2013). Serotypes determined by Quellung reaction were considered the reference method for comparisons. Serotypes were grouped into two categories: vaccine serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) and nonvaccine serotypes (serotypes not included in PCV13).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using agar dilution and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines' breakpoints on 1713 isolates. Ten antibiotics penicillin, cefotaxime, meropenem,

erythromycin, tetracycline, chloramphenicol, cotrimoxazole, levofloxacin, rifampin and vancomycin were tested [7]. MIC was considered the reference method for comparisons. Penicillin resistance was defined as MIC of ≥0.12 µg ml^{-1}, according to meningitis break-points. Similarly, for cefotaxime, intermediate resistance and resistance were defined as MIC of 1, and ≥2 µg ml^{-1}, respectively. *S. pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 were used as quality-control strains. Double disc diffusion assay using erythromycin (15 µg) and clindamycin (2 µg) discs was performed to evaluate the inducible or constitutive expression of the MLS_B phenotype [7]. Isolates with intermediate or full resistance were defined as non-susceptible (NS). Multidrug resistance (MDR) was defined as non-susceptibility to three or more classes of antimicrobial agents [8].

Molecular characterization

A subset of 413 isolates that was randomly selected from the overall collection (*n*=1713) was sequenced on an Illumina HiSeq platform to produce paired-end reads of 151 base pairs in length and raw data were deposited European Nucleotide Archive (ENA) (Supplementary Material – metadata). WGS data was processed as previously described [9]. Briefly, we derived the serotype [10], multilocus sequence types (MLSTs) [11], pilus determinants and resistance profiles for seven antibiotics, including penicillin, ceftriaxone (resistance due to mutations within gene *pbp*1a, *pbp*2b, *pbp*2x) [12, 13], chloramphenicol (presence of gene *cat*), cotrimoxazole (I100L mutation in *fol*A and indel between 56-67th amino acid in *fol*P), erythromycin (presence of *erm*B and *mef*A), tetracycline (presence of *tet*(M), *tet*(O) and *tet*(S/M), vancomycin (presence of *van*A, *van*B, *van*C, *van*D, *van*E and *van*G), from the genomic data using the sequencing reads and the published tools developed by the USA Centers for Disease Control and Prevention (CDC) and the Wellcome Sanger institute, UK [14–16]. STs are included within the same clonal complex (CC) only if they share at least 6/7 identical alleles with at least one other ST in the group using eBURST as previously described [9, 17].

Population structure of pneumococci before and after PCV introduction

The population structure was defined by assigning Global Pneumococcal Sequence Cluster (GPSC) [9] to each isolate using a Kmer-based clustering method PopPUNK [18] and a reference list of pneumococcal isolates (*n*=34780) in the Global Pneumococcal Sequencing (GPS) database (https://www.pneumogen.net/gps/assigningGPSCs.html) and published genomes.

We defined the status of a lineage as vaccine type (VT) GPSC (100% PCV13 serotypes), non-VT GPSC (100% non-PCV13 serotypes) and GPSC with both VT and non-VT isolates, on the basis of its serotype composition detected in the whole study period.

Phylogenetic analysis

Phylogenetic analysis was performed on the 413 isolates by constructing a maximum-likelihood tree using FastTree based on SNPs extracted from an alignment generated by mapping reads to the reference genome of *S. pneumoniae* ATCC 700669 (NCBI accession number FM211187). The metadata and analysis results can be interactively visualized online using the Microreact tool at https://microreact.org/project/GPS_Argentina.

Statistics

Changes in serotype distribution in the overall collection were detected by comparison of the proportion of serotypes of all the isolates recovered in each of the three study periods. Similarly, in the collection of 413 isolates selected for WGS, we evaluated significant changes of VT/ non-VT in proportion to all VT/ non-VT in each GPSC and prevalence of antibiotic resistance between vaccine periods using Fisher's exact test.

We calculated the number of samples that we need to achieve an 80% statistical power with significant level of 0.05 using R package pwr which contains functions for basic power calculation from Cohen (https://cran.r-project.org/web/packages/pwr/pwr.pdf). If our observational number of samples was insufficient to detect the changes at 80% statistical power, Fisher's exact test was not carried out. Two-sided *P* values of <0.05 were considered statistically significant. Multiple testing was adjusted using the Benjamini-Hochberg false discovery rate of 5%. The statistical analysis was carried out in R version 3.5.2 and R scripts used for analyses were deposited at https://github.com/StephanieWLo/Genomic-Surveillance.

RESULTS

Serotype distribution

Among the 1713 *S. pneumoniae* isolates, 54 different serotypes were found. The most common serotypes were 14 (21.8%), 1 (13.6%), 5 (12.6%), 7F (5.4%), 6B (5.1%), 19A (4.8%) with 48 other serotypes causing the remaining 36.7% (Fig. 1a). Serotypes included in PCV7 vaccine represented 38.3%, serotypes included in PCV13 vaccine but not in PCV7 vaccine (1, 3, 5, 6A, 7F and 19A) 44.0% and non-vaccine serotypes 17.7%.

Compared with pre-PCV, serotypes included in the PCV7 showed a significant decrease from 52.8–39.3% in PCV7, and 23.8% in PCV13 periods (*P*<0.05 for all comparisons). Compared with pre-PCV, serotypes included in PCV13 but not in PCV7 vaccines (1, 3, 5, 6A, 7F and 19A) increased significantly from 35.1–46.3% in PCV7 and 47.6% in PCV13 periods (*P*<0.05 for both comparisons). Non-PCV13 serotypes increased from 12.0% in pre-PCV and 14.5% in PCV7 periods to 28.6% in PCV13 period (*P*<0.05 between pre-PCV and PCV13 and between PCV7 and PCV13). Compared to pre-PCV, PCV7 serotypes 6B, 14 and 19F showed a significant decrease in PCV13 (Fig. 1b). Significant changes observed in PCV13 additional serotypes between pre-PCV and PCV13 periods were: increases in serotypes 7F (2.8–6.7%, *P*=0.0052) and 3 (0.7–5.2%, *P*<0.001). Among non-VTs, significant changes were only observed in serogroup 24, which increased from 0% in pre-PCV to 5.3% in PCV13 (*P*<0.001). Increase in serotype

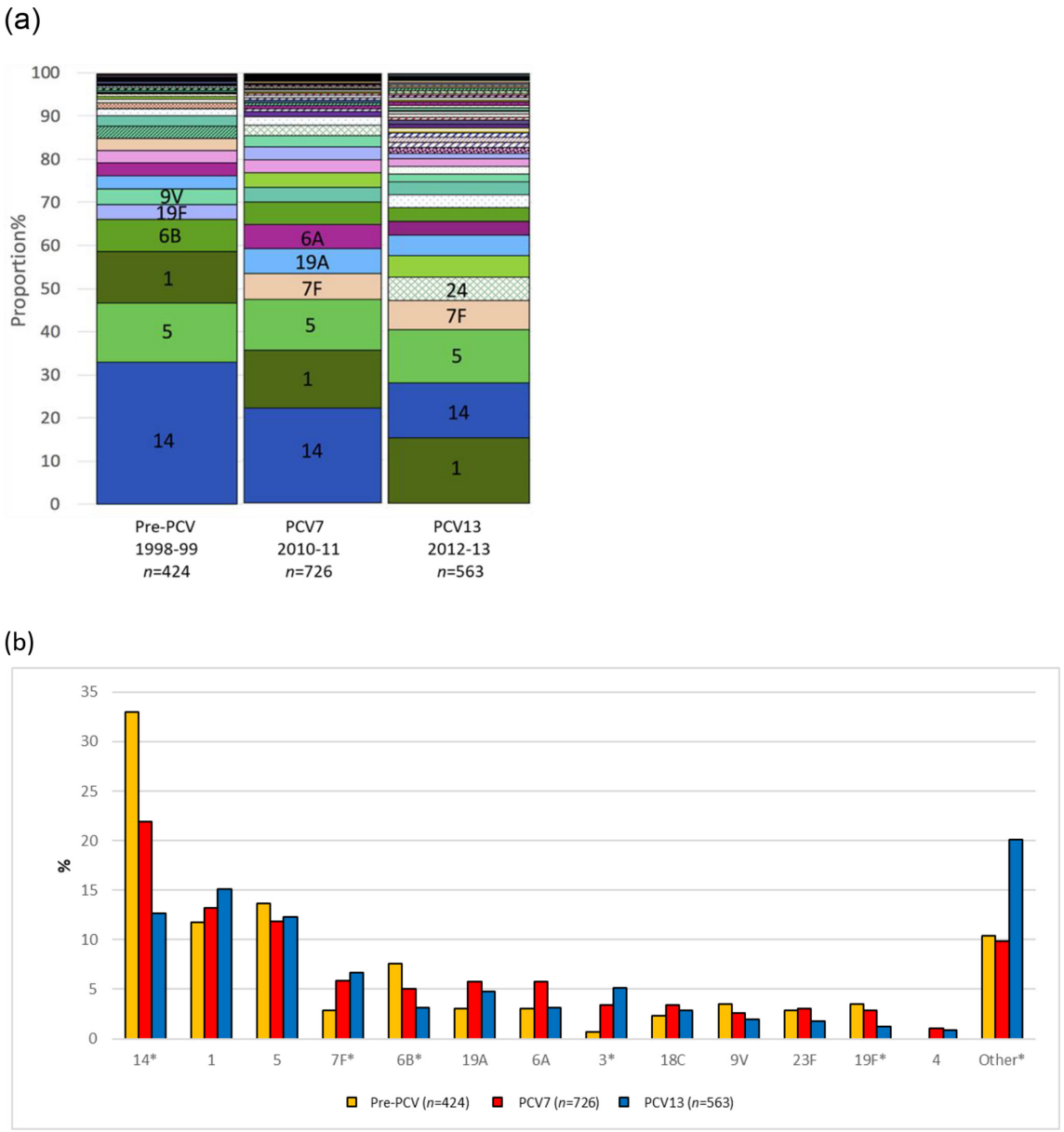

Fig. 1. (a) Serotype distribution among 1713 invasive pneumococcal isolates from children aged ≤5 years in Argentina in pre-PCV, PCV7 and PCV13 periods. Vaccine serotypes are represented by solid fill while non-VT by coloured hatched patterns. The six most prevalent serotypes for each period are labelled. (b) Distribution of PCV13 serotypes among 1713 invasive pneumococcal isolates from children aged ≤5 years in Argentina in pre-PCV, PCV7 and PCV13 periods. The asterisks indicate statistically significant changes between periods.

12F was also observed throughout the study period, although it was not statistically significant (P=0.211).

Antimicrobial resistance

Of the 1713 isolates, penicillin resistance was found in 598 (34.9%) (n=28 had MICs ≥4 µg ml^{-1}), cefotaxime-NS in 181 (10.6%), meropenem-NS in 84 (4.9%) and cotrimoxazole-NS in 770 (45%) of the isolates. A total of 368 isolates (21.5%) were resistant to erythromycin, 263 (15.4%) to tetracycline and 7 (0.4%) to chloramphenicol. All the isolates were susceptible to levofloxacin, rifampin and vancomycin. Compared with the pre-PCV period, penicillin and cotrimoxazole resistance significantly decreased in the PCV13 period; while erythromycin and tetracycline resistance increased significantly (Table 1). In the PCV13 period, VTs were still the major contributors for all antibiotic resistance and multidrug resistance, especially serotype 14 (Table S1). Among non-VT isolates, significant increases were observed in penicillin, erythromycin, tetracycline, cotrimoxazole and multidrug resistance (Table 1); serogroup 24 is the major non-VT associated with these antibiotic resistances and MDR in the PCV13 period, accounting for 29% (5/17) of penicillin resistance, 83% (5/6) erythromycin resistance, 42%

Table 1. Changes in antibiotic non-susceptiblity between vaccine periods in the overall collection (*n*=1713) from Argentina, 1998–2013

	No. of isolates (%)		*P* value comparing pre-PCV and PCV7 periods	No. of isolates (%)	*P* value comparing pre-PCV and PCV13 periods	*P* value comparing PCV7 and PCV13 periods
	pre-PCV period	PCV7 period		PCV13 period		
Overall (*n*)	424	726	–	563	–	–
Penicillin	165 (38.9%)	252 (34.7%)	0.1621	181 (32.1%)	0.0310	0.3420
Erythromycin	28 (6.6%)	212 (29.2%)	<0.0001	128 (22.7%)	<0.0001	0.0091
Tetracycline	22 (5.2%)	135 (18.6%)	<0.0001	98 (17.4%)	<0.0001	0.6099
Cotrimoxazole	260 (61.3%)	260 (35.8%)	<0.0001	250 (44.4%)	<0.0001	0.0019
Chloramphenicol	6 (1.4%)	1 (0.1%)	0.0118	0	0.0061	1
Multidrug resistance	17 (4.0%)	127 (17.5%)	<0.0001	91 (16.2%)	<0.0001	0.7075
NVT isolates only (*n*)	51	97	–	156	–	–
Penicillin	2 (3.9%)	25 (25.8%)	0.0007	61 (39.1%)	<0.0001	0.0403
Erythromycin	1 (2.0%)	16 (16.5%)	0.0066	33 (21.2%)	0.0008	0.4153
Tetracycline	2 (3.9%)	20 (20.6%)	0.0066	42 (26.9%)	0.0003	0.2942
Cotrimoxazole	5 (9.8%)	28 (28.9%)	0.0117	71 (45.5%)	<0.0001	0.0116
Chloramphenicol	0	1 (1.0%)	1	0	1	0.3834
Multidrug resistance	0	16 (16.5%)	0.0013	31 (19.9%)	0.0001	0.6183

*Multidrug resistance was defined as isolates non-susceptible to three ore more antibiotics detected in this study.
PCV: pneumococcal conjugate vaccine. VT: vaccine serotype.

(5/12) tetracycline resistance, 31% (4/13) cotrimoxazole resistance and 83% (5/6) MDR.

Correlation between phenotype and genotype

Among the 413 isolates whole-genome sequenced, we found 100% concordance when comparing the serotype determined by Quellung and WGS-based serotyping.

Genomic inference of antibiotic resistance was reliable with 94.7–100% of categorical (S/I/R) agreement (Table 2). Compared to agar dilution penicillin MICs, MICs predicted by PBP type showed an essential agreement (MICs agree within one dilution) of 96.6% and categorical agreement (interpretive results agree) of 100%. Based on the *pbp*1a, *pbp*2b and *pbp*2x, 82 PBP allele combinations were detected in 413 genomes. Twelve of them (*n*=23) had new allele combinations.

Of the 72 isolates phenotypically resistant to erythromycin, 41 were positive for *mefA* alone, 23 for *ermB* alone, 8 for *ermB* plus *mefA,* and neither *ermB* nor *mefA* were detected in five isolates. D-test was performed in all the

Table 2. Agreement between the phenotype and genotype in antimicrobial resistance to five antibiotics

Antibiotics	No. of isolates	Concordance	Discordance		
			Minor discrepancy*	Major discrepancy†	Very major discrepancy‡
Penicillin	413	413 (100%)	0	0	0
Chloramphenicol	413	412 (99.8%)	0	1 (0.7%)	0
Erythromycin	413	411 (99.5%)	0	2 (0.5%)	0
Tetracycline	413	404 (97.8%)	0	7 (1.7%)	2 (0.5%)
Cotrimoxazole	413	391 (94.7%)	22 (5.3%)	0	0
Total	2065	2031 (98.4%)	22 (1.0%)	10 (0.5%)	2 (0.1%)

*Intermediate by phenotypic methods but inferred as susceptible by WGS or susceptible by phenotype but inferred intermediate by WGS.
†Susceptible by phenotypic methods but inferred as resistant by WGS.
‡Resistant by phenotypic methods but inferred as susceptible by WGS.

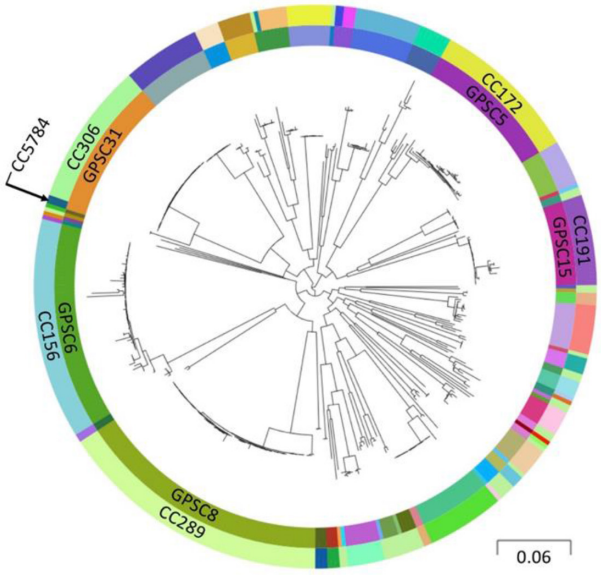

Fig. 2. Phylogeny of 413 pneumococcal isolates. Inner ring shows the global pneumococcal sequence clusters (GPSCs) and outer ring clonal complex (CC). The figure shows 52 GPSCs, and 40 CCs + 30 singletons. Each colour represents either a GPSC or CC or singleton. The five most predominant GPSCs were labelled. This figure can be visualized at https://microreact.org/project/GPS_Argentina/7b62128d.

erythromycin-resistant isolates from PCV7 and PCV13 periods (*n*=66). From 30 isolates resistant to erythromycin and clindamycin with constitutive MLS_B phenotype, 22 carried the *ermB* gene alone and eight carried both *ermB* plus *mefA* genes. All isolates with M phenotype (*n*=36) carried the *mefA* gene. In addition, 58/60 tetracycline-resistant isolates were positive for *tet*(M), and the two *tet*(M)-negative isolates were subject to BLAST against a collection of 146 *tet* genes in ResFinder Database (last updated on 8 April 2020) with a cutoff of identity >80% and coverage >60%. No *tet* was detected in these isolates. The four isolates chloramphenicol-resistant were positive for the *cat* gene, while one isolate positive for *cat* but phenotypically categorized as chloramphenicol-susceptible had elevated MIC (4 µg ml^{-1}). Their promoter and ribosomal binding regions of *cat* were intact, indicating this gene should be functional. Of the 185 isolates phenotypically NS to cotrimoxazole, 178 contained 1–2 codon insertions within the dihydropteroate synthetase gene *folP* gene (intermediate phenotype, MIC 1–2 µg ml^{-1}), while 93 of them also contained the I100L substitution in dihydrofolate reductase gene *folA* (resistant phenotype, MIC ≥4 µg ml^{-1}).

Population structure

Seventy-three known and 36 novel sequence types (STs) were identified in 413 isolates belonging to 38 clonal complexes (CCs) and 25 singletons. CCs correlated well with the genome-based clusters, GPSCs (Fig. 2). Overall, 52 GPSCs were identified, and the most prevalent were GPSC8 (CC289 expressing serotype 5, Colombia[5]-19 PMEN clone), GPSC6 (CC156, serotype 14 and 9V, Spain^{9V}-3 PMEN clone), GPSC31 (CC306 and CC5784, serotype 1), GPSC5 (CC172, serotype 19A and 23B) and GPSC15 (CC191, serotype 7F), accounting for 52% of the whole collection (Table 3). Of 52 GPSCs, 28 (54%) were VT lineages, 18 (35%) were non-VT lineages and 6 (11%) were lineages (GPSC 5, 7, 10, 16, 43, 657) with both VT and non-VT isolates. It is of note there are ten GPSCs (GPSC657, 714, 715, 716, 820, 821, 822, 823, 825, 826) uniquely found in Argentina. Using their STs to query the MLST database, we found that all related strains were only found in Latin America (Table S2).

In addition, all eight globally-spreading lineages recognized in the previous GPS study [9] were found in the current collection with a prevalence of 13.1% (54/413, GPSC6, CC156), 4.6% (19/413, GPSC18, CC15, England[14]-9 PMEN clone), 2.7% (11/413, GPSC12, CC180), 2.4% (10/413, GPSC32, CC218), 2.2% (9/413, GPSC16, CC81), 1.9% (8/413, GPSC1, CC320), 0.7% (3/413, GPSC7, CC439) and 0.7% (3/413, GPSC23, CC273), respectively.

Compared with the pre-PCV period, we detected a significant decrease in two GPSCs: GPSC6 (CC156) expressing serotype 14 and 9V (*P*<0.001) and GPSC16 (CC66) mainly expressing serotype 9 N (*P*=0.0027) in the PCV13 period; while significant increase in two GPSCs: GPSC5 (CC172) expressing 19A in the PCV7 period and GPSC31 (CC306 and CC5784) expressing serotype 1 in the PCV13 period (*P*<0.001 for both comparisons) (Fig. 3). Notably, another serotype 1 lineage GPSC2 (CC615) was seen to decrease, however, the change was not significant after adjusting for multiple testing. Clonal replacement within a serotype is not uncommon [19, 20]. In this study, we observed clonal replacement in serotype 1 (GPSC2 ->GPSC31, Fig. S2A) and serotype 14 (GPSC6 ->GPSC9 and GPSC18, Fig. S2B). Unlike GPSC6 (CC156), isolates belonged to GPSC9 (CC63) and GPSC18 (CC15) were all, apart from one, resistant to erythromycin mainly through expression of *mefA* (74%, 25/34), followed by *ermB* (24%, 8/34) (Fig. 3). GPSC9 (CC63) was additionally resistant to tetracycline mediated by *tet*(M).

Two years after PCV13 introduction, two PCV13 serotypes 3 and 7F increased and they were mainly mediated by GPSC12 (CC180) and GPSC15 (CC191), respectively (Fig. 3). Both lineages were generally susceptible to antibiotics, except for 64% (7/11) of isolates in GPSC12 that were tetracycline resistant. The only non-PCV13 serotype that significantly increased was serogroup 24. All serogroup 24 isolates, six 24F (ST230, Denmark[14]-32 PMEN clone) and one 24A, were clustered in GPSC10 (CC230), together with a serotype 19A isolate. They were all recovered from children aged under 3 who had pneumonia (*n*=3), meningitis (*n*=1, 19A), sepsis (*n*=1), and unknown disease with febrile symptom (*n*=3). The earliest GPSC10 isolate expressing serotype 24F was identified in 2010 in Santa Cruz, which preceded the GPSC10 serotype 19A isolate that was recovered in Santa Fe, 2011, indicating that the emergence of serogroup 24 is unlikely due to a recent capsular switching event. GPSC10 was a lineage that was multidrug resistant to penicillin, erythromycin (*ermB*) and tetracycline *tet*(M) and

Table 3. Association between serotypes, GPSCs, CCs and STs and their distribution by period of study of 413 isolates randomly selected from the overall collection (*n*=1713) from Argentina, 1998–2013

Serotype (no. isolates)	GPSC	CC	Sequence type	Pre-PCV	PCV7	PCV13
				1998–1999	2010–2011	2012–2013
14 (83)	6	CC156	ST156	31	5	4
			ST162	3	0	0
			ST370	1	0	0
			ST14426 (Novel ST)	1	0	0
			ST14491 (Novel ST)	1	0	0
			ST14525 (Novel ST)	1	0	0
			ST14448 (Novel ST)	1	0	0
	18	CC15	ST9	2	5	5
			ST 15183 (Novel ST)	0	1	2
			ST15	2	0	0
			ST 15177 (Novel ST)	0	1	0
			ST13	1	0	0
	9	CC63	ST782	0	5	5
			ST2678	0	1	3
	822	Singleton	ST797	1	0	0
5 (67)	8	CC289	ST289	21	21	20
			ST15180 (Novel ST)	2	0	0
			ST15179 (Novel ST)	1	0	0
1 (56)	31	CC306	ST306	0	10	23
			ST15186 (Novel ST)	0	1	0
			ST8415	0	1	0
	31	CC5784	ST304	2	0	0
	2	CC615	ST615	11	2	5
19A (45)	5	CC172	ST1131	0	19	10
			ST172	0	1	0
			ST14428 (Novel ST)	0	1	0
			ST14588 (Novel ST)	0	1	0
	4	CC199	ST876	0	4	0
			ST199	0	1	0
			ST15181 (Novel ST)	1	0	0
	1	CC320	ST320	0	2	1
			ST1451	0	1	0
			ST2410	0	0	1
	10	CC230	ST276	0	1	0
	714	Singleton	ST4062	0	1	0
7F (22)	15	CC191	ST191	4	7	8

Continued

Table 3. Continued

Serotype (no. isolates)	GPSC	CC	Sequence type	Pre-PCV	PCV7	PCV13
				1998–1999	2010–2011	2012–2013
			ST1062	0	1	0
			ST4665	0	0	1
6B (17)	47	CC315	ST315	1	3	2
	37	CC751	ST751	1	0	2
			ST14566 (Novel ST)	1	0	0
			ST14567 (Novel ST)	0	1	0
	23	CC273	ST94	0	0	1
	23	Singleton	ST1121	1	0	0
	23	Singleton	ST14563 (Novel ST)	1	0	0
	820	Singleton	ST1123	1	0	0
	13	CC473	ST14514 (Novel ST)	1	0	0
12F (14)	32	CC218	ST218	3	3	4
	26	CC989	ST989	0	0	3
18C (13)	50	CC113	ST113	3	0	1
			ST1923	0	1	1
			ST2429	1	0	0
			ST14470 (Novel ST)	1	0	0
			ST15184 (Novel ST)	0	1	0
			ST15182 (Novel ST)	0	0	1
	657	Singleton	ST14585 (Novel ST)	0	0	1
6A (12)	13	CC473	ST473	0	3	2
			ST1876	0	1	2
	821	CC385	ST15178 (Novel ST)	1	0	0
	823	Singleton	ST747	0	1	0
	29	Singleton	ST2611	0	1	0
	37	CC751	ST14565 (Novel ST)	0	0	1
3 (12)	12	CC180	ST180	0	3	8
	51	CC458	ST458	0	0	1
19F (11)	44	CC177	ST179	0	2	1
	1	CC320	ST236	0	1	1
			ST763	1	0	0
	9	CC63	ST2100	0	1	0
	657	Singleton	ST14487 (Novel ST)	0	0	1
	290	CC306	ST14493 (Novel ST)	1	0	0
			ST13887 (Novel ST)	0	1	0
	716	Singleton	ST14486 (Novel ST)	0	1	0

Continued

Table 3. Continued

Serotype (no. isolates)	GPSC	CC	Sequence type	Pre-PCV	PCV7	PCV13
				1998–1999	2010–2011	2012–2013
24F (6)	10	CC230	ST230	0	2	4
24A (1)			ST14499 (Novel ST)	0	0	1
9 N (7)	16	CC66	ST66	7	0	0
9V (7)	6	CC156	ST162	1	0	3
			ST156	0	2	0
	43	CC2987	ST2240	0	1	0
23F (7)	113	CC775	ST775	0	2	1
	7	CC439	ST14523 (Novel ST)	0	1	0
			ST804	1	0	0
	16	CC81	ST81	1	0	0
	715	Singleton	ST779	0	0	1
33F (5)	3	CC2223	ST2223	0	1	2
	3	CC100	ST100	0	1	0
	3		ST717	0	0	1
16F (4)	386	Singleton	ST7438	0	2	1
	386	Singleton	ST14458 (Novel ST)	0	0	1
18A (4)	95	CC241	ST241	1	0	3
4 (4)	70	Singleton	ST259	0	2	0
	70	Singleton	ST7026	0	0	2
23B (4)	5	CC172	ST387	0	0	3
			ST14492 (Novel ST)	0	0	1
22F (3)	61	CC698	ST6403	1	0	0
			ST698	0	0	1
13 (3)	657	Singleton	ST14585 (Novel ST)	1	0	1
	16	Singleton	ST14460 (Novel ST)	1	0	0
11A (3)	43	CC280	ST280	0	1	0
	269	Singleton	ST4063	0	0	1
		Singleton	ST14520 (Novel ST)	0	0	1
27 (2)	226	Singleton	ST1475	1	0	0
38 (2)	38	CC393	ST393	0	0	2
23A (2)	7	CC439	ST15176 (Novel ST)	1	0	0
15B/15C (2)	48	Singleton	ST3201	0	0	1
	131	CC3669	ST3669	1	0	0
2 (1)	96	CC74	ST3744	0	1	0
10A (1)	266	CC5472	ST3539	0	0	1
15A (1)	140	Singleton	ST15185 (Novel ST)	0	1	0

Continued

Table 3. Continued

Serotype (no. isolates)	GPSC	CC	Sequence type	Pre-PCV	PCV7	PCV13
				1998–1999	2010–2011	2012–2013
17F (1)	826	Singleton	ST14495 (Novel ST)	0	0	1
33B (1)	825	Singleton	ST1135	0	0	1
35B (1)	59	CC558	ST14528 (Novel ST)	0	0	1
35F (1)	267	Singleton	ST14449 (Novel ST)	0	0	1

was found in five different cities in Argentina. Across the three vaccine periods, GPSC8 was the only cluster ranked in the top five pneumococcal lineages (Table 4) and its prevalence did not significantly change (Fig. 3). It accounted for all serotype 5 isolates and which were susceptible to all the antibiotics tested, except cotrimoxazole (91% NS) (Fig. 3).

Antibiotic-resistant pneumococcal lineages and pilus genes

Among pneumococcal lineages with more than five isolates, ine GPSCs (GPSC1, 4, 5, 6, 9, 10, 13, 37 and 47) had >80% isolates that were penicillin-resistant. Most of them were VT lineages, except for GPSC5 (19A and 23B1) and GPSC10 (19A and 24) (Fig. 3). Four pneumococcal lineages (GPSC1, 9, 10, 26 and 47) were multidrug resistant, with GPSC10 (CC230) and GPSC26 (CC989) mainly composed of non-PCV13 serotypes, serotype 24 and 12F, respectively (Fig. 4).

The presence of PI-1 or both of the pilus loci was associated with certain clonal complexes (CCs) and serotypes. Overall, pilus islet 1 (PI-1) was identified in 16% (67/413) isolates, pilus islet 2 (PI-2) in 17% (72/413) isolates and 1.5% (6/413) positive for both PI1 and PI-2. PI-1 was mainly associated with GPSC6 (CC156) expressing 14 and 9V; PI-2 was found in all isolates within GPSC31 (CC306 and CC5784) expressing serotype 1, 95% (20/21) GPSC15 (CC191) expressed serotype 7F, and 72% (13/18) GPSC2 (CC615) expressing serotype 1. Isolates harboured both PI-1 and PI-2 were only observed in GPSC1 (CC320) expressing serotype 19A (n=4) and 19F (n=2) (Fig. 4).

DISCUSSION

This study demonstrated the usefulness of genomic surveillance for monitoring early changes in pneumococcal population causing invasive disease in Argentina after PCV7 and

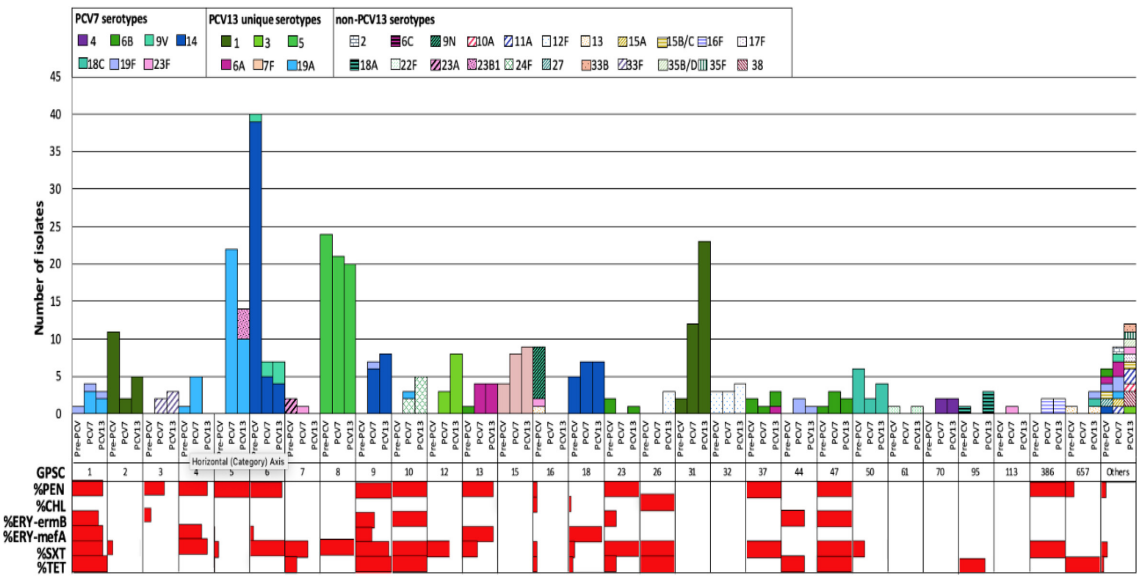

Fig. 3. Dynamics of Global Pneumococcal Sequence Clusters (GPSCs) among invasive isolates from children aged ≤5 years over vaccine periods in Argentina. The number of invasive disease isolates is plotted by GPSCs with stratification into three vaccine periods and coloured by serotypes. PCV13 serotypes are represented by solid fill while non-PCV13 serotypes by coloured hatched patterns. The horizontal bars in red indicate the percentage of isolates that are predicted to be non-susceptible to antibiotics based on genomic data. PEN, penicillin; CHL, chloramphenicol; COT, cotrimoxazole; ERY, erythromycin; TET, tetracycline.

Table 4. The five most prevalent lineages and their associated serotypes in a collection of disease isolates (*n*=413) from Argentina in pre-PCV, PCV7 and PCV13 periods

	Pre-PCV period (1998–1999) *n*=122			PCV7 period (2010–2011) *n*=134			PCV13 period (2012–2013) *n*=157		
Rank	**GPSC (CC)**	***N* (%)**	**Associated serotype (*n*)**	**GPSC (CC)**	***N* (%)**	**Associated serotype (*n*)**	**GPSC (CC)**	***N* (%)**	**Associated serotype (*n*)**
First	6 (CC156)	40 (33%)	**14** (39), **9V** (1)	5 (CC172)	22 (16%)	**19A** (22)	31 (CC306)	23 (15%)	**1** (23)
Second	8 (CC289)	24 (20%)	**5** (24)	8 (CC289)	21 (16%)	**5** (21)	8 (CC289)	20 (13%)	**5** (20)
Third	2 (CC615)	11 (9%)	**1** (11)	31 (CC306)	12 (9%)	**1** (12)	5 (CC172)	14 (9%)	**19A** (10), **23B** (4)
Fourth	16 (CC66)	9 (7%)	9 N (7), 13 (1), **23F** (1)	15 (CC191)	8 (6%)	**7F** (8)	15 (CC191)	9 (6%)	**7F** (9)
Fifth	50 (CC113)	5 (4%)	**18C** (5)	9 (CC63)	7 (5%)	**14** (6), **19F** (1)	9 (CC63)	8 (5%)	**14** (8)
							12 (CC180)	8 (5%)	**3** (8)

Vaccin-types are shown in bold.

PCV13 introduction. The integration of WGS into surveillance has an added value of observing changes beyond serotype to identify pneumococcal lineages that are driving serotype replacement and changes in antibiotic resistance after PCV. The use of both standardized genome definition of strain typing (GPSC) and ST/CC combinations in this study has also allowed us to understand the circulation of previously recognized global-spreading strains [9] and native strains of Argentina, and place the findings in a global context. In addition, this study also highlighted the accuracy of using genomic data to infer serotype and antibiotic resistance among isolates from Argentina; such high concordance is similar to pneumococcal isolates from elsewhere [9, 12–15].

Although PCV7 was not included in the national immunization programme in Argentina and was only available in the private market from 2000, a significant decrease in the PCV7 serotypes was observed over time, as well as a significant increase in PCV13 and non-vaccine serotypes. A significant decrease in serotype 14 was observed, however it remained

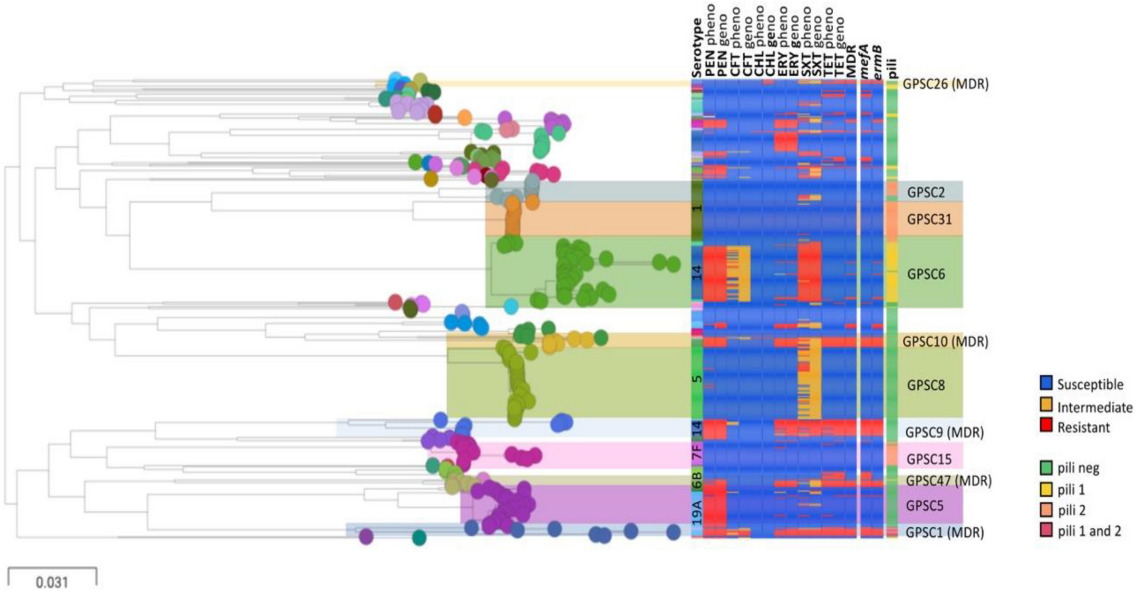

Fig. 4. Maximum-likelihood tree constructed with all pneumococcal genomes in this study (*n*=413). The nodes of the tree were coloured by the Global Pneumococcal Sequencing Clusters (GPSCs). Serotype and antibiotic resistance were indicated in the metablock. The phenotypic antimicrobial susceptibility was measured by agar dilution and interpreted based on CLSI guideline. Penicillin resistance was predicted based on the *pbp*1a, *pbp*2b, *pbp*2x sequences; tetracycline and erythromycin resistance were predicted based on the presence of *tet*(M), *tet*(O) and *tet*(S/M), and *erm*(B) and *mef*(A), respectively. Cotrimoxazole resistance was predicted based on the presence of mutation I100L in *fol*A and any indel within amino acid residue 56–67 in *fol*P while presence of either mutation predicted to be cotrimoxazole-intermediate. PEN, penicillin; CFT, cefotaxime; CHL, chloramphenicol; ERY, erythromycin; SXT, cotrimoxazole; TET, tetracycline; MDR, multidrug resistant. This figure can be visualized at https://microreact.org/project/GPS_Argentina/21de0fb9.

one of the predominant serotypes through 2013 and was a major contributor of antibiotic resistance in the early PCV13 period. In this study, the major serotype 14 clones were GPSC6 (CC156), GPSC9 (CC63) and GPSC18 (CC15). Clonal replacement of GPSC6 (CC156) by GPSC9 (CC63) and GPSC18 (CC15) could be due to their trait of macrolide resistance.

In previous studies, GPSC9 (CC63) and GPSC18 (CC15) were reported as major pneumococcal clones accounting for macrolide resistance in Argentina [21, 22]. In 1995–2001, PMEN England[14]-9 clone (or ST9 encompassed in CC15 or GPSC18) represented 42% of the isolates resistant to macrolides isolated in Argentina from children with IPD, mainly associated with efflux mechanism and the presence of the *mefA*/*E* genes [21]. PMEN England[14]-9 clone and Sweden[15A]-25 clone (or ST782 encompassed in CC63 and GPSC9) accounted for most of the macrolide-resistant isolates recovered from children with acute otitis media between May 2009 and August 2010 in Argentina [22]. In Argentina, the defined daily dose per 1000 individuals (DDD) for macrolide in 2000 was 346 DDD and it almost doubled to 770 DDD in 2015 (https://resistancemap.cddep.org/), suggesting that antibiotic-selective pressure could be one of the factors contributing to clonal replacement in serotype 14, while vaccine-selective pressure was unlikely as all isolates in these lineages are PCV7 serotypes.

Serotype 5 GPSC8 and CC289 was the second most predominant serotype in number of isolates in our collection and was present in the three vaccine periods. The prevalence of serotype 5 decreased notably several years after the introduction of PCV13, accounting for less than 2% since 2014 until present.

Clonal replacement was observed in serotype 1 in this study. The emergence of CC306 (GPSC31) observed before the introduction of PCV13 suggests that factors other than vaccine introduction may have caused this clonal replacement. It is of note that 100% (37/37) of serotype 1 isolates in GPSC31 had pili2 while 68%(13/19) in GPSC2 had pili2 genes, although carriage isolates were not available in this study to measure the potential differences in invasiveness between the two GPSCs. In the Gambia, clonal replacement of serotype 1 was also observed [20]. Genomic and *in vivo* infection model demonstrated the emerging clone (ST3081, GPSC2) is more virulent and is carried at higher density during nasopharyngeal carriage, as compared to clone ST618 (GPSC2). A fixed mutation in a gene encoding toxin pneumolysin *ply* was demonstrated to be associated with increased haemolytic activity in the emerging clone, highlighting invasiveness and virulence at the genetic background beyond capsule.

A significant increase in serotype 19A in GPSC5 was detected. GPSC5 was resistant to penicillin and susceptible to other antibiotics tested. Although GPSC5 was not detected in the pre-PCV period in this study, previous studies showed that ST1131 (encompassed in CC172 and GPSC5) was first detected in Argentina in 1997 and has increased over time, accounting for most of the 19A isolates recovered from colonization, IPD and acute otitis media infection [Corso personal communication, 22, 23]. Globally, this lineage associated with 15 serotypes (5VT and 10 non-VT) [9] is responsible for the expansion of serotype 35B/D in South Africa after PCV13 [24].

The increase in serotype 7F detected in this study could be transient, as a similar initial increase was observed in Italy during the shift between PCV7 and PCV13 and then later declined [25]. In contrast, future decline in serotype 3 could be limited due to low efficacy of PCV13 observed against serotype 3 [26, 27]. Globally, the majority of serotype 3 isolates are CC180 (GPSC12; Netherlands[3]-31 clone), with the exception of Africa where non-CC180 clones are prevalent [28]. Recently, a study was conducted on 301 serotype 3 CC180 isolates from 24 countries over 20 years, to explore epidemiological, genotypic and phenotypic factors associated with the persistence of serotype 3 in carriage and disease. The authors found that the recent success of CC180 was related to an emerging clade II, different in antigenic composition, antibiotic susceptibility and competence, which was first reported in Asia in 1999, but is now globally distributed. South America, together with Asia, Africa and North America made up a large proportion of clade-II samples in the study [28]. However, in a study from the US, which includes strains recovered during 2015–2016 through Active Bacterial Core surveillance, non-clade-II serotype 3 was also prevalent [29]. In the present study, serotype 3/CC180 was detected in 11 isolates post-PCV introduction with four belonging to clade Ia and seven to clade II (https://microreact.org/project/AE8Lqwsds/381ea183). A single serotype 3 isolate belonged to GPSC51 ST458, the ST dominant in South Africa [30]. A detailed genomic analysis of the GPSC12 (or CC180 phylogeny) can also be found in a recent study by Gladstone *et al.* [31].

Two years after PCV13 introduction, PCV13 serotypes remained in the top five serotypes with one emerging non-vaccine serogroup 24 suggesting early indications of restructuring of the population toward its new equilibrium. In a more recent follow-up study, we found serogroup 24 has continued to be the major non-PCV13 serotype in children under 2 years old with IPD, ranking the first in this study in 2013 and reaching 16.2% in 2016 with a high proportion of MDR [32]. Increases in serogroup 24 after PCV13 introduction was also observed in Denmark [33], France [34], Italy [25], Japan [35, 36], Spain [37, 38], the UK [39] and reported to be one of the predominant non-vaccine serotypes causing IPD in Germany [40] and Portugal [41]. A meta-analysis showed that serotype 24F was at the upper end of the invasiveness spectrum and appeared to be prone to cause meningitis [42]. Based on case reports, serotype 24F was associated with significant invasive disease potential [36] and clinical severity [43]. Among the six serotype 24F isolates in this study, none were recovered from meningitis and mainly associated with pneumonia and an unknown disease with febrile symptom. Further investigation is needed to better understand its propensity to cause acute and severe infections. Globally,

serotype 24F isolates mainly belong to three lineages: ST162 (GPSC6), CC230 (GPSC10) and CC72 (GPSC16), in which GPSC10 expressing serotype 24F was penicillin- and multidrug-resistant, while the other two lineages were not [33, 38]. CC230 (GPSC10) was a multidrug-resistant lineage that had been notorious for driving the increase in serotype 19A after PCV7 and serotype 24F after PCV13 in Spain [38, 44].

Serotype 12F associated with MDR lineage GPSC26 (CC989) and pan-susceptible lineage GPSC32 (CC218) was not significantly increased in this dataset. A recent follow-up study in Argentina 2017–2018 showed that serotype 12F and 24F were the most frequent cause of IPD in children under 5 (Sireva II Argentina; http://antimicrobianos.com.ar/ATB/wp-content/uploads/2019/10/Tablas-vigilancia-SIREVA-II-Spn-2018-1.pdf). Importantly, the treatment of choice for pneumonia in children remains to be penicillin or ampicillin, as well as cefotaxime or ceftriaxone for meningitis and bacteremia in infants.

A continuous genomic surveillance will enable us to track these two emerging non-vaccine serotypes.

A limitation of the present work is that it represents proportional changes in the distribution of serotypes, but that the real changes in the incidence of each serotype were not evaluated because the data on disease burden is not available.

This study, describing early changes in pneumococcal disease epidemiology, demonstrated that the pneumococcal population is still evolving after the introduction of PCV13. Continuous surveillance post-PCV13 introduction is essential to evaluate further evolution of pneumococcal infection, which is vital for informing future vaccine formulation and implementation and antibiotic treatment options.

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Author contributions
P.G.: conceptualization, formal analysis, investigation, data curation, writing – original draft preparation, visualiation. S.W.L.: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft preparation, visualization, project administration. P.A.H.: investigation, data curation, writing – review and editing, project administration. R.A.G.: methodology, formal analysis, data curation, writing – review and editing, visualization, project administration. M.R.: investigation, resources. D.F.: writing – review and editing. SIREVA-Argentina group: resources. K.P.K.: conceptualization, funding. R.F.B.: conceptualization, writing – review and editing. L.M.: conceptualization, methodology, resources, writing – review and editing, supervision, project administration, funding. S.D.B.: conceptualization, writing – review and editing, supervision, project administration, funding. A.C.: conceptualization, resources, writing – original draft preparation, visualization, supervision, project administration.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The isolates for this study were selected from the SIREVA-Argentina collection as part of laboratory surveillance. No tissue material or other biological material was obtained from humans. All information on these isolates was anonymized.

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